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Bernd Nilius
Veit Flockerzi *Editors*

Mammalian Transient Receptor Potential (TRP) Cation Channels

Volume I

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Volume 222

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Preface

When we edited in 2007 the first issue on transient receptor potential channel in the Handbook of Experimental Pharmacology, we were all very excited by the progress in this field although only one decade after cloning the first TRP channel had passed. At this time, somewhat less than 5,000 papers were published on TRP channel (1/1/1960 until 31/12/2006). If we check now the period (1/1/2007 until 13/1/2014), additional 9,300 papers can be found in a PubMed search.[1] Needless to say, the general interest on these 28 members of the Trp gene family which encode ion channels is nearly exponentially growing. Therefore, it seemed to be indicated, although many excellent books on TRP channels have been published meanwhile, to jump into a new adventure editing a comprehensive source book in this successful Springer Handbook series again on the same topic. This is not only an update of the 2007 book but also an impressive introduction of novel areas which TRP channels have entered. The 2007 view that TRP channels are mainly cell sensors with an intriguing variability concerning the modes of activation has dramatically extended into the evolutionary field, the structural approach, and especially the advent of the important role of TRP channels in hereditary and acquired diseases. Important new data concerning the role of TRP channels in intracellular compartments are included. We also refer to the still controversial topic how TRP channel is involved in store-dependent Ca^{2+} entry. Indeed, the TRP field expansion did not lose the fast speed. It is extending into so far unexpected areas. The *gain of knowledge* has reached such an extent that we have not been able to restrict the source book into a single volume; rather, we had to agree on a two-volume publication. In the first volume, we go through all the known TRP channels. Leading experts in the field summarize features of individual TRP channels starting with the description of the gene, the expression patters, associated proteins, biophysical and biochemical function properties, and transgenic animal models and

[1] The used search string was ("transient receptor potential" OR trpa* OR trpc* OR trpm* OR trpp* OR trpv* OR PKD* OR stim1 OR stim2 OR orai1 OR orai2 OR orai3 OR trpa*). Note that this search included also the main players of store-operated Ca^{2+} entry, because of the still-often-reported links to TRP as also discussed in Volume 2.

closing with cellular TRP functions, dysfunctions, and their role in diseases. The second volume starts with a chapter on sensor properties and functions of TRP channels. This was highlighted in the 2007 book but is not very much extended. Surprising new features are reported, e.g., new insights into thermo- and light-sensing, novel roles or TRPs in taste perception and chemesthesis, and especially their functional importance as chemosensors for gasotransmitters, including oxygen sensing, which was evidenced only in the last 5 years. In the second part, more general topics related to TRP functions and features are discussed such as channel structure; TRPs as targets of pharmacological modulation, including a wealth of natural compounds; and the exciting discovery of novel channel toxins. New aspects are discussed concerning the role of TRPs as important players in the physiology of reproduction and in neural networks which control reproductive behavior opening a *TRP window* into neuroendocrinology, i.e., their role in hormone-secreting cells. We finish this book with some critical remarks on the current state of TRP research, controversies, and surprises.

We hope that this book will guide a large reader community through the fascinating world of the TRP channel family from basic science to pathophysiology and disease. May this voluminous source/textbook also help to establish interactions between the fundamental and clinical research and the research in drug discovery and development! We are convinced that this book is "translational" in the best meaning of this word. Despite the many advances in the understanding of the molecular mechanisms and function features of TRP channel, there is still a tremendous need for more in-depth understanding of the structure of TRP channels, their implementation in diverse signal cascades, more mechanistic insight into channel function at the molecular and systemic level, as well as the need for identifying selective pharmacological tools, new therapeutic targets, and developing new treatment options. We hope this book stimulates further research. Finally, we may conclude that we might be still in a period of the end of the beginning rather than the beginning of the end! The editors wish to thank all authors for excellent contribution and also Wilma McHugh (Springer) for all expert support and very helpful editorial advice!

Leuven — Bernd Nilius
Homburg — Veit Flockerzi

Contents for Volume I

Contents for Volume II

TRPs: Truly Remarkable Proteins

Veit Flockerzi and Bernd Nilius

Abstract

The family of transient receptor potential cation channels has received in the last 10 years a tremendous interest because members of this family are involved in a plethora of cell functions and have been identified as causal for many hereditary and acquired diseases. We shortly introduce these channels, summarize nomenclature and chromosomal location of the 28 mammalian *Trp* genes, and list the available *Trp*-deficient mouse lines.

Keywords

TRP cation channel proteins • TRP phylogenetic tree • *Trp* gene • Chromosomal location • *Trp* gene knockout • *Trp*-deficient mouse strain • Mouse (animal) model

The "transient receptor potential" (*trp*) gene was cloned from the Drosophila fly in 1989 (Montell and Rubin 1989), the molecular identification and functional characterization of the mammalian TRPC members occurred in 1995 (Wes et al. 1995; Zhu et al. 1995; Nilius and Owsianik 2011), and we are now, in 2014, probably just at the beginning to get a deeper understanding of the molecular structure, the biophysical properties, the functional role, and the pathophysiological impact of the 28 mammalian (27 human) members of this superfamily. The number of publications on this topic rises explosively ranging from molecular biology and crystallography to clinical research, food production, and cosmetics. So far, more

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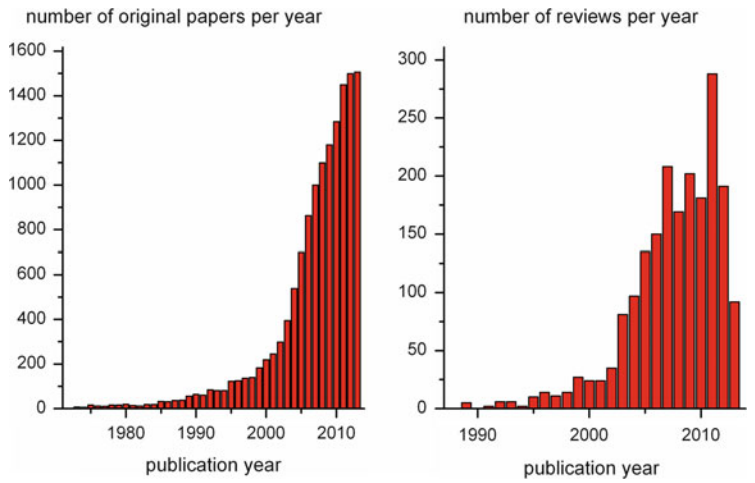

Fig. 1 Publications in the TRP field (adapted from PubMed, November 3, 2013)

than 13,000 publications and 2,000 reviews have been published about TRPs (Fig. 1). The increase in the last 20 years is nearly exponential! This flood of new information justifies hopefully a comprehensive source book which covers the state of the art in TRP research.

Transient receptor potential (TRP) cation channels have been extensively studied and described as polymodal cell sensors (Gees et al. 2010, 2012; Nilius and Owsianik 2011; Wu et al. 2010). They fall into six subfamilies (Fig. 2): TRPC for "canonical" (TRPC1–7), TRPV for "vanilloid" (TRPV1–6), TRPM for "melastatin" (TRPM1–8), TRPP for "polycystin" (TRPP2, TRPP3, TRPP5), TRPML for "mucolipin" (TRPML1–3), and TRPA for "ankyrin" (TRPA1). All TRP gene products are intrinsic membrane proteins with six putative transmembrane spans (S1–S6) and a cation-permeable pore region between S5 and S6. The length of the intracellular amino (N) and carboxy (C) termini and structural domains (e.g., ankyrin) they encompass varies significantly between members of the TRP channel subfamilies (Owsianik et al. 2006). The cytoplasmic domains are involved in the regulation and modulation of channel function and trafficking. Functional TRP channels consist of four identical or similar TRP subunits.

Table 1 summarizes the nomenclature used in this chapter and lists the chromosomal locations of the 28 *Trp* channel genes present in human and mouse. Whereas some chromosomes carry one up to three or four *Trp* genes (Table 2), only the *Trpv6* and *Trpv5* genes and the *Trpv1* and *Trpv3* genes are located side by side and in the same transcriptional orientation in single human and mouse chromosomes (Table 2) suggesting recent gene duplication events in the TRPV subfamily (Abramowitz and Birnbaumer 2007). In human *Trpv3* is located in chromosome 17 immediately before *Trpv1* (separated by 7.45 kbps). Similarly in mouse *Trpv3* is located in chromosome 11 immediately before *Trpv1* (separated by 6.15 kbps). The *Trpv6* gene is located in human chromosome 7 and mouse chromosome

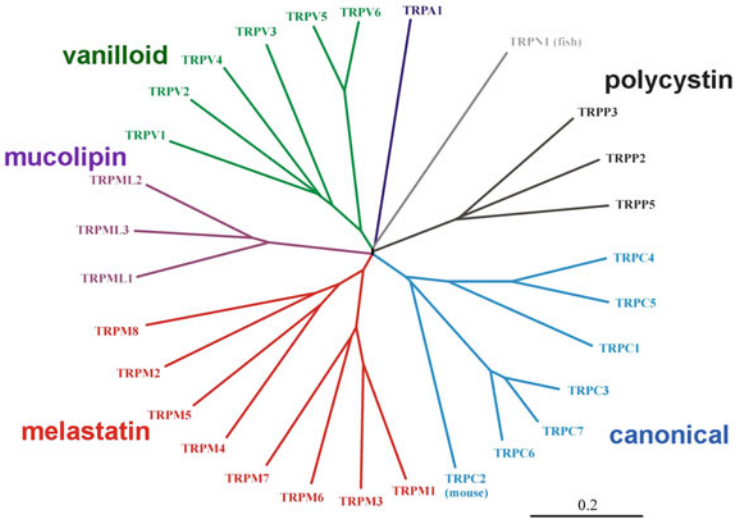

Fig. 2 The phylogenetic tree of the TRP family [adapted from Nilius and Owsianik (2011) with permission]

6, respectively, immediately in front of the *Trpv5* gene (separated by 21.76 kbps in human and by 16.37 kbps in mouse).

This handbook is published 7 years after the first TRP *Handbook of Experimental Pharmacology* (HEP) (Flockerzi and Nilius 2007) with the intention to focus our view on the most important achievements. We cover in detail all the 28 mammalian members of the TRP family, describing the gene, expression, channel functions, functional properties, interaction partners of TRP channels, diverse and complex signaling cascades, lessons from knockout models, and their impact on human diseases.

Especially TRP gene knockout animals, obtained by deletion of individual *Trp* genes in embryonic stem cells through homologous recombination, have made it possible to identify TRP channel functions and their relationship to physiological and pathophysiological processes in the living organism (Freichel et al. 2011). Since 2007 additional knockouts for 16 *Trp* genes have been published [compared to 10 before 2007 Freichel and Flockerzi (2007)], and at present only two *Trp* genes remain to be deleted, *Trpml2* and *Trpp5* (Table 3). Among the wealth of information obtained by phenotyping wild-type animals in comparison to the corresponding *Trp* gene-deficient animals (and described in the various chapters of this volume), two findings are striking: (1) With the exception of *Trpm7*-deficient (Jin et al. 2008), *Trpm6*-deficient (Walder et al. 2009), and *Trpp2*-deficient animals (Wu et al. 1998), all other knockouts are viable. $Trpm7^{-/-}$ embryos died before day 7.5 of embryogenesis, $Trpm6^{-/-}$ mice never survived to weaning and mostly died by embryonic day 12.5, and $Trpp2^{-/-}$ embryos died later than embryonic day 15. (2) Although several TRPs have been associated with fertility and reproduction,

Table 1 TRP nomenclature and chromosomal location of TRP channel genes in the human and mouse genomes

	Human			Mouse		
TRP	Gene	Chromosome	Location	Gene	Chromosome	Location
Trpa1	ENSG00000104321	8	72,932,152–72,987,852	ENSMUSG00000032769	1	14,872,648–14,918,862
Trpc1	ENSG00000144935	3	142,442,916-142,526,730	ENSMUSG00000032839	9	95,706,627-95,750,358
Trpc2	ENSG00000182048	11	3,631,131–3,658,789	ENSMUSG00000070425	7	102,065,511–102,096,864
Trpc3	ENSG00000138741	4	122,800,182–122,872,909	ENSMUSG00000027716	3	36,620,482–36,690,167
Trpc4	ENSG00000133107	13	38,210,773–38,444,562	ENSMUSG00000027748	3	54,156,057–54,318,470
Trpc5	ENSG00000072315	x	111,017,543–111,326,004	ENSMUSG00000041710	x	144,381,671–144,688,180
Trpc6	ENSG00000137672	11	101,322,295–101,743,293	ENSMUSG00000031997	9	8,544,196–8,680,565
Trpc7	ENSG00000069018	5	135,548,999–135,732,730	ENSMUSG00000021541	13	56,773,113–56,895,789
Trpm1	ENSG00000134160	15	31,293,264–31,453,476	ENSMUSG00000030523	7	64,153,835–64,269,775
Trpm2	ENSG00000142185	21	45,770,046–45,862,964	ENSMUSG00000009292	10	77,907,722–77,970,563
Trpm3	ENSG00000083067	9	73,149,949–74,061,820	ENSMUSG00000052387	19	22,139,119–22,989,884
Trpm4	ENSG00000130529	19	49,660,998–49,715,093	ENSMUSG00000038260	7	45,303,155–45,333,780
Trpm5	ENSG00000070985	11	2,425,745–2,444,275	ENSMUSG00000009246	7	143,069,153–143,094,642
Trpm6	ENSG00000119121	9	77,337,411–77,503,010	ENSMUSG00000024727	19	18,749,983–18,892,510
Trpm7	ENSG00000092439	15	50,844,670–50,979,012	ENSMUSG00000027365	2	126,791,565–126,876,230
Trpm8	ENSG00000144481	2	234,826,043–234,928,166	ENSMUSG00000036251	1	88,277,661–88,389,293
Trpv1	ENSG00000196689	17	3,468,738–3,500,392	ENSMUSG00000005952	11	73,234,292–73,261,242
Trpv2	ENSG00000187688	17	16,318,856–16,340,317	ENSMUSG00000018507	11	62,574,486–62,600,515
Trpv3	ENSG00000167723	17	3,413,796–3,461,289	ENSMUSG00000043029	11	73,267,388–73,300,363
Trpv4	ENSG00000111199	12	110,220,890–110,271,212	ENSMUSG00000014158	5	114,622,152–114,658,421
Trpv5	ENSG00000127412	7	142,605,267–142,630,905	ENSMUSG00000036899	6	41,652,770–41,680,723
Trpv6	ENSG00000165125	7	142,568,956–142,583,507	ENSMUSG00000029868	6	41,620,621–41,636,405
Trpml1	ENSG00000090674	19	7,587,512–7,598,895	ENSMUSG00000004567	8	3,500,457–3,515,232
Trpml2	ENSG00000153898	1	85,391,268–85,462,796	ENSMUSG00000011008	3	146,149,833–146,195,513

Trpml3	ENSG00000055732	1	85,483,765–85,514,182	ENSMUSG00000036853	3	146,117,459–146,141,806
Trpp2	ENSG00000118762	4	88,928,820–88,998,929	ENSMUSG00000034462	5	104,459,450–104,505,819
Trpp3	ENSG00000107593	10	102,047,903–102,090,243	ENSMUSG00000037578	19	44,147,637–44,192,442
Trpp5	ENSG00000078795	5	137,223,657–137,278,436	ENSMUSG00000014503	18	34,409,423–34,442,789

Gene resource: http://www.ensembl.org

Table 2 Know thy neighbor: chromosomal location of human and mouse *Trp* genes

Chromosome	Homo sapiens	Mus musculus
1	*Trpml2+ Trpml3−*	*Trpa1− Trpm8+*
2	*Trpm8+*	*Trpm7−*
3	*Trpc1+*	*Trpc3− Trpc4+ Trpml3+ Trpml2+*
4	*Trpp2+ Trpc3−*	
5	*Trpp5+ Trpc7−*	*Trpp2+ Trpv4−*
6		*Trpv6−/Trpv5−*
7	*Trpv6−/Trpv5−*	*Trpm4− Trpm1+ Trpc2+ Trpm5−*
8	*Trpa1−*	*Trpml1+*
9	*Trpm3− Trpm6−*	*Trpc6+ Trpc1−*
10	*Trpp3−*	*Trpm2−*
11	*Trpm5− Trpc2+*	*Trpv2+ Trpv1+/Trpv3+*
12	*Trpc6− Trpv4−*	
13	*Trpc4−*	*Trpc7−*
14		
15	*Trpm1− Trpm7−*	
16		
17	*Trpv3−/Trpv1− Trpv2+*	
18		*Trpp5+*
19	*Trpml1+ Trpm4+*	*Trpm6+ Trpm3+ Trpp3−*
20		
21	*Trpm2+*	
22		
x	*Trpc5−*	*Trpc5−*
y		

Back to back are *Trpv6/Trpv5* (human, chromosome 7, and mouse, chromosome 6) and *Trpv3/Trpv1* (human, chromosome 17, and mouse, chromosome 11); +, forward strand; −, reverse strand

only TRPV6 channels have been shown to be essential for (mouse male) fertility (Weissgerber et al. 2011, 2012).

So far half of the published knockouts are conventional or global knockouts (Table 3). The gene deletion is unrestricted and animals inherit the genetic deletion in all of their cell types. In these animals, it may be difficult to exclude the possibility that developmental defects or compensatory upregulation of other genes contributes to the phenotype observed in adult animals. In addition, this global gene deletion might make it difficult to attribute abnormal phenotypes to a particular type of cell. The other half are conditional knockouts (Table 3) which allow regional and temporal control of TRP gene expression and that restrict deletions to cells in a specific tissue or at specific points in an animal's development. More of these conditional TRP-deficient mouse models are required.

Other strategies allow visualizing TRP-expressing cells. These strategies include generation of TRP-dependent reporter-tagged null mutations as shown for TRPA1 [*alkaline phosphatase* (Kwan et al. 2006)], TRPM8 [*eGFP* (Dhaka et al. 2007) or *lacZ* (Colburn et al. 2007)], and TRPM3 [*lacZ* (Vriens et al. 2011)]. Whereas the

Table 3 *Trp*-deficient mice

Gene	Deletion of exon(s)	Conditional	References
Trpa1	23 (part.)	No	Bautista et al. (2006)
	22–24, replaced by *IRES-alkaline phosphatase-polyA*-cassette	No	Kwan et al. (2006)
Trpc1	8	No	Dietrich et al. (2007)
Trpc2	7–10	No	Stowers et al. (2002)
	6–11	No	Leypold et al. (2002)
Trpc3	7	Yes	Hartmann et al. (2008)
	7–8	Yes	Hirschler-Laszkiewicz et al. (2012)
Trpc4	6	No	Freichel et al. (2001)
Trpc5	5	Yes	Riccio et al. (2009)
	4	Yes	Xue et al. (2011)
Trpc6	7	No	Dietrich et al. (2005)
Trpc7	1	Yes	Perez-Leighton et al. (2011)
	5	Yes	Xue et al. (2011)
Trpv1	9, 10, 11 (part.)	No	Caterina et al. (2000)
Trpv2	10–13	Yes	Park et al. (2011)
Trpv3	14–15	No	Moqrich et al. (2005)
Trpv4	4	No	Suzuki et al. (2003)
	12	Yes	Liedtke and Friedman (2003)
Trpv5	13	Yes	Hoenderop et al. (2003)
Trpv6	9–15	No	Bianco et al. (2007)
	13–15	Yes	Weissgerber et al. (2012)
Trpm1	2–4	No	Morgans et al. (2009)
	3 (part.)–5	No	Shen et al. (2009)
	4–6	No	Koike et al. (2010)
Trpm2	20–21	No	Yamamoto et al. (2008)
Trpm3	19 (part.) replaced by *IRES- lacZ-neo*-cassette	No	Vriens et al. (2011)
Trpm4	15–16	Yes	Vennekens et al. (2007)
	3–6	Yes	Barbet et al. (2008)
Trpm5	15–19	No	Zhang et al. (2003)
	Promoter, 1–4	No	Damak et al. (2006)
Trpm6	5–7	No	Walder et al. (2009)
	2–3 replaced by *IRES- lacZ-neo*-cassette	No	Woudenberg-Vrenken et al. (2011)
Trpm7	Intron 1 (insertion)	No	Jin et al. (2008)
	17	Yes	Jin et al. (2008)
	32–36 (kinase)	No	Ryazanova et al. (2010)
Trpm8	1–2, replaced by *lacZ-neo*-cassette	No	Colburn et al. (2007)
	Knock-in of *eGFP-polyA*-cassette into exon 5, 27 nt following start	No	Dhaka et al. (2007)
	13–14	No	Bautista et al. (2007)

(continued)

Table 3 (continued)

Gene	Deletion of exon(s)	Conditional	References
Trpp2	1 (insertion)	No	Wu et al. (1998)
	1	No	Pennekamp et al. (2002)
Trpp3	3–9	No	Horio et al. (2011)
Trpp5	n.d.		
Trpml1	3–5 (part.)	Yes	Venugopal et al. (2007)
Trpml2	n.d.		
Trpml3	11	Yes	Jors et al. (2010)
	7–8	Yes	Castiglioni et al. (2011)

Part. partial, *n.d.* not described, *nt* nucleotides; *conditional, no,* refers to conventional or global gene deficiency

TRPA1 and TRPM8 mice have been generated by gene targeting approaches, the TRPM3 mouse and one of the TRPM6 mouse strains were generated by gene trapping. The visualization of TRP-expressing cells could also be accomplished by mouse lines, which carry an internal ribosome entry site (IRES) followed by the cDNA of a Cre recombinase within the *Trp* gene. In this strategy the IRES element will result in transcription of a bicistronic messenger RNA from which the TRP and cre recombinase are independently translated. These TRP-IRES-Cre animals can be bred to reporter mice where the "reporter," the cDNA of a marker gene (e.g., *LacZ*, *GFP*, *CFP*, or *YFP*), is expressed only following Cre-mediated recombination (Fig. 3). So far, no such TRP-IRES-Cre mouse lines obtained by homologous recombination have been published, but several are in various pipelines. By these strategies, TRP-positive cells can be directly visualized and additionally manipulated in various ways depending on the properties of the "reporter" genes (channelrhodopsin, diphtheria toxin, calcium indicators, etc.) used.

In this volume, the special progress of studying TRP channels in intracellular organelles, such as TRPML channels in endosomes and lysosomes, will also be highlighted. In addition, we provide in detail an overview on special cellular functions of these channels such as photoreception, hearing, olfaction, taste, and somatosensation such as nociception, mechanoreception, temperature sensing, chemosensing, i.e., all the classical Aristotle's senses which are so much depending on TRPs. TRPs were first considered as unique cell sensors which are involved in all our Aristotle's senses by which we discover the world (Damann et al. 2008). However, TRP channels have a much higher functional importance than just acting as sensory channels. They play an important role in many homeostatic functions. These aspects will also be discussed in this book. In addition, some exciting new developments, e.g., the modulation of TRPs by a plethora of natural compounds, the role of TRPs in endocrinology and metabolic control, the exciting interaction with STIM, ORAI, components of the molecular machinery which constitutes store-operated Ca^{2+} entry.

From the point of view of fundamental research, TRPs show a unique promiscuity of gating mechanisms which came as a surprise even for channel maniacs [for

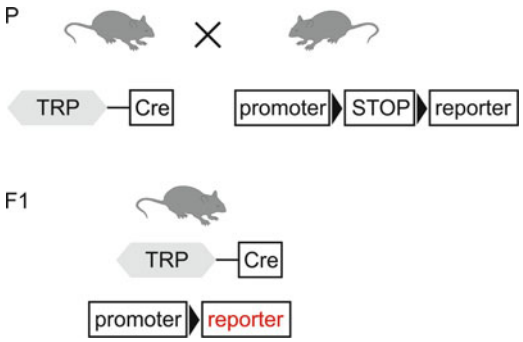

Fig. 3 Strategy to visualize and manipulate TRP-expressing cells. Mice carrying a targeted integration of the cDNA of cre recombinase (Cre) within a *Trp* gene (TRP) are mated with mice carrying a (floxed) strong transcriptional termination sequence (STOP) followed by the cDNA of the "reporter" under the control of a (constitutively and ubiquitously expressed) promoter (reporter strain). P, parental mice. After Cre-mediated recombination, the floxed "STOP" cDNA is excised allowing the promoter to drive expression of the reporter. Black triangles represent loxP target sites for Cre-mediated recombination. A multitude of strains have been created using the cDNAs of fluorescent proteins (channelrhodopsin2, lacZ, diphtheria toxin A, halorhodopsin, and reverse tetracycline transactivator, to name only a few) by targeted insertion of a reporter gene in the ubiquitously expressed Rosa26 locus (Soriano 1999)

a comprehensive review, see Gees et al. (2012)]. TRPs are probably expressed in all cells of our body. It was therefore not unexpected that TRP channels are involved in several, still not well-understood diseases and have therefore triggered a huge hope for the development of new drug targeting these channels (Moran et al. 2011; Nilius et al. 2007; Nilius and Voets 2013).

We hope that this book will provide the most actual overview on the different faces of these channels written by world leaders in this field. Another—maybe more sophisticated—answer is that we know a lot about TRPs but we understand a lot less about the 28 mammalian members of this channel superfamily than of other ion channels. It is therefore important to reevaluate and reinterpret even the well-known data under the view of all new achievements. Hopefully this book issue will serve this important task to describe in a really up-to-date fashion these *truly remarkable* TRP *proteins*!

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Part I

The TRPC Subfamily

TRPC1

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Contents

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Abstract

The TRPC1 ion channel was the first mammalian TRP channel to be cloned. In humans, it is encoded by the *TRPC1* gene located in chromosome 3. The protein is predicted to consist of six transmembrane segments with the N- and C-termini located in the cytoplasm. The extracellular loop connecting transmembrane segments 5 and 6 participates in the formation of the ionic pore region. Inside the cell, TRPC1 is present in the endoplasmic reticulum, plasma membrane, intracellular vesicles, and primary cilium, an antenna-like sensory organelle functioning as a signaling platform. In human and rodent tissues, it shows an almost ubiquitous expression. TRPC1 interacts with a diverse group of proteins including ion channel subunits, receptors, and cytosolic proteins to mediate its effect on Ca^{2+} signaling. It primarily functions as a cation nonselective channel within pathways controlling Ca^{2+} entry in response to cell surface receptor activation. Through these pathways, it affects basic cell functions, such as proliferation and survival, differentiation, secretion, and cell migration, as well as cell type-specific functions such as chemotropic turning of neuronal growth cones and myoblast fusion. The biological role of TRPC1 has been studied in genetically engineered mice where the *Trpc1* gene has been experimentally ablated. Although these mice live to adulthood, they show defects in several organs and tissues, such as the cardiovascular, central nervous, skeletal and muscular, and immune systems. Genetic and functional studies have implicated TRPC1 in diabetic nephropathy, Parkinson's disease, Huntington's disease, Duchenne muscular dystrophy, cancer, seizures, and Darier–White skin disease.

Keywords

Channel • TRP • Calcium signaling • Disease

1 Introduction

G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) comprise a very large group of cell surface receptors that elicit their physiological responses through the production of inositol-1,4,5-trisphosphate (IP_3) (Berridge and Irvine 1984) (Fig. 1). Receptor stimulation results in the activation of PLC-β or γ isoforms, which catalyze the formation of IP_3 and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2). Newly synthesized IP_3 acts on IP_3 receptors (IP_3Rs) to trigger a rapid increase in the intracellular Ca^{2+} concentration

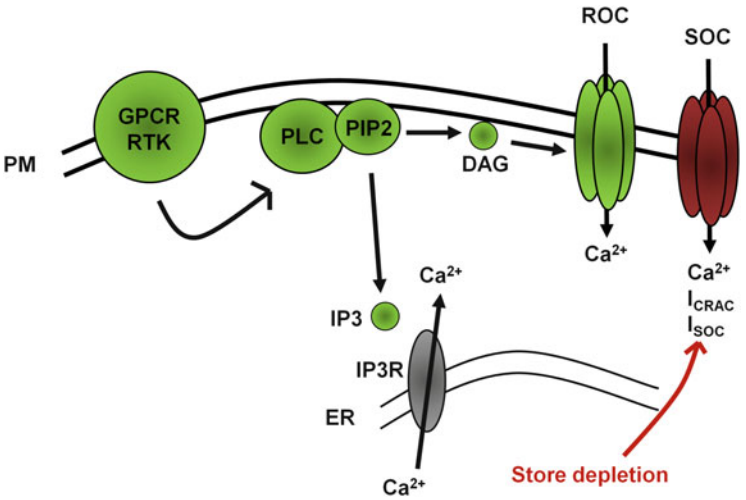

Fig. 1 Diagram illustrating activation mechanisms of receptor- and store-operated Ca^{2+} entry channels. Agonist stimulation of a G-protein-coupled receptor (GPCR) or receptor tyrosine kinase (RTK) in the plasma membrane (PM) results in the activation of phospholipase C-β or γ, respectively, and production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 induces a rapid Ca^{2+} release from the endoplasmic reticulum (ER) by acting through the IP_3 receptors (IP3Rs) on the ER. Ca^{2+} release causes a rapid depletion of Ca^{2+} in the ER resulting in the activation of store-operated Ca^{2+} (SOC) entry channels (shown in *burgundy*) in the PM. SOCs can mediate the highly Ca^{2+}-selective, Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) or the less Ca^{2+}-selective, store-operated channel current (I_{SOC}). Receptor-operated channels (ROC, shown in *green*) are activated by second messengers generated in response to the activation of a given GPCR or RTK, but not by store depletion

by releasing free Ca^{2+} from intracellular stores (Burgess et al. 1984). Intracellular Ca^{2+} concentration returns to normal levels by extrusion of cytoplasmic Ca^{2+} to the extracellular space mediated by plasma membrane Ca^{2+}-ATPases and Na^{+}–Ca^{2+} exchangers, readmission of Ca^{2+} into the endoplasmic reticulum (ER) mediated by the SERCA pump, and Ca^{2+} entry via the store- and receptor- operated Ca^{2+} channels (Fasolato et al. 1994; Putney and McKay 1999). Store- and receptor-operated Ca^{2+} entry maintains Ca^{2+} homeostasis and keeps the cell in a Ca^{2+} signaling-competent stage. Both processes are needed for diverse cellular functions (Parekh and Penner 1997) ranging from cell proliferation and gene expression in T lymphocytes (Lewis 2001) to endothelial cell function (Nilius and Droogmans 2001) and regulation of the acrosome reaction in germ cells (Wassarman et al. 2001).

In general, store-operated channels are the channels defined by their ability to open in response to the depletion of the internal Ca^{2+} stores, but the term receptor-operated channels is loosely defined (also discussed in (Patterson et al. 2002)). According to one of the two widely used definitions, receptor-operated channels are the channels activated in response to the activation of an IP_3-coupled receptor. Therefore, the term is inclusive of both store-operated and second messenger-

activated channels (Barritt 1999). The other usage of the term is applied to describe channels activated directly by second messengers (i.e., DAG or cell-permeant derivatives such as 1-oleoyl-2-acetyl-sn-glycerol, OAG) and not by store depletion, following receptor stimulation (Patterson et al. 2002). To avoid confusion, here we will adopt the latter definition (Fig. 1). Therefore, cell surface receptor stimulation will result in the activation of both store- and receptor-operated channels, whereas depletion of internal stores will result in the activation of only the store-operated channels (Fig. 1).

Store-operated Ca^{2+} entry channels can be divided into several types based on their biophysical properties (Parekh and Putney 2005). The prototypical Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) was first described in mast cells (Hoth and Penner 1992), and its biophysical and pharmacological properties have been characterized in great detail (Hoth and Penner 1992; Zweifach and Lewis 1995). It is mediated by the CRAC channel which consists of the pore-forming subunits Orai1–3 (or CRAC modulator 1–3) and requires the Ca^{2+} sensors, STIM1 and STIM2, for activation (Hogan et al. 2010; Soboloff et al. 2012; Vig and Kinet 2009). In addition to I_{CRAC}, there are other store depletion-activated currents whose properties deviate to various degrees from the ones ascribed to I_{CRAC}. These currents are mediated by channels that show lower selectivity to Ca^{2+} compared to CRAC channel and called store-operated channels (SOCs) (Birnbaumer 2009; Lee et al. 2010; Parekh and Putney 2005; Worley et al. 2007). TRPC1 has been shown to modulate I_{CRAC} (Mori et al. 2002; Ong et al. 2013) and I_{SOC} (Cheng et al. 2008, 2011a; Huang et al. 2006; Liao et al. 2007, 2008; Shi et al. 2012; Singh et al. 2002; Yuan et al. 2003, 2007; Kiselyov et al. 1998; Zhu et al. 1996).

Receptor-operated channels (ROCs) fall into a group of ion channels activated by diverse stimuli generated in response to the activation of a cell surface receptor (Fig. 1). Second messengers that are generated in response to the activation of a receptor coupled to the phosphoinositide pathway can activate ion channels independently of store depletion. These messengers can include are DAG, PIP_2 hydrolysis, protein kinase C activation, increase in intracellular Ca^{2+} concentration following Ca^{2+} release from the ER, products of certain types of phospholipases, etc. (Fig. 1). A subgroup of the TRPC subfamily, such as TRPC3/6/7, is considered typical receptor-operated channels since they are activated by DAG/OAG (Hofmann et al. 1999). As it will be discussed later, TRPC1 has been shown to modulate Ca^{2+} influx mediated by the CRAC, SOC, and ROC channels.

2 Gene Structure

The primary amino acid sequence of TRPC1 was deduced almost 20 years ago (Zhu et al. 1995; Wes et al. 1995). The *TRPC1* gene is located in human chromosome 3q23. It consists of 13 distinct introns bearing GT-AG borders and 13 exons and spans a total of 83.84 kb of genomic sequence, from 142442902 to 142526737 (NCBI 37, August 2010), on the direct strand. Exon 1 contains the translation initiation start site. The *Trpc1* gene generates at least five reported splice variants in

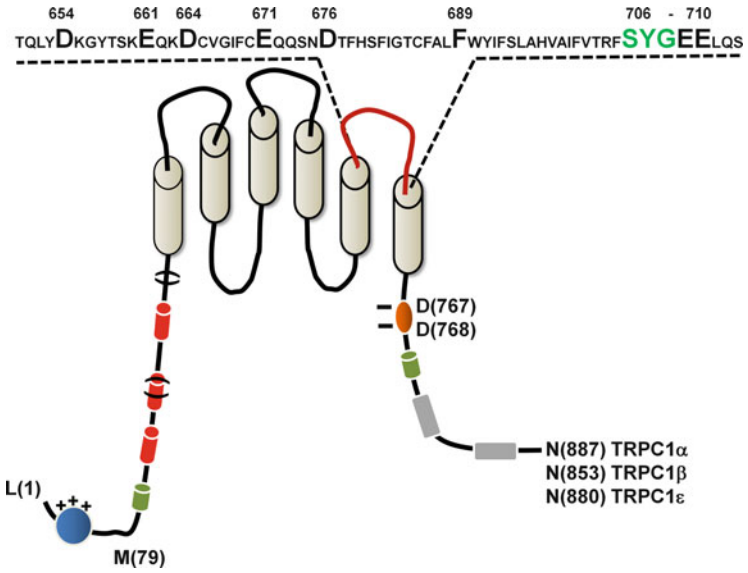

Fig. 2 Diagrammatic representation of TRPC1. TRPC1α (accession number for mRNA, U73625; for annotated protein, AAB50622.1) is shown with six transmembrane segments and cytosolic N- and C-termini. Translation is initiated from a leucine (L) residue, located 78 amino acid residues upstream of the first predicted methionine (M) in mouse TRPC1α. Note that this methionine is not conserved in human TRPC1 isoforms. Ankyrin repeats are shown as *red cylinders*, Homer and FKBP binding sites are shown as *green cylinders*, and Ca^{2+}/CaM binding sites are shown as *gray cylinders*. A putative positively charged domain in the N-terminal extension (amino acid residues 1–78) is shown as a *blue sphere*. Negatively charged residues (aspartic acids, D767 and D768) mediating binding to STIM1 are shown as a *brown oval*. Primary amino acid sequence of the putative pore-forming region is shown. Within this region, negatively charged residues (aspartic acid (D)654, glutamic acid (E)661, D664, E671, D676, E709, and E710), phenylalanine (F689), and the putative selectivity filter (serine–tyrosine–glycine (SYG) shown in *green*) are shown enlarged. *Brackets* indicate deletion of 7 amino acid residues between the third ankyrin repeat and transmembrane segment 1 in TRPC1ε or deletion of 34 residues within ankyrin repeat 2 in TRPC1β isoform. Total length of mouse TRPC1α, TRPC1β, or TRPC1ε is predicted to be 887, 853, or 880 residues. Numbering is according to mouse TRPC1α (AAB50622.1 plus the 78 residues of the N-terminal extension)

rodents (α–ε) and four in humans (α–δ). Isoforms α, β, and ε have been functionally validated, whereas it is currently unknown whether isoforms γ and δ form functional channels (Fig. 2).

3 Expression

Detection of TRPC1 protein using TRPC1-specific antibodies has been challenging. Different antibodies have detected TRPC1 protein with sizes varying from 60 to 100 kDa (Ong et al. 2002). It appears that a monoclonal antibody described in

Ma et al. (2003) recognizes a band of about 80–90 kDa that is non-detectable in tissues from *Trpc1* knockout mice (Seth et al. 2009). Using this and other antibodies, TRPC1 has been detected in the kidney (Bai et al. 2008a), liver (Ong et al. 2002), skeletal muscle (Ma et al. 2003), brain (Sinkins et al. 2004; Ma et al. 2003), cells derived from the salivary glands (Brazer et al. 2003), and numerous other tissues and cell lines (Bon and Beech 2013). Inside the cell, TRPC1 shows strong immunoreactivity in the ER (Alfonso et al. 2008), intracellular vesicles (Cheng et al. 2011a), plasma membrane (Brazer et al. 2003; Xu and Beech 2001), neuronal dendrites (Martorana et al. 2006), and the primary cilium (Bai et al. 2008a; Raychowdhury et al. 2005). The primary cilium is an antenna-like organelle composed mainly by microtubules. It functions as a sensory organelle housing a large number of receptors and ion channels. Expression of several TRPC channels including TRPC1 has been documented in the sperm flagellum, a specialized form of the cilium (Trevino et al. 2001). The functional role of TRPC1 in the primary cilium and flagellum is unknown.

4 Channel Protein Including Structural Aspects

4.1 Pore-Forming Domain

TRPC1 is predicted to have six transmembrane segments (M1–M6) flanked by cytosolic N- and C-termini (Fig. 2). It shows the highest primary amino acid sequence homology with a subgroup of the TRPC subfamily containing TRPC2, TRPC4, and TRPC5. As expected for a cation channel bearing six transmembrane segments, the extracellular loop between transmembrane segments M5 and M6 shows an α-helical structure that could form all or part of the ionic pore (Dohke et al. 2004). The importance of this domain in the function of TRPC1 is supported by site-directed mutagenesis experiments whereby mutating phenylalanine (F)689 (formerly cited as F561A) to alanine (A) abolished TRPC1 activity (Kim et al. 2003; Yildirim et al. 2012). Further, mutagenesis experiments neutralizing negatively charged residues (aspartic acid (D)654, glutamic acid (E)661, D664, E671, D676, E709, and E710) reduced Ca^{2+}, but not Na^{+} permeability supporting the idea that this domain functionally contributes to the ionic pore (Liu et al. 2003). Finally, antibodies raised against the extracellular loop between M5 and M6 segments suppressed TRPC1 activity in transfected and native cells (Antoniotti et al. 2002; Kim et al. 2003; Xu and Beech 2001).

4.2 N- and C-Terminal Cytosolic Fragments

A region in TRPC1 located in the C-terminal cytosolic tail has been identified to bind calmodulin (CaM) in the presence of Ca^{2+} (Ca^{2+}/CaM) and inactivate TRPC1-mediated store-operated currents, suggesting that this domain mediates the Ca^{2+}-dependent inactivation of TRPC1-mediated currents (Singh et al. 2002). Still in the

C-terminal cytosolic domain, two negatively charged residues (aspartic acid, D) were identified to mediate a direct protein–protein interaction with positively charged residues in STIM1 allowing activation of TRPC1 in response to store depletion through STIM1 (Kiselyov et al. 2000). Thus, these residues mediate the gating of TRPC1 by STIM1 in response to store depletion. TRPC1 has also been reported to homomultimerize (Kobori et al. 2009). Homomultimerization is likely to be mediated through domains with homology to ankyrin protein, called ankyrin repeats, as was seen with other TRPC members (Lepage et al. 2006). Lipid bilayer reconstitution and atomic force spectroscopy revealed that TRPC1 can form homotetramers (Barrera et al. 2007). Overall, the N- and C-termini of TRPC1 have been implicated in gating, Ca^{2+}-dependent inactivation, and homomultimerization.

4.3 N-Terminal Extension

Until very recently, it was assumed that translation of mouse or human TRPC1 is initiated by the first methionine in exon 1. Ong et al. (2013) showed that translation of mouse or human TRPC1 is more efficiently initiated by an upstream leucine (L) adding 78 amino acid residues to all known TRPC1 isoforms. Close inspection of this N-terminal extension reveals the presence of a stretch of glycine–serine (G–S) residues suggesting the presence of a highly flexible hinge region connecting the N-terminal extension with the rest of the molecule. More importantly, a stretch of positively charged residues is present upstream of the flexible hinge region. The clustering of positive charges in the N-terminal extension along with the flexible region is highly suggestive of a "ball-and-chain" structure in TRPC1, which is likely to have important consequences on channel function.

5 Interacting Proteins

5.1 Channel Subunits

5.1.1 TRPP2

TRPP2 (also called polycystin-2, gene: polycystic kidney disease 2 (*PKD2*)) belongs to the polycystin subgroup of TRP superfamily (Delmas 2005; Tsiokas et al. 2007; Giamarchi et al. 2006; Cantiello 2004) and was originally cloned because of its involvement in autosomal dominant polycystic kidney disease (ADPKD) (Mochizuki et al. 1996). Naturally occurring mutations in TRPP2 are responsible for ~15 % of all cases of ADPKD, one of the most common genetic diseases affecting primarily the kidney. TRPP2 formed a cation conductive channel requiring interaction with the auxiliary subunit, polycystin-1 (PKD1) (Delmas et al. 2004; Hanaoka et al. 2000). The TRPP2 channel is highly versatile, being involved in EGF-mediated currents (Ma et al. 2005; Bai et al. 2008b), intracellular Ca^{2+} release (Koulen et al. 2002), and mechanodetection (Nauli et al. 2003). TRPP2

was shown to physically interact with TRPC1, and the interaction was mapped to specific residue D886 of human TRPP2 (Tsiokas et al. 1999). This residue was not required for the interaction with PKD1 (Tsiokas et al. 1999), indicating that TRPP2 binds PKD1 or TRPC1 through different residues. Notably, this very same residue was identified as the pathogenic mutation in an affected ADPKD family (Reynolds et al. 1999), suggesting that the TRPP2/TRPC1 interaction may be relevant to the pathophysiology of ADPKD. The TRPP2/TRPC1 complex in transfected cells was demonstrated using TRPC1 constructs containing the N-terminal extension and the native TRPP2/TRPC1 complex was detected in the membrane fraction of kidney lysates with both proteins being localized in the primary cilium of kidney epithelial cells (Bai et al. 2008a). Lipid bilayer reconstitution experiments and atomic force spectroscopy confirmed the formation of TRPP2/TRPC1 at the single molecule level (Zhang et al. 2009b; Kobori et al. 2009). Overall, the TRPC1/TRPP2 interaction has been independently documented biochemically and functionally at the single cell and single molecule levels; however, its determination of its biological role remains outstanding.

5.1.2 TRPC Subunits

Structurally, TRPC1 is closer to TRPC2/TRPC4/TRPC5 compared to TRPC3/6/7. It has been shown that TRPC1 formed a functional heteromultimer with TRPC3 in transfected or native HEK293 cells (Wu et al. 2004; Liu et al. 2005; Zagranichnaya et al. 2005). Strubing et al. (2001) and Goel et al. (2002) showed that TRPC1 can also form a complex with TRPC4 or TRPC5. The interactions of TRPC1 and TRPC4 or TRPC5 were shown in transfected cells and in the adult brain, whereas TRPC1/TRPC3 interactions were shown exclusively in the embryonic brain (Strubing et al. 2003). Mice lacking TRPC5 alone (Riccio et al. 2009) or TRPC1/TRPC4 together show strong brain phenotypes (Phelan et al. 2013) supporting functional interactions first described in cell culture. However, recently it was shown that formation of functional complexes between TRPC1 and TRPC4 is isoform-specific, as TRPC1β did not form a functional complex with TRPC4 (Kim et al. 2013). It remains unknown whether TRPC1 containing the N-terminal extension forms biochemical and functional complexes with other TRPC subunits.

5.1.3 TRPV6

TRPC1β lacking the N-terminal extension was shown to physically interact with TRPV6 in transfected cells (Schindl et al. 2012). The interaction was mediated by the N-terminal ankyrin repeats of the two proteins and resulted in the reduction of TRPV6-mediated currents in transfected HEK293 cells. The mechanism by which TRPC1 suppressed TRPV6-mediated currents was through the sequestration of TRPV6 inside the cell and away from the plasma membrane (Schindl et al. 2012). However, TRPC1 suppressed TRPV6 activity in *Xenopus laevis* oocytes by a mechanism different than cytosolic sequestration, as TRPV6 was detected in the plasma membrane of frog oocytes in the presence of TRPC1 (Courjaret et al. 2013). It was suggested that the mechanism of TRPC1-mediated inhibition of TRPV6 might be species-specific. Nevertheless, understanding the

mechanism(s) responsible for the negative regulation of TRPV6 is important in deciphering the molecular function of endogenous TRPV6, whose activity at the endogenous level is still elusive. Based on these results, it is conceivable that native TRPV6 is under negative regulation by TRPC1 and mechanisms that downregulate TRPC1 may represent modes of TRPV6 activation (Courjaret et al. 2013). In this regard, *Trpc1* knockout mice should be helpful in delineating endogenous TRPV6-mediated currents and cellular function(s).

5.1.4 TRPV4

In endothelial cells, TRPC1 has been shown to interact with TRPV4 in a 2:2 stoichiometry to form a channel complex activated in response to store depletion in vascular endothelial cells (Ma et al. 2010a, 2011b). Consistently, thapsigargin-induced Ca^{2+} entry was reduced in endothelial cells derived from *Trpv4* or *Trpc1* knockout mice (Ma et al. 2011a). Mechanistically, it appeared that TRPC1/TRPV4 was localized in intracellular vesicles, whose plasma membrane insertion required CRAC channel activity, a model that was previously suggested for TRPC1 (Cheng et al. 2011a). The same channel complex was found to mediate fluid flow-induced Ca^{2+} entry in human endothelial cells in cell culture (Ma et al. 2010b). Interestingly, these data raise the possibility that store-operated Ca^{2+} entry and fluid flow-induced Ca^{2+} entry may share common molecular mechanisms, at least in endothelial cells.

5.1.5 CRAC Channel (Orai and STIM1 Proteins)

TRPC1 has been shown to physically interact with both Orai1 (Galan et al. 2011; Lu et al. 2010; Ong et al. 2007; Liao et al. 2007) and STIM1 (Yuan et al. 2007; Zeng et al. 2008). Although the TRPC1/Orai1 interaction was recently shown under native conditions (Zhang et al. 2013; Lu et al. 2010), its functional role in store-operated Ca^{2+} entry is still unclear. In contrast, the TRPC1/STIM1 interaction has been shown to be required for the gating of TRPC1 by STIM1 in response to store depletion (Zeng et al. 2008).

5.2 Cell Surface Receptors

5.2.1 Metabotropic Glutamate Receptor 1

The metabotropic glutamate receptor 1 (mGluR1) is a Gq-coupled receptor present in the perisynaptic region of postsynaptic hippocampal and cerebellar Purkinje neurons. It is activated by glutamate and induces two types of conductance, one that is dependent on phospholipase C activity and another one called excitatory postsynaptic conductance (EPSC). TRPC1 has been shown to co-localize and physically interact with mGluR1 in the postsynaptic terminals of Purkinje cells (Kim et al. 2003). Functional experiments in cell culture revealed that TRPC1 was necessary and sufficient for mGluR1-induced EPSC, whereas in mice lacking TRPC1 the mGluR-mediated slow EPSC in Purkinje cells was unaffected (Hartmann et al. 2008). The mechanism of TRPC1 activation by mGluR1 is unknown. However, co-expression of TRPC1 and TRPC5 increased the size of

EPSC in CHO-K1 cells suggesting that in vivo, TRPC1 may require interaction with TRPC5 or the closely related TRPC4 to mediate EPSC. Consistently, mice lacking TRPC1, TRPC4, or both showed reduced epileptiform activity induced by activation of mGluRs in hippocampal CA1 (Phelan et al. 2013) or lateral septal neurons (Phelan et al. 2012).

5.2.2 Fibroblast Growth Factor Receptor

Fibroblast growth factor receptor (FGFR-1) belongs to the receptor tyrosine kinase superfamily of cell surface receptors. Antoniotti et al. reported the cloning and identification of TRPC1α and β isoforms in bovine endothelial cells (Antoniotti et al. 2002). It was also shown that basic fibroblast growth factor (bFGF) induced Ca^{2+} influx independently of store depletion. Block of TRPC1 using a neutralizing antibody recognizing the predicted pore-forming region of TRPC1 between transmembrane segments 5 and 6 suppressed the bFGF-induced Ca^{2+} entry suggesting the involvement of endogenous TRPC1 in the mitogenic and, thus, angiogenic effect of bFGF on these cells. Subsequently, Fiorio et al. (2005) isolated rat embryonic neural stem cells and showed similar effects. Basic FGF-induced Ca^{2+} entry or an inward cation current in these cells was required for cell proliferation. Downregulation of TRPC1 by an antisense TRPC1-specific mRNA suppressed Ca^{2+} entry, cation conductance, and proliferation. Fiorio et al. showed that endogenous FGFR-1, the receptor for bFGF in these cells, physically interacted with endogenous TRPC1. Basic FGF-induced cation conductance was independent of store depletion and OAG. These data suggested that TRPC1 had a necessary role in bFGF-induced Ca^{2+} entry in early neuroprogenitors, most likely acting in a receptor-operated fashion located in a very close proximity to FGFR-1.

5.2.3 Tumor Necrosis Factor Receptor Superfamily Member 1α

Tumor necrosis factor receptor superfamily member 1α (TNFRSF1α) or TNF-R1 belongs to the cytokine receptor superfamily of cell surface receptors and is not coupled to the Ca^{2+}/IP3 pathway. It is coupled to the JAK-STAT pathway. It has been shown that TNFα can augment GPCR-mediated Ca^{2+} signaling (Amrani et al. 1995; Tiruppathi et al. 2001) which has implications in mechanisms of bradykinin-induced inflammation. TNFRSF1α physically interacted with TRPC1/4/5, but not TRPC3/6/7 (Mace et al. 2010). Moreover, the cytosolic TNFRSF1α-interacting protein TRUSS clustered TNFRSF1α and TRPC1/4/5 channels into a multiprotein complex. Co-expression of TNFRSF1α, TRUSS, and TRPC1 augmented Gq-coupled receptor-induced Ca^{2+} entry and Ca^{2+} capacity of the intracellular stores. These data have implications in the role of inflammatory cytokines in Ca^{2+} signaling.

5.2.4 Bone Morphogenetic Protein Receptor 2

Bone morphogenetic protein receptor 2 (BMPR2) is activated by BMPs and functions as a serine/threonine kinase. It is coupled to the SMAD pathway and does not directly cause changes in the intracellular Ca^{2+} concentration. Using an unbiased proteomics approach, TRPC1 was identified as an interacting protein of

the BMPR2 through its C-terminal cytosolic tail in C2C12 myoblasts (Hassel et al. 2004). Although no functional data are available to support the BMPR2–TRPC1 interaction, they implied a possible role of TRPC1 in BMP-induced signaling.

5.3 Cytosolic Proteins

5.3.1 I-mfa

Inhibitor of myogenic family (I-mf) is the founding member of a group of related proteins with a cysteine-rich domain (Thebault and Mesnard 2001). I-mfa was first identified as an interacting protein with MyoD in a yeast two-hybrid screen. I-mf exists in three isoforms (a, b, and c) produced by alternative splicing (Chen et al. 1996), with each isoform containing a common N-terminal and a variable C-terminal region (a, b, or c) (Chen et al. 1996). All three transcripts encoded small cytoplasmic proteins lacking any predicted transmembrane segments or nuclear localization signals (Chen et al. 1996). I-mfa, the a-isoform, inhibited the transcriptional activity of MyoD and other basic helix-loop-helix (bHLH) transcription factors such as myogenin, Myf5, Mash2, and Hand1 by preventing their nuclear localization (Chen et al. 1996; Kraut et al. 1998). During development, I-mfa was detected in mouse somites and the notochord and at lower levels in the neural tube, limb buds, heart, branchial arches, and head mesenchyme. Despite its original identification as an interacting protein of myogenic factors, I-mfa mRNA was not expressed in the myotome (Chen et al. 1996), and I-mfa-deficient mice did not show muscle defects (Kraut et al. 1998; Ong et al. 2013). I-mfa protein was found in the adult kidney, liver, and lung (Ma et al. 2003). It shares remarkable identity (77 %) with another protein called HIC for *h*uman *I*-mfa domain-*c*ontaining protein (Thebault and Mesnard 2001; Thebault et al. 2000). HIC exists in two isoforms, p40 and p32, which are expressed in the spleen, thymus, prostate uterus, small intestine, and peripheral blood leukocytes (Thebault et al. 2000).

I-mfa was identified as an interacting partner of the TRPC1 through its C-terminal tail through a yeast two-hybrid screen from two independent cDNA libraries (Ma et al. 2003). Biochemical experiments confirmed the interaction in transfected cells, native cell lines, and intact rat tissues. Functional experiments showed that I-mfa overexpression suppressed native store-operated conductance in CHO-K1 cells while RNA interference (RNAi) showed that native I-mfa suppressed store-operated currents in A431 cells. These complementary gain- and loss-of-function experiments in combination with co-immunoprecipitation experiments in native tissues provided evidence for a physiological role of I-mfa to suppress store-operated currents in native systems (Ma et al. 2003). Recently, it was shown that I-mfa was induced in hematopoietic progenitors in response to macrophage colony-stimulating factor (M-CSF) and downregulated in response to receptor activator of NF-kB ligand (RANKL) in myeloid precursors (Ong et al. 2013). TRPC1α and TRPC1ε isoforms were co-expressed in these cells with I-mfa. Mice lacking I-mfa showed a marked increase in osteoclast numbers and

surface and an osteopenic phenotype, which was corrected in double mutant mice lacking both TRPC1 and I-mfa. Myeloid precursors derived from I-mfa-null mice showed enhanced store-operated currents, which were normalized in precursors derived from TRPC1/I-mfa mutant mice (Ong et al. 2013). These data were consistent with the function of I-mfa as an inhibitor of TRPC1 and altogether supported the functional and biological role of the I-mfa-mediated inhibition of TRPC1 in osteoclastogenesis.

5.3.2 Calmodulin

Two calmodulin (CaM)-binding domains have been mapped in the C-terminal cytosolic tail of TRPC1 (Singh et al. 2002). Deletion of the second domain encompassing the 35 most C-terminal residues resulted in the loss of Ca^{2+}-dependent inactivation of store-operated currents in human salivary gland cells (Singh et al. 2002). These results suggested the TRPC1 can show Ca^{2+}-dependent inactivation.

5.3.3 Caveolin

Ca^{2+} signaling complexes are clustered in specialized microdomains within the plasma membrane termed lipid raft domains (LRDs) (Isshiki and Anderson 2003; Pani and Singh 2009; Ong and Ambudkar 2011). These domains are rich in cholesterol, PIP_2, PIP_3, and sphingolipids (Simons and Toomre 2000). Caveolin 1 (Cav-1) binds cholesterol and numerous proteins accumulate in LRDs. TRPC1 was shown to bind Cav-1 through three distinct sites, one in the N-terminus and two in the C-terminus (Brazer et al. 2003; Lockwich et al. 2000; Kwiatek et al. 2006). Binding of TRPC1 to Cav-1 was proposed to recruit TRPC1 to LRDs, where it could form signaling complexes with the PMCA pump, Homer, and other proteins (Ong and Ambudkar 2011). The presence of TRPC1 in LRDs has been documented in salivary gland cells (Brazer et al. 2003; Lockwich et al. 2000), skeletal muscle cells (Formigli et al. 2009; Sabourin et al. 2009a), blood cells (Kannan et al. 2007; Brownlow and Sage 2005; Berthier et al. 2004), endothelial cells (Murata et al. 2007), and sperm (Trevino et al. 2001). Consistent with a function as a protein regulating store-operating Ca^{2+} entry, disruption of LRDs by pharmacological means attenuated store-operated Ca^{2+} entry (Pani et al. 2008).

5.3.4 Homer Proteins

Homer proteins are cytosolic proteins that have the ability to recognize and bind to proline-rich domains in a diverse group of proteins involved in signal transduction, such as the group 1 metabotropic glutamate receptors, inositol trisphosphate receptors (IP_3Rs), ryanodine receptors, and others. In addition to the target recognition site, Homer proteins have the ability to homomultimerize through a coiled-coil domain. Therefore, Homer proteins organize channels and receptors into multiprotein complexes facilitating signal specificity and efficiency. Yuan et al. identified a proline-rich domain in the C-terminal tail of TRPC1 and showed that Homer 3 interacted with TRPC1 (Yuan et al. 2003). The TRPC1–Homer interaction seems to involve two binding sites in TRPC1, one in its C-terminus

and another one in its N-terminus. Given the ability of Homer 3 to homomultimerize, it has been proposed that Homer 3 brings together the N- and C-termini of TRPC1 preventing constitutive activation. Disruption of the TRPC1/Homer interaction resulted in constitutive activation of TRPC1, suggesting that the interaction may mediate the gating of TRPC1 in response to store depletion. The functional role of the TRPC1/Homer 3 was studied in the context of the store-operated mode of TRPC1 through IP3Rs. However, the gating of TRPC1 by IP3R seems unlikely in all cell types. Therefore, whether the TRPC1/Homer 3 interaction is functionally relevant to the store-operated mode of TRPC1 is unknown, but it is possible to regulate either other modes of TRPC1 activation or the store-operated mode of TRPC1 through components of the CRAC channel (i.e., STIM and Orai proteins). The later possibility is supported by experiments in mice lacking TRPC1 (Zanou et al. 2010), STIM1 (Stiber et al. 2008a; Li et al. 2012), or Homer 1 (Stiber et al. 2008b), which all show defects in skeletal muscle function.

5.3.5 Immunophilins

Immunophilins comprise a group of cytosolic enzymes that catalyze the *cis*/*trans* isomerization of prolyl-peptide bonds. Their peptidyl-prolyl isomerase (PPlase) activity is blocked by binding to FK506, and hence, they are called FK506-binding proteins or FKBPs. One of their physiological roles is to facilitate protein folding. Similarly to Homer proteins, FKBP52 was shown to bind TRPC1 (Goel et al. 2001; Sinkins et al. 2004) and cross-link its N- and C-termini (Shim et al. 2009). The FKBP52 binding sites overlapped with these of Homer 3, but distinct residues mediated interactions between TRPC1 and Homer3 and TRPC1 and FKBP52. Co-expression of TRPC1 and FKBP2 led to increased activation of TRPC1 by a Gq-protein-coupled receptor agonist. A mutant form of FKBP52 that lacked PPlase activity was unable to enhance TRPC1 activity. An array of biochemical, biophysical, and mutagenesis experiments showed that FKBP12 isomerization was required for TRPC1 gating in response to receptor activation. One of the biological roles of the FKBP-mediated regulation of TRPC1 was the modulation of the chemoattraction of growth cones of spinal or hippocampal neurons toward a Netrin gradient (Shim et al. 2005; Wang and Poo 2005; Gomez 2005; Song and Poo 1999). Netrin acts through its cognate receptor, deleted in colorectal cancer (DCC), and involves a Ca^{2+} signaling pathway that depends on PLC-γ, PI3K, MAPK, and Rho GTPases (Song and Poo 1999). FKBP52 activity is essential in the Netrin-induced activation of TRPC1 in mediating chemoattraction (Shim et al. 2009). Overall, FKBPs function downstream of DCC activation by Netrin and upstream of TRPC1 in chemotropic guidance of neuronal growth cones.

A comprehensive list of TRPC1 interacting proteins can be found in http://trpchannel.org/summaries/TRPC1.

6 Channel Function, Permeation, and Gating

6.1 Activation by Store Depletion

6.1.1 Function

There is strong evidence in support of a role of TRPC1 in the pathways of store-operated Ca^{2+} entry. TRPC1 has been directly linked to I_{CRAC} (Mori et al. 2002; Ong et al. 2013), whereas there are numerous reports showing the requirement of TRPC1 in I_{SOC} in transfected or native cells (Zhu et al. 1996; Cheng et al. 2011a; Worley et al. 2007; Yuan et al. 2003, 2007; Huang et al. 2006; Shi et al. 2012; Zeng et al. 2008; Ong et al. 2013). However, it remains unknown whether the CRAC and SOC channels are different entities or the SOC channel is a modified form of the CRAC channel containing TRPC1. Current models support both possibilities (Fig. 3).

Model 1 (Fig. 3): This model was proposed by Chung et al. (2011a), and it is based on data showing that Orai1 forms a TRPC1-independent assembly in the plasma membrane in resting conditions. TRPC1 under these conditions is localized in intracellular vesicles. Upon store depletion, STIM1 binds, clusters, and activates Orai1 allowing Ca^{2+} entry into the cell through the CRAC channel. This local increase in Ca^{2+} induces the translocation of TRPC1 to the plasma membrane where it can be gated by STIM1 to form an independent channel complex allowing Na^{+} and Ca^{2+} into the cell. TRPC1 is induced to open by STIM1 through electrostatic interactions between residues in the C-terminus of TRPC1 and the polybasic domain of STIM1. The domain in STIM1 that gates TRPC1 is distinct from the CAD/SOAR domain required to activate Orai1. This model explains the dependence of TRPC1 on Orai1 and STIM1; does not require assembly of a heteromultimeric channel of Orai1/TRPC1; supports the formation of a functional ionic pore by TRPC1, which is consistent with data showing that pore mutants of TRPC1 are not functional; and can be consistent with data showing that TRPC1/TRPV4 heteromultimers are dependent on store depletion. However, it is not consistent with data showing spontaneous activity of TRPC1, i.e., TRPC1 mutants lacking binding to Homer proteins or in cells where TRPC1 is co-expressed with FKBP12. If TRPC1 requires STIM1/Orai1 activity in order to be inserted into the plasma membrane, then TRPC1 mutants lacking binding to Homer proteins or overexpression of FKBP12 should not make active channels in the membrane, as they would be trapped inside the cell in intracellular vesicles. It is also unknown whether properly translated TRPC1, containing the N-terminal extension, is induced to traffic to the plasma membrane in a manner similar to truncated TRPC1 versions lacking the N-terminal extension.

Model 2 (Fig. 3): Ong et al. (2013) showed that a deletion of seven amino acids generated by alternative splicing in TRPC1 can profoundly change the biophysical/biochemical properties of TRPC1 and by extrapolation, the CRAC channel. These residues are located within the N-terminus of TRPC1, which has not been implicated in the regulation of the cell surface expression of TRPC1. Consistently, surface biotinylation experiments showed that indeed TRPC1α and TRPC1ε were

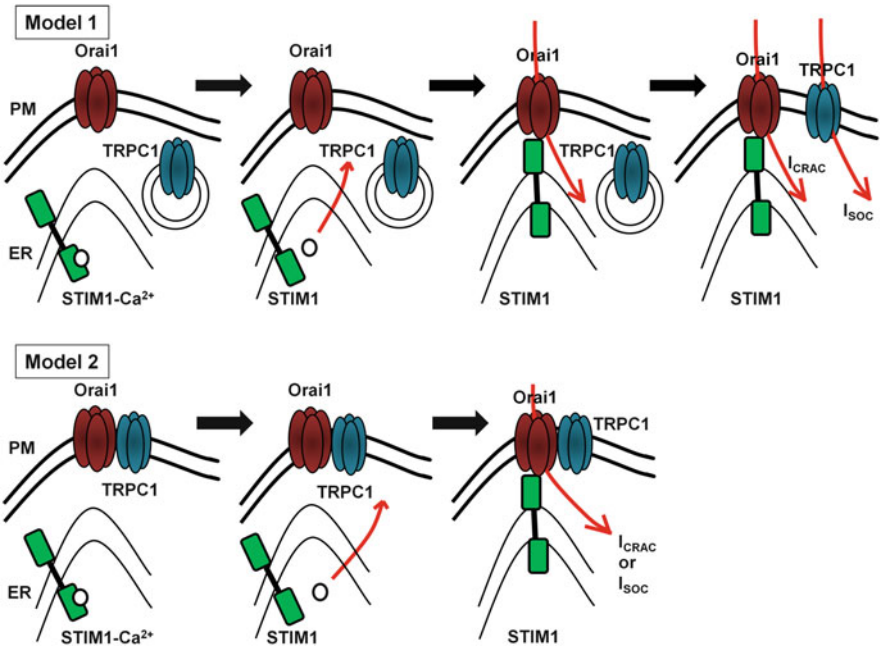

Fig. 3 Hypothetical models showing the contribution of TRPC1 to store-operated Ca^{2+} entry. In model 1, in resting conditions, TRPC1 (blue) resides in intracellular vesicles, whereas Orai1 (*burgundy*) and STIM1 (*green*) reside in the plasma membrane (PM) or membrane of the endoplasmic reticulum (ER), respectively. Upon store depletion, STIM1 captures and activates Orai1 at punctae. Local Ca^{2+} entry mediated by Orai1 induces the insertion of TRPC1 into the PM. TRPC1 inserted in the PM is gated (activated) through a physical interaction with STIM1 (not shown) to mediate I_{SOC}. In this model, I_{CRAC} and I_{SOC} occur independently. In model 2, in resting conditions, TRPC1 can physically interact with Orai1. TRPC1 can be located in the PM, membrane of the ER, and/or other intracellular sites and interact with Orai1 through their cytosolic segments. Upon store depletion, TRPC1 modifies Orai1-mediated I_{CRAC} by interfering with the gating and activation of Orai1 by STIM1. Depending on the TRPC1 isoform present in the cell and the way that each isoform interacts with Orai1 and/or STIM1, TRPC1 can augment I_{CRAC} (TRPC1ε) or modify I_{CRAC} to generate I_{SOC} (TRPC1α or TRPC1β)

present at equal amounts in the plasma membrane (Ong et al. 2013). Further store depletion did not cause a significant increase in the expression of TRPC1α in the plasma membrane. Thus, it was proposed that TRPC1, which can be localized either at the plasma membrane, ER membrane, and/or any other location in close proximity to Orai1, could modify the function of STIM1/Orai1 through physical interactions involving soluble (cytosolic) domains of TRPC1 and STIM1 or Orai1. Since it is known that STIM1 can regulate not only gating but also selectivity of Orai1 (Scrimgeour et al. 2009; McNally et al. 2012) and homomultimerization of Orai1 can regulate its own selectivity (Thompson and Shuttleworth 2013), protein–protein interactions between the N-terminal extension of TRPC1α/TRPC1ε isoforms and Orai1 could indirectly influence the selectivity of the Orai1 channel.

This model is consistent with the idea that the SOC channel is a modified version of the CRAC channel and is supported by biochemical experiments showing interactions between Orai1 and TRPC1 in transfected and native cells.

Despite the gap in our knowledge of the exact molecular mechanism by which TRPC1 contributes to store-operated Ca^{2+} entry, Ong et al. suggested several possibilities that could explain the presence of I_{CRAC} and/or I_{SOC} in any given cell type (Ong et al. 2013). In cells expressing STIM and Orai proteins, the predominant store-operated current should be I_{CRAC}. However, in cells expressing STIM/Orai and TRPC1, there should be a mix of I_{CRAC} and I_{SOC}. The contribution of I_{SOC} to whole cell store-operated current can be further determined by proteins that inhibit TRPC1, such as I-mfa and/or the presence of TRPC1ε, and possibly other isoforms with similar effects on the CRAC channel.

6.1.2 Gating

Mutagenesis experiments have shown that store-operated TRPC1 is gated through electrostatic interactions with STIM1 (Zeng et al. 2008). However, additional models have been proposed. One model involves the dynamic interaction of TRPC1 and IP3Rs (Yuan et al. 2003). In the closed state, the N- and C-termini of TRPC1 are held together through physical interactions with Homer proteins. Upon store depletion a dynamic interaction between TRPC1 and the IP3Rs results in the dissociation of the two termini of TRPC1 leading to channel activation. Another mode of TRPC1 gating in response to store depletion is through protein kinase Cα (Albert 2011). In human glomerular mesangial cells and vascular smooth muscle cells, store-operated Ca^{2+} entry channels were shown to be activated by store-independent means such as EGF, vasoconstrictors, and phorbol esters. These channels were inhibited by PKC inhibitors and were found to be composed of TRPC1/TRPC4 heteromultimers. TRPC1 was also found to be activated by PIP_2 in portal vein and coronary artery vascular smooth muscle cells (Albert 2011). However, this type of regulation might be an indirect effect of PIP_2 on STIM1, since STIM1 depends on PIP_2 for anchoring to the plasma membrane.

6.2 Activation by Receptor Stimulation

6.2.1 Function

Association of TRPC1 with other TRP channel subunits has been shown to result in the formation of receptor-operated channel complexes. These channel complexes show distinct biophysical properties from individual channels, and some of them have been documented in native cells and tissues.

Association with TRPP2 was shown to result in a channel complex with a unique properties compared to the properties of individual TRPP2 or TRPC1 (Bai et al. 2008a). The TRPP2/TRPC1 channel was shown to be activated in response to GPCRs and displayed single channel conductance of 40 pS which was different from the single channel conductance of TRPC1 expressed alone (16 pS), amiloride sensitivity, and ion permeability different from these of TRPP2 or TRPC1 alone.

Native TRPP2/TRPC1 activity was identified in kidney cells by complementary gain- and loss-of-function experiments. Given that TRPP2 was shown to be activated by the depletion of PIP_2 (Ma et al. 2005) and TRPP2/TRPC1 activity was suppressed by PLC inhibition, it is likely that activation of TRPP2/TRPC1 is also mediated by PIP_2 depletion in response to activation of a Gq-coupled receptor.

Association of TRPC1 with TRPC4 or TRPC5 was shown to profoundly change the activity of TRPC4 or TRPC5 in a voltage-dependent manner (Strubing et al. 2001). Specifically, overexpression of constructs lacking the N-terminal extension of TRPC1α yielded that TRPC1α alone was unable to form a store- or a receptor-activated channel but when co-expressed with TRPC4 or TRPC5 suppressed inward currents and augmented outward currents mediated by TRPC5 alone activated by receptors coupled to Gq proteins. These dramatic changes in the biophysical properties of TRPC4 or TRPC5 by TRPC1, along with the ability of these proteins to physically interact, suggested that TRPC1 may form a heteromultimeric channel complex with TRPC4 or TRPC5, which is most likely to be physiologically relevant in excitable cells and tissues such as neurons.

Formation of functional complexes containing TRPC1 and TRPC3/6/7 has also been shown. Overexpression of human TRPC1α lacking the N-terminal extension led to Na^+-permeable channel activated by OAG (Lintschinger et al. 2000). This conductance was blocked in the presence of extracellular Ca^{2+}. Overexpression of TRPC3 showed some permeability to Ca^{2+} and was also activated by OAG. Co-expression of TRPC1 and TRPC3 induced a conductance with reduced permeability to receptor-activated Ca^{2+} entry and suppressed OAG-activated currents. These data suggested that TRPC1/TRPC3 complex is sensitive to phospholipase C activity and extracellular Ca^{2+} (Lintschinger et al. 2000). Storch et al. (2012) followed a similar approach and found that TRPC1 could not form a functional channel by itself, but it did so when it was co-expressed with all other members of the TRPC subfamily. In agreement with Lintschinger et al. (2000), they showed that transfection of TRPC1 reduced the Ca^{2+} permeability of other TRPC members suggesting the formation of heteromultimers. In gonadotropin-releasing neurons that express endogenously TRPC1, 2, 5, and 6, TRPC1 knockdown reduced calcium permeability of endogenous channels activated by carbachol acting through Gq-coupled receptors (Storch et al. 2012) and cell migration, suggesting that TRPC1 suppresses Ca^{2+} permeability of heteromultimeric channels involved in neuronal migration. In freshly isolated smooth muscle cells, TRPC1 functioned together with TRPC3 (Chen et al. 2009). This channel complex was activated by UTP, an activator of endogenous Gq-coupled receptor, and inhibited by protein kinase G (PKG). The PKG-mediated inhibition of the TRPC1/TRPC3 complex was critical for the nitric oxide-mediated vasorelaxation in these cells. TRPC1 interacted with TRPC3, but not TRPC4 in human parotid gland ductal cells (Liu et al. 2005). The TRPC1/TRPC3 complex was activated by store depletion and OAG.

6.2.2 Gating

Although there is strong evidence in support of the formation of functional complexes containing TRPC1 that respond to cell surface receptor stimulation, a unifying theory of the mechanism by which a second messenger generated in response to cell surface receptor activation can open TRPC1 is lacking. One possibility is that a PKCα-mediated phosphorylation of TRPC1 could gate receptor-operated TRPC1 similarly to store-operated TRPC1 (Albert 2011). Another possibility is through the hydrolysis of PIP_2 and/or concomitant generation of DAG. Finally, gating can occur through the action of immunophilins as was shown for FKBP52 and the Netrin/DCC receptor system.

6.3 Activation by Mechanical Forces

6.3.1 Function

In addition to the roles of TRPC1 in store- and receptor-operated Ca^{2+} entry pathways, TRPC1 was shown to mediate native stretch-activated currents in *X. laevis* oocytes and also in TRPC1-transfected CHO-K1 cells (Maroto et al. 2005; Barritt and Rychkov 2005), whereas TRPC1 was not functional in CHO-K1 cells in another study (Gottlieb et al. 2008). TRPC1 was found to mediate mechanosensitive currents in neuronal cells (Kerstein et al. 2013), migrating Madin–Darby canine kidney (MDCK) cells (Fabian et al. 2008, 2012), mouse brain microvessel endothelial cells (Berrout et al. 2012), and primary afferent sensory neurons (Garrison et al. 2012).

6.3.2 Gating

The activation mechanism of mechanosensitive TRPC1 is unknown.

6.4 Permeation-Single Channel Conductance

The biophysical properties of TRPC1 such as permeation and single channel conductance are difficult to be determined unequivocally because of the numerous protein–protein interactions of TRPC1 with other channel subunits, modes of activation, and the lack of a specific TRPC1 inhibitor. Cation substitution experiments using heterologously expressed TRPC1 in CHO-K1 cells showed similar permeability to Na^+ and Ca^{2+} and a single channel conductance of 16 pS (Zitt et al. 1996). Further support for the formation of a functional ionic pore passing Ca^{2+} was obtained by mutagenesis experiments whereby Ca^{2+}, but not Na^+, permeability was reduced by neutralizing negatively charged residues in the extracellular domain connecting transmembrane segments 5 and 6 (Liu et al. 2003) (Fig. 2). These and other results mainly in TRPV channels assisted in the theoretical modeling of the architecture of the putative pore-forming region of TRPC1 (Owsianik et al. 2006). Purified TRPC1 reconstituted in lipid bilayers supported formation of a functional cation nonselective pore with unitary conductance of

22 pS (Zhang et al. 2009b). Outside-out patches of cells transfected with mouse TRPC1 revealed a single channel conductance of 16 pS or 40 pS in association with TRPP2 activated in response to bradykinin, a Gq-coupled receptor agonist (Bai et al. 2008a). Other preparations including inside-out patches showed unitary conductance of 2 pS (Albert 2011). Mechanosensitive TRPC1 showed a nonlinear I–V curve with a unitary conductance of 50 pS at negative potentials (Maroto et al. 2005). In regard to ion selectivity, the vast majority of studies are consistent with the fact that TRPC1 alone or in combination with other TRP subunits makes a cation nonselective channel. However, there are two exceptions: TRPC1ε co-expressed with STIM1 and Orai1 formed a Ca^{2+}-selective channel (Ong et al. 2013), and deletion of TRPC1 in DT40 cells suppressed the highly Ca^{2+}-selective I_{CRAC} (Mori et al. 2002). Overall, when TRPC1 is successfully reconstituted in cells or cell-free systems, it forms a cation nonselective or a Ca^{2+}-selective channel of a single channel conductance between 2 and 50 pS, depending on preparation, interacting proteins, and cell context.

7 Physiological Functions in Native Cells, Organs, and Organ Systems

7.1 Cardiovascular System

Through its role as a store-, receptor-, and mechanically activated channel, TRPC1 has been shown to affect basic and cell type-specific functions of the major cell types of the cardiovascular system, such as cardiomyocytes, vascular smooth muscle cells, endothelial cells, and platelets. Cardiomyocytes respond to pressure or mechanical overload by increasing cell size, a phenomenon called hypertrophy. Cardiomyocytes derived from mouse hearts subjected to hemodynamic stress showed an upregulation of TRPC1 and the appearance of a cation nonselective whole cell current, which was eliminated in cells derived from *Trpc1*$^{-/-}$ mice. Consistently, cardiac hypertrophy in response to hemodynamic stress was not seen in these mice (Seth et al. 2009). Using a different approach, Wu et al. (2010) showed that cardiac-specific expression of dominant negative constructs of TRPC3, TRPC6, or TRPC4, which should functionally abrogate complexes containing TRPC1, showed reduced hypertrophic response induced by pressure overload or neuroendocrine agonists. At the cellular level, deletion of TRPC1 or overexpression of dominant negative alleles of TRPC3, TRPC6, or TRPC4 resulted in reduced activity of the NFAT transcription factor, which is linked to Ca^{2+} signaling and cardiac hypertrophy, suggesting that TRPC1-mediated activation of NFAT is required for cardiac hypertrophy.

Several reports show that TRPC1 in various types of vascular smooth muscle cells mediates vasoconstriction through its effect on store-operated Ca^{2+} entry. Bergdahl et al. showed that the potent vasoconstrictor, endothelin-1, activated store-operated Ca^{2+} entry in endothelium-denuded caudal arteries, which was inhibited by cholesterol or a TRPC1-specific antibody (Bergdahl et al. 2003,

2005). Conversely, overexpression of TRPC1 in rat pulmonary artery enhanced vasoconstriction (Kunichika et al. 2004). Despite these acute effects of the manipulation of TRPC1 function or expression on vascular smooth muscle cells, aortic smooth muscle cells derived from *Trpc1*-null mice did not show differences in vasoconstriction and store-operated Ca^{2+} entry (Dietrich et al. 2007, 2010). The reasons for these discrepancies are unknown. In addition to its possible effect on vasoconstriction, TRPC1 has been shown to be involved in neointimal hyperplasia, a condition characterized by the switching of VSMCs from a differentiated and non-proliferative phenotype to an invasive and proliferative phenotype. Phenotypic switching of VSMCs caused an upregulation of TRPC1 levels accompanied with enhanced Ca^{2+} entry and proliferation (Kumar et al. 2006). All these effects were blocked by a TRPC1-specific antibody raised against the extracellular domain of TRPC1 forming the ionic pore (Kumar et al. 2006), suggesting that in VSMCs TRPC1 can contribute to cell proliferation and phenotypic switching.

Endothelial cells form a thin layer of cells connecting the vasculature with the blood stream. TNF-α was shown to induce expression of TRPC1 through a mechanism involving NF-κB, enhanced store-operated Ca^{2+} entry, and endothelial permeability (Paria et al. 2003, 2004, 2006). Using endothelial cells derived from single *Trpc1*-null mice, or *Trpc1/Trpc4* double knockout mice, TRPC1 was found to control endothelial permeability through an interaction with TRPV4 (Ma et al. 2011a) or TRPC4 and STIM1 (Sundivakkam et al. 2012). A role of zebrafish TRPC1 in endothelial sprouting has been shown. TRPC1 was found to be an essential component of VEGF signaling in angiogenesis in developing zebrafish. Specifically, TRPC1 acted downstream of VEGF in inducing angiogenic sprouting of intrasegmental vessels in an endothelial cell-autonomous manner (Yu et al. 2010). TRPC1 was required for filopodia extension, migration, and proliferation in zebrafish endothelial cells.

TRPC1 is expressed in platelets and megakaryocytes (Hassock et al. 2002; Berg et al. 1997), but its role in these cells, and in particular in platelets, is controversial (Dietrich et al. 2010). While it has been shown that it participated in store-operated Ca^{2+} entry pathways affecting platelet activation and thrombus formation (Rosado and Sage 2001; Lopez et al. 2013; Jardin et al. 2012; Rosado et al. 2002), studies using platelets derived from the *Trpc1* knockout mice could not show an effect on either store-operated Ca^{2+} entry or platelet function, such as activation, aggregation, hemostasis, and thrombosis (Varga-Szabo et al. 2008).

7.2 Skeletal Muscle

TRPC1 is expressed in myoblasts and myotubes (Sabourin et al. 2009a, b) and participates in a program controlling myoblast differentiation, migration, and regeneration (Louis et al. 2008; Zanou et al. 2012). Muscle differentiation and regeneration was delayed in $Trpc1^{-/-}$ muscles subjected to cardiotoxin-induced injury accompanied with a reduction in phosphatidylinositol 3 kinase (PI3K) activity. The TRPC1-mediated effect on PI3K required extracellular Ca^{2+} (Zanou

et al. 2012). Myoblast migration precedes myoblast fusion to form multinucleated myotubes. TRPC1 activity was shown to be required for myoblast migration and fusion. This effect was mediated through the activation of Ca^{2+}-sensitive enzymes, m- or mu-calpains, which in turn cleaved myristoylated alanine-rich C-kinase substrate (MARCKS), an actin-binding protein (Louis et al. 2008). In addition to the identification of a role of TRPC1 in myoblast migration and fusion, these experiments identified calpains as downstream effectors of TRPC1. The role of TRPC1 in myoblast fusion is interesting as it may not be restricted to myoblasts but also to osteoclasts. In support of this idea, TRPC1 was required in osteoclast formation (Ong et al. 2013), which also proceeds through cell migration and fusion. The exact Ca^{2+} pathway, by which TRPC1 affects myoblast differentiation, is not clear, as there is evidence supporting its role as both a store-dependent (Louis et al. 2008; Sabourin et al. 2009b) and store-independent channel (Zanou et al. 2010).

7.3 Neurons

TRPC1 is expressed in hippocampal and spinal neurons and mediates chemoattraction or repulsion of developing growth cones toward a Netrin or myelin-associated glycoprotein (MAG) gradient, respectively (Shim et al. 2005, 2009; Wang and Poo 2005). This pathway seems to involve the function of immunophilins. However, the possibility that TRPC1 can suppress growth cone turning of *X. laevis* spinal neurons through its role as a mechanosensitive channel has also been proposed (Kerstein et al. 2013). In this case, TRPC1 functions in a mechanosensitive pathway, whereby it locally activates calpain to cleave talin leading to a reduction of Src-dependent axonal outgrowth.

7.4 Salivary Gland Cells

The majority of the studies supporting the function of TRPC1 as a store-operated Ca^{2+} entry channel have been done in salivary gland cells (Cheng et al. 2011b). In these cells, STIM1-stimulated TRPC1 mediates a cation nonselective current needed for Ca^{2+}-induced activation of K^+ channels leading to salivary fluid secretion (Cheng et al. 2011a). These data are supported by experiments in *Trpc1*-null mice where agonist-induced store-operated entry channels and salivary gland fluid secretion are both suppressed in salivary gland cells derived from these mice (Liu et al. 2007). Further, naturally occurring mutations in STIM1 in humans lead to Sjogren's syndrome, an autoimmune disease characterized by exocrine gland defects (Cheng et al. 2012).

7.5 Bone

There is evidence that TRPC1 influences the differentiation of the two major cell types of long bones, the osteoblasts and osteoclasts. The role of TRPC1 in chondrogenesis is unknown. *Trpc1* mRNA was detected in several human osteoblastic cell lines such as MG-63, SaOS, and U2 OS (Abed et al. 2009). Knockdown of TRPC1 in MG-63 by RNAi showed a reduction in store-operated Ca^{2+} entry and platelet-derived growth factor BB (PDGFB)-induced proliferation (Abed et al. 2009). Suzuki et al. showed expression of TRPC1/3/4/6 in human SaM-1 osteoblastic cells, and treatment with 2-APB, Gd^{3+}, and La^{3+} inhibited bradykinin-induced Ca^{2+} signaling in these cells (Suzuki et al. 2011). Ong et al. examined osteoblast numbers and surface in $Trpc1^{-/-}$ long bones, and although there was a significant reduction in both of these parameters, dynamic bone formation and bone architecture was unchanged compared to control mice (Ong et al. 2013). These data suggest that reduction of mature osteoblast numbers in $Trpc1^{-/-}$ mice may be accompanied by increased and/or accelerated differentiation in vivo. In sum, while there are several reports demonstrating expression of mainly *Trpc1* mRNAs in osteoblasts, not much is known regarding its role in osteoblastic differentiation.

Osteoclasts are the bone-resorbing cells derived from hematopoietic stem cells residing in the bone marrow. They are constantly made throughout life through a series of complex events depending on extracellular stimuli and the microenvironment. The first step in the differentiation process of hematopoietic stem cells to osteoclasts is the formation of myeloid precursors in response to M-CSF, a receptor tyrosine kinase (Hamilton 2008). Myeloid precursors differentiate into early osteoclasts in response to RANKL acting through its cognate receptor, RANK, a typical receptor of the TNFR subfamily of cell surface receptors. In the continuing presence of RANKL, mononucleated pre-osteoclasts migrate toward each other and fuse to form large multinucleated or mature osteoclasts that efficiently resorb bone. One of the early events in RANKL-mediated signaling is Ca^{2+} oscillations that precede the massive induction of NFTAc1, among other Ca^{2+}-sensitive transcription factors to mediate the conversion of myeloid precursors to pre-osteoclasts (Negishi-Koga and Takayanagi 2009). Store-operated Ca^{2+} entry has been suggested to have an early role of this process to maintain Ca^{2+} oscillations rendering the cell competent for NFATc1 activation (Negishi-Koga and Takayanagi 2009; Takayanagi et al. 2002). Consistently deletion of *Orai1* in mice or Orai1 depletion in cell lines led to inhibition of osteoclast differentiation (Robinson et al. 2012; Hwang and Putney 2012). Ong et al. showed that hematopoietic stem cells express TRPC1α and TRPC1ε in response to M-CSF (Ong et al. 2013). In addition, M-SCF induced the expression of I-mfa, an inhibitor of TRPC1 as a store-operated Ca^{2+} channel. Mice lacking I-mfa had a severe osteopenic phenotype characterized by high numbers of mature osteoclasts and erosion surface. However, this defect was completely corrected in mice lacking TRPC1 and I-mfa suggesting that I-mfa controlled osteoclastogenesis by keeping TRPC1 in check (Ong et al. 2013). Myeloid precursors derived from TRPC1 knockout mice had no difference in osteoclast differentiation and store-operated

currents consistent with the idea that TRPC1 was inhibited by I-mfa. However, in myeloid precursors lacking I-mfa, osteoclastogenesis was enhanced along with the magnitude of store-operated currents. These currents returned to normal levels in cells lacking both TRPC1 and I-mfa. These data suggested that TRPC1 enhanced, whereas I-mfa suppressed, store-operated currents increasing the dynamic range of the CRAC channel under physiological conditions.

8 Lessons from Knockout Mice

Trpc1 knockout mice are fertile and live to adulthood (Dietrich et al. 2010), suggesting that the *Trpc1* gene is not absolutely essential for life in mice. However, examination of several organs and tissues, guided by experiments in cell culture, has revealed pathologies of the exocrine, cardiovascular, skeletal and muscular, and central nervous systems. Some of these defects are consistent with results from experiments done in cell culture, while some are at variance with in vitro studies. Characterization of *Trpc1* knockout mice has also led to the identification of unanticipated roles of TRPC1 in osteoclasts, retina, and the immune system.

As discussed above, experiments in cell culture showed that TRPC1 could function as a store-operated Ca^{2+} entry channel. Given the role of store-operated Ca^{2+} entry channels in exocytosis and secretion (Parekh and Putney 2005), *Trpc1* knockout mice were examined for defects in exocrine glands (Liu et al. 2007). *Trpc1*-null mice show reduced secretion of salivary fluid, a function that is consistent with its role as a store-operated channel (Liu et al. 2007). These findings are supported by the identification of loss-of-function mutations in *STIM1*, associated with Sjogren's syndrome (Cheng et al. 2012), characterized by reduced salivary gland secretion. Guided by the potential role of TRPC1 as a receptor- and/or mechanically activated channel, a possible role of TRPC1 in cardiac hypertrophy was examined (Seth et al. 2009). *Trpc1* knockout mice show a reduced response to hemodynamic stress in terms of developing cardiac hypertrophy (Seth et al. 2009). Vascular smooth muscle cells derived from the thoracic aorta of *Trpc1*-null mice showed increased vasoconstriction in response to phenylephrine (Kochukov et al. 2013). Mice lacking *Trpc4* gene showed reduced store-operated Ca^{2+} entry and increased endothelial permeability, and TRPC1 was shown to associate with TRPC4 in these cells (Sundivakkam et al. 2012). Heterologous co-expression of TRPC1 and TRPC4 in HEK293 cells resulted in a channel complex with strong outward rectification and very little activity at resting negative potentials, implying a potential role of the TRPC1/TRPC4 channel in excitable cells, such as neurons (Strubing et al. 2001). Consistently, TRPC1/TRPC4 or TRPC5 complexes were identified in adult and embryonic brain (Strubing et al. 2003). Further, TRPC1 associated with the group 1 mGluR1 and mediated excitatory postsynaptic current in hippocampal neurons. All these data indicated a potential role of TRPC1 alone or in association with other TRPC subunits in the central nervous system. In agreement with this idea, compound mice lacking *Trpc1* and *Trpc*4 lacked epileptiform

bursting secondary to metabotropic glutamate receptor activation in hippocampal CA1 neurons (Phelan et al. 2013) or suppressed by 74 % in lateral septal neurons in single *Trpc1* mutant mice (Phelan et al. 2012). Similar effects were seen in olfactory granule cells (Stroh et al. 2012). Following previous cell culture work (Ma et al. 2003), Ong et al. showed that TRPC1-enhanced store-operated currents in myeloid precursors and mice lacking *Trpc1* showed a modest decrease in osteoclastogenesis and an increase in bone mass (Ong et al. 2013). Overall, studies using *Trpc1* knockout mice and cells derived from these mice have provided support for the hypothesis that TRPC1 can augment store-operated channel in salivary glands, myeloid precursors, and endothelial cells. Experiments in hippocampal, septal neurons, and possibly cardiomyocytes support a role of TRPC1 as a receptor-operated channel. Since cardiac hypertrophy can be induced by mechanical overload, it is conceivable that TRPC1 can also modulate mechanosensitive currents in these cells.

In contrast to studies providing support for TRPC1 as a store-operated channel in the above-mentioned cell types, there are studies showing the lack of an effect of TRPC1 on store-operated Ca^{2+} entry in vascular smooth muscle cells, platelets, and rod cells. Specifically, *Trpc1*-null vascular smooth cells did not show differences in their response to various vasoconstrictors and their store-operated currents (Dietrich et al. 2007). Platelet activation, aggregation, or thrombus formation and store-operated Ca^{2+} entry were also indistinguishable between cells derived from wild-type mice and *Trpc1*-null mice (Dietrich et al. 2010; Varga-Szabo et al. 2008). In mouse retina, TRPC1 was expressed in rods and cones and rod store-operated Ca^{2+} entry was unaffected in *Trpc1*-null mice, indicating that TRPC1 does not regulate light-evoked responses at the mouse rod output synapse (Molnar et al. 2012). However, downregulation of TRPC1 by RNAi did suppress store-operated Ca^{2+} entry in rods (Szikra et al. 2008). The reasons for these discrepancies are unknown but could stem from inherent differences between acute (RNAi in vitro) and chronic (knockout in vivo) gene ablation. Alternatively, endogenous TRPC1 inhibitors, such as I-mfa, Homer proteins, and possibly others, can complicate data obtained from single knockout mice. In this regard, experiments in cell culture using RNAi demonstrated a role of TRPC1 in skeletal muscle differentiation through an effect on store-operated Ca^{2+} entry channel (Louis et al. 2008). Experiments using adult muscles from *Trpc1* knockout mice revealed similar defects, but could not demonstrate an effect on store-operated Ca^{2+} entry pathways (Zanou et al. 2010). One of the explanations for the apparent discrepancy was that store-operated TRPC1 was most likely inhibited by I-mfa in adult muscles, whereas this inhibition could have been lifted in myoblasts through dynamic interactions among TRPC1, I-mfa, and myogenic factors. As discussed above, a similar concept was shown in pre-osteoclasts, where TRPC1 remained inhibited by I-mfa and, thus, did not contribute to store-operated Ca^{2+} entry pathways in these cells (Ong et al. 2013). However, when I-mfa was deleted, TRPC1 had a large effect on store-operated currents in these cells. A similar type of regulation could be possible with the TRPC1/Homer interaction(s), as Homer 1a can function as a dominant negative allele and it is an inducible immediate early gene (Yuan et al. 2003). The

identification of endogenous proteins functioning as TRPC1 inhibitors raises interesting questions about their physiological significance. One possibility is that they generate a pool of TRPC1 that can become available under certain circumstances. For example, I-mfa has been shown to be induced by M-CSF (Ong et al. 2013) and vitamin D3 (Tsuji et al. 2001), whereas suppressed by RANKL/RANK in myeloid precursors (Ong et al. 2013). Therefore, the contribution of TRPC1, at least to store-operated Ca^{2+} entry, appears to be regulated. The effect of I-mfa on receptor- or mechanically activated TRPC1 is currently unknown. The realization that TRPC1 channel remains inhibited under physiological conditions suggests that TRPC1 channel function can be best revealed under conditions that induce some sort of stress (hemodynamic or pressure overload), mechanisms of defense (infection-induced M-CSF production), etc. Current *Trpc1* knockout animal models point to this direction.

A role of TRPC1 in the immune system was recently shown. *Trpc1* deletion resulted in reduced numbers of T helper type 2 (Th2) cells in response to allergen (methacholine) challenge in vivo. In vitro, $Trpc1^{-/-}$ splenocytes showed reduced T cell proliferation and T cell receptor-induced IL-2 production. Increased levels of circulating immunoglobulins were indicative of dysregulated B cell function. It was suggested that TRPC1 has a pro-inflammatory role and could be therapeutically targeted in asthma and immune diseases (Yildirim et al. 2012). In contrast to previous reports showing a contribution of TRPC1 to mast cell degranulation (Suzuki et al. 2010; Cohen et al. 2009), Medic et al. showed that TRPC1 deficiency did not affect degranulation, but *Trpc1*-null mice showed a delayed recovery of antigen-induced anaphylaxis (Medic et al. 2013). They also noted higher production of inflammatory cytokines, such as TNF-α, that accounted for the delayed anaphylaxis.

9 Role in Hereditary and Acquired Diseases

9.1 Diabetic Nephropathy

Thus far, there is no hereditary disease directly linked to TRPC1. However, genetic polymorphisms in the *TRPC1* gene have been identified in diabetic nephropathy in type 1 and type 2 diabetes mellitus in the Han Chinese population (Chen et al. 2013). Reduced *Trpc1* mRNA expression in the kidney of diabetic rat models has also been reported (Niehof and Borlak 2008). However, in a separate study, no TRPC1 polymorphisms were found to be associated with diabetic nephropathy in a mixed population consisted mainly of European Americans (Zhang et al. 2009a), but a significant reduction in *Trpc1* mRNA was detected in affected individuals (Zhang et al. 2009a). Therefore, the involvement of TRPC1 in diabetic nephropathy may be related to its downregulation.

9.2 Parkinson's Disease

A possible involvement of TRPC1 in Parkinson's disease has been proposed (Selvaraj et al. 2012; Mattson 2012). Specifically, *Trpc1*$^{-/-}$ mice showed an increased unfolded protein response and a reduced number of dopaminergic neurons in the substantia nigra, a condition closely resembling Parkinson's disease. Consistently, brain lysates of patients with Parkinson's disease showed decreased levels of TRPC1.

9.3 Huntington's Disease

Huntington's disease (HD) is a neurodegenerative disease caused by mutations in Huntingtin. Neurons obtained from both transgenic *Drosophila* and mouse models of Huntington's disease displayed elevated store-operated Ca^{2+} entry, which was suppressed by a class of quinazoline-derived compounds. These compounds corrected the neurodegenerative phenotype of HD flies and required the expression of TRPC1 for their effects. It was suggested that the effects of these compounds may have targeted heteromultimeric complexes of TRPC1 with other components of the machinery mediating store-operated Ca^{2+} entry in neurons (Wu et al. 2011). These data implicate TRPC1 through its role in store-operated Ca^{2+} entry in the pathophysiology of HD.

9.4 Cancer

Expression studies identified several overexpressed TRP channels, including TRPC1, in human breast ductal adenocarcinoma. TRPC1 mRNA levels along with TRPM7 and TRPM8 positively correlated with proliferative markers in these cells. These data suggest that TRPC1 could be used as a diagnostic marker and perhaps as a therapeutic target in invasive forms of breast ductal adenocarcinoma (Dhennin-Duthille et al. 2011). Consistent with a causal role of TRPC1 in cancer invasion, Cuddapah et al. showed that TRPC1 functioned upstream of Cl^- channels and downstream of EGFR activation in promoting cell migration of glioma cells (Cuddapah et al. 2013).

9.5 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in dystrophin, a cytosolic protein. It is characterized by proximal muscle weakness eventually leading to muscle degeneration. Mdx mice lacking the dystrophin gene phenocopy DMD in mice. TRPC1 is found predominantly in the sarcolemma of adult muscle fibers, where it co-localizes and physically interacts with dystrophin and α1-syntrophin, another dystrophin-interacting protein (Vandebrouck

et al. 2002, 2007). Deletion of dystrophin in mdx mice causes the downregulation of α1-syntrophin and increased activity of TRPC1 functioning in either a store-dependent (Vandebrouck et al. 2006) or store-independent manner in association with TRPC4 (Sabourin et al. 2009b). A similar effect is shown in mice lacking Homer 1, a TRPC1-interacting protein keeping TRPC1 in the closed state (Yuan et al. 2003; Stiber et al. 2008b). Overall, it is believed that dystrophin organizes a costameric macromolecular complex including TRPC1 at the sarcolemma. Disruption of this complex in DMD patients leads to increased TRPC1-mediated Ca^{2+} entry promoting proteolysis and muscle necrosis (Sabourin et al. 2009a).

9.6 Seizures and Epilepsy

Neurotoxicity often follows seizure activity in epilepsy. TRPC1 in association with TRPC4 has been shown to mediate neuronal cell death in response to seizures in lateral septal and hippocampal neurons (Phelan et al. 2012, 2013). It is believed that activation of the TRPC1/TRPC4 heteromultimeric complex is initially triggered by the metabotropic glutamate receptors and further activated by neuronal firing leading to epileptiform bursting in the lateral septum (Phelan et al. 2012).

9.7 Darier–White Disease

Darier–White disease (DD) is a rare autosomal dominant genetic disease of the skin. Affected individuals develop warty papules and plaques in central trunk, flexures, scalp, and forehead, palmoplantar pits, and nail abnormalities. Severe forms of the disease can lead to painful erosions and blistering (OMIM124200). Mutations in the *ATP2A2* gene encoding the sarcoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) are responsible for DD. Pani et al. found that TRPC1 was upregulated in patients with DD leading to increased cytosolic Ca^{2+} concentration and proliferation (Pani et al. 2006). A similar upregulation of TRPC1 was seen in $Atp2a2^{+/-}$ mice (Pani et al. 2006). It was suggested that loss-of-function mutations in *ATP2A2* gene leads to an upregulation of TRPC1 promoting cell proliferation and keratosis in DD patients (Pani et al. 2006; Pani and Singh 2008).

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TRPC2

Barbara A. Miller

Contents

Abstract

Trp2 was the second ortholog of the Drosophila trp gene to be identified. Whereas full-length TRPC2 transcripts have been cloned in a number of species including mice, rats, and New World monkeys, TRPC2 is a pseudogene in humans, apes, Old World monkeys, and in a number of other vertebrates. TRPC2 is highly expressed in the rodent VNO. It is also detectable at the protein level in murine erythroblasts, sperm, and brain and has been detected in other tissues by RT-PCR. Its activation by DAG and by erythropoietin has been described in greatest detail, and inhibition by Ca^{2+}-calmodulin has been reported. The major demonstrated functions of TRPC2 are regulation of pheromone-evoked signaling in the rodent VNO, regulation of erythropoietin-stimulated calcium influx in murine erythroid cells, and ZP3-evoked calcium influx into sperm. Depletion of TRPC2 in knockout mice resulted in changes in behavior including altered sex discrimination and lack of male–male aggression. The red cells of TRPC2 knockout mice showed increased mean corpuscular

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volume, mean corpuscular hemoglobin, and hematocrit and reduced mean corpuscular hemoglobin concentration. TRPC2-depleted red cells were resistant to oxidative stress-induced hemolysis.

Keywords
TRPC2 • VNO • Pheromones • Oxidative stress • Red cell volume regulation • Erythropoietin • Sperm acrosome reaction

1 Genes, Promoters, and Splicing

Trp2 was the second of the mammalian orthologs of the Drosophila trp gene to be discovered and was identified first in humans using an expressed sequence tag database (Wes et al. 1995). Mouse (Zhu et al. 1996; Vannier et al. 1999; Hofmann et al. 2000; Yildirim et al. 2003), bovine (Wissenbach et al. 1998), and rat (Liman et al. 1999) genes are among a large number subsequently cloned (Frankenberg et al. 2011). TRPC2 is a pseudogene in humans because of the introduction of a number of stop codons within the open reading frame (Zhu et al. 1996; Vannier et al. 1999; Yildirim et al. 2003). In contrast, full-length transcripts were identified in mouse and rat. Four N-terminal splice variants of TRPC2 were initially identified in mice, called clone 14 or a, clone 17 or b, α, and β (Fig. 1) (Vannier et al. 1999; Hofmann et al. 2000; Yildirim et al. 2003). The clone 14 splice variant has 1,172 aa and a molecular weight of 131–135 kDa on SDS/PAGE gels and clone 17 1,072 aa and a molecular weight of 120 kDa (Vannier et al. 1999; Chu et al. 2004). These N-terminal splice variants differ in that clone 17 is missing the first 111 amino acids of clone 14 and its first 11 amino acids are unique. Domains that are absent from clone 17 but present on clone 14 include glycosylation, myristoylation, and protein kinase C phosphorylation sites. TRPC2 α is missing the first 286 amino acids of clone 14 (886 aa, 99 kDa) (Hofmann et al. 2000). The α isoform lacks the putative nuclear localization signal, arginine-rich regions, and phospholipase Cγ Src homology-binding site present on clones 14 and 17. TRPC2 β (890 aa, 100 kDa) was exclusively found in the vomeronasal organ (VNO) (Hofmann et al. 2000). An additional N-terminal TRPC2 splice variant was subsequently identified called "Similar to mouse TRPC2" (smTRPC2), consisting of 213 amino acids largely coincident with the N-terminus of TRPC2 clone 17. This splice variant lacked all six TRPC2 transmembrane domains and the calcium pore but was able to inhibit calcium influx through full-length TRPC2 (Chu et al. 2005).

TRPC2 is an essential component of vomeronasal chemoreception. TRPC2 gene functionality has been studied in a number of terrestrial and marine animals, and its pseudogenization is thought to provide evidence for deterioration of vomeronasal pheromone transduction and its role across evolution (Yu et al. 2010; Grus and Zhang 2009; Zhao et al. 2011). The VNO is expressed in New World monkeys but

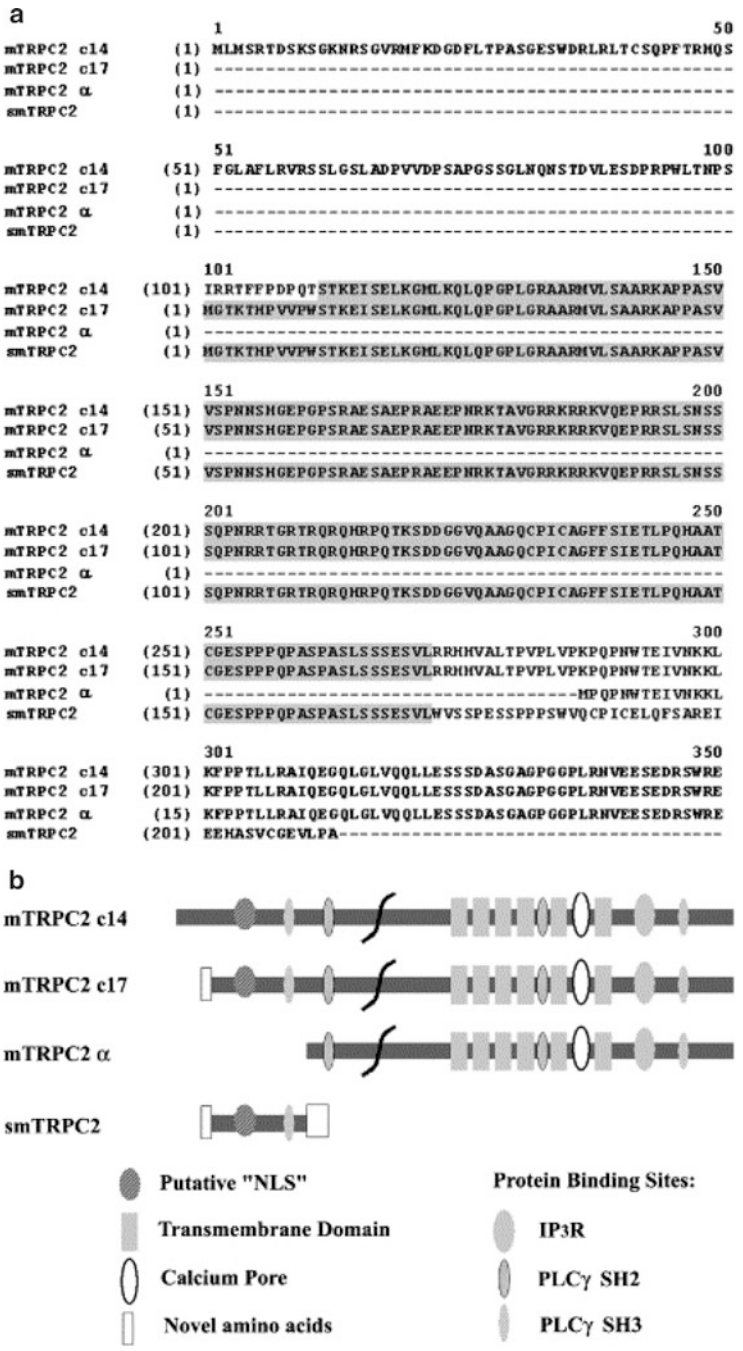

Fig. 1 Structural features of murine TRPC2. (**a**) Deduced amino acid sequence of the N-terminus of TRPC2 clone 14, clone 17, α, and smTRPC2. *Shading* indicates regions of homology with smTRPC2. (**b**) Predicted functional domains of TRPC2 isoforms (reprinted from Cell Calcium 37, Chu et al. 173–182, 2005, with permission from Elsevier)

became vestigial in the common ancestor of Old World monkeys and apes, where TRPC2 is a pseudogene (Liman and Innan 2003; Cai and Patel 2010).

2 Expression

Murine TRPC2 transcripts were detected in the VNO, testis, brain, spleen, heart, and in rat thyroid cells using RT-PCR or 5′-terminal RACE (Hofmann et al. 2000; Wissenbach et al. 1998; Liman et al. 1999; Chu et al. 2002; Sukumaran et al. 2012). With Western blotting or immunofluorescence, TRPC2 protein was found in the VNO, sperm, and brain (Vannier et al. 1999; Liman et al. 1999; Chu et al. 2002; Jungnickel et al. 2001). Expression of TRPC2a (clone 14) was also demonstrated in murine primary erythroblasts and murine erythroid cell lines by RT-PCR and Western blotting (Chu et al. 2002, 2004; Hirschler-Laszkiewicz et al. 2012). Low-level TRPC2 expression in other tissues is possible but has not yet been demonstrated. For example, ESTs have been cloned from trp2 mRNA from kidney as well as spleen (Vannier et al. 1999). TRPC2 expression on the plasma membrane has been documented in the VNO and in murine erythroblasts (Liman et al. 1999; Chu et al. 2002; Hirschler-Laszkiewicz et al. 2012; Tong et al. 2004).

TRPC2 is highly expressed in mice and rat VNO microvilli and is necessary for pheromone sensing in rodents (Liman et al. 1999; Stowers et al. 2002; Kiselyov et al. 2010). In the rat, TRPC2 is exclusively expressed in mature VNO neurons (Liman et al. 1999). The VNO became vestigial in the common ancestor of Old World monkeys and apes (Liman and Innan 2003), explaining the reduced importance of functional VNO transduction components such as TRPC2 in humans (Liman et al. 1999).

3 The TRPC2 Channel Protein and Structural Aspects

TRPC2 is closest phylogenetically in sequence similarity to TRPC1. While there is a lack of domain architecture information for TRPC2, like other TRP channels, TRPC2 sequence analysis predicts six membrane-spanning domains, a putative pore between the fifth and sixth transmembrane segments, an intracellular N-terminus with ankyrin repeats, and calmodulin-binding domains (Kiselyov et al. 2010). There are similarities as well as distinct differences with other TRPC channels. Critical functional domains of TRPC2 include the transmembrane domain 5 and 6 containing the putative calcium pore and a sequence motif Glu-Trp-Lys-Phe-Ala-Arg shared among all TRP genes. Similar to TRPC3, 6, and 7, there are three peripheral lipid-binding signals found in TRPC2, characteristic of diacylglycerol (DAG)-sensitive TRPCs (Kiselyov et al. 2010). Up to seven calmodulin-binding domains have been predicted on TRPC2, and they occur primarily within peripheral lipid-binding sites, also consistent with other DAG-sensitive TRPC (Kiselyov et al. 2010). Calmodulin-binding and phosphorylation of lipid-binding domains have been proposed to disrupt N- and C-terminal binding of TRPC2 to the plasma

membrane, inactivating the channel and preparing it for endocytosis (Kiselyov et al. 2010). The N-terminus of TRPC2 binds calmodulin at a region around 104–195 aa and does so in a Ca^{2+}-dependent manner (Yildirim et al. 2003). However, TRPC2 has only three ankyrin repeats unlike the rest of murine TRPC channels which have four. Specific domain alignments comparing TRPC2 to other murine TRPC have been published (Kiselyov et al. 2010). A detailed analysis of threedimensional TRPC2 structure has not been carried out.

4 Interacting Proteins

TRP channels assemble as homo- or heterotetrameric ion channels, and subunit composition has an important influence on biophysical and regulatory properties (Xu et al. 1997, 2000; Strubing et al. 2001; Hoenderop et al. 2003; Hofmann et al. 2002). TRPC2 and TRPC6 are both expressed in the plasma membranes of primary murine erythroblasts, and their ability to coassociate was demonstrated with immunoprecipitation in both a heterologous expression system and in primary murine erythroblasts (Chu et al. 2002, 2004). Erythropoietin modulated calcium influx through TRPC2 isoforms, but not through TRPC6, and when coexpressed with TRPC2, TRPC6 inhibited erythropoietin-stimulated calcium influx through TRPC2 (Chu et al. 2004). This interaction is not completely unexpected because of the close phylogenetic relationship between TRPC2 and TRPC6 (Hofmann et al. 2002). Multimeric channel formation has been reported for a number of TRPC channels, but this is the only report of TRPC2 directly interacting with another TRPC. The major organ in which TRPC2 function has been investigated is the VNO, and expression of additional TRPC has not been detected in that organ.

Naturally occurring N-terminal splice variants of TRP channels have been demonstrated which bind to and alter the activity of their full-length proteins (Xu et al. 2000, 2001; Balzer et al. 1999; Zhang et al. 2003). Many of these have been shown to inhibit full-length channel function. One such physiological N-terminal splice variant of TRPC2, smTRPC2, was identified in murine primary erythroblasts and was shown to colocalize with full-length TRPC2 by confocal microscopy and coassociate with immunoprecipitation (Chu et al. 2005). Expression of smTRPC2 inhibited Epo-modulated calcium influx through full-length TRPC2 function. The expression or a functional role for this TRPC2 splice variant in the VNO or in mouse sperm function has not been shown. In murine erythroblasts, a novel role for TRPC2 in red cell (RBC) volume regulation has been reported (Hirschler-Laszkiewicz et al. 2012). The Ca^{2+}-activated potassium channel Gardos channel is a key determinant of RBC volume. TRPC2 and the Gardos channel were shown to coassociate with reciprocal immunoprecipitation and to colocalize with confocal microscopy (Hirschler-Laszkiewicz et al. 2012). It was hypothesized that TRPC2 function is required for activation of the Gardos channel, and in the absence of TRPC2, the Ca^{2+}-sensitive Gardos channel is not activated, contributing to increased RBC volume in the TRPC2 knockout (Hirschler-Laszkiewicz et al. 2012).

Homer family proteins are scaffolding proteins, and disruption of Homer binding to TRPC1 or expression of dominant-negative Homer altered the activity of TRPC1, making it spontaneously active (Kiselyov et al. 2010; Yuan et al. 2003). The chaperones receptor transporting protein 1 (RTP1) and Homer (particularly Homer 1b/c and 3) are expressed in the rat VNO (Mast et al. 2010). Homer and RTP1 were shown to interact with TRPC2 (Mast et al. 2010). In addition, RTP1 increased cell-surface expression of TRPC2 in in vitro assays (Mast et al. 2010), and this was associated with an increase in ATP-stimulated whole-cell current in an in vitro patch-clamp assay. This data demonstrate that chaperone and scaffolding protein interactions play an important role in TRPC2 regulation and therefore in chemosignaling.

TRPC2 has also been shown to interact with the inositol 1,4,5-triphosphate receptor type 3 (IP3R) (Tong et al. 2004; Mast et al. 2010). Data suggest that in the VNO, a complex exists between TRPC2, Homer 1b/c, and the IP3R (Mast et al. 2010), and in murine erythroid cells, between TRPC2, the erythropoietin receptor, phospholipase Cγ, and the IP_3R (Tong et al. 2004). Signaling pathways mediated through these interactions are discussed below.

STIM1 is a protein which translocates to the plasma membrane upon depletion of ER calcium, where it interacts with TRPC channels and with Orai, a channel with characteristics similar to I_{CRAC} (Putney 2009). STIM1 mediates activation of both Orai and TRP channels through store depletion. Both STIM1 and Orai have been shown to bind to TRPC2, but the functional importance of this interaction is not known (Kiselyov et al. 2010). TRPC2 clones a and b have Ca^{2+}-dependent calmodulin-binding sites in their N-terminus (Yildirim et al. 2003), and calmodulin has been shown to colocalize with TRPC2 in VNO microvilli (Spehr et al. 2009). Inhibition of pheromone-sensitive TRPC2 channel activation in the VNO by Ca^{2+}-calmodulin is discussed below (Spehr et al. 2009).

5 Biophysical Description of Channel Function, Permeation, and Gating

TRPC2 has several activation mechanisms, and the best described mechanisms involve those which occur in the VNO and those that are activated by erythropoietin. TRPC3, 6, and 7 are the other DAG-sensitive TRPCs. In the VNO, TRPC2 has been shown to be activated by the lipid messenger diacylglycerol (DAG) rather than by Ca^{2+} store depletion or by inositol 1,4,5-trisphosphate (Kiselyov et al. 2010; Lucas et al. 2003; Zufall et al. 2005). Techniques developed to allow physiological recordings from large numbers of VNO including local field potentials and excitatory electrical responses demonstrated that the VNO can transduce specific pheromone ligands into electrical membrane signals through TRPC2 (Zufall 2005). Evidence supports the model that pheromones activate specific G-protein-coupled receptors in the VNO, which leads to generation of DAG or its analogs through activation of phospholipase C (Zufall 2005). DAG activates gating of TRPC2, and this signal is then terminated through activity of DAG kinase (Kiselyov et al. 2010;

Lucas et al. 2003). DAG-activated whole-cell current was greatly diminished at all concentrations in the vomeronasal system of TRPC2−/− mice, providing strong evidence for the role of DAG in its regulation (Lucas et al. 2003; Zufall 2005). However, deletion of TRPC2−/− did not fully abolish activation of a DAG-gated channel in the VNO, suggesting that other channels have a role (Lucas et al. 2003; Zhang et al. 2010). These other channels may include the calcium-activated potassium channel SK3 and the G-protein-activated potassium channel GIRK (Kim et al. 2012). The "DAG effect" may include increased channel expression at the plasma membrane, which had been shown for TRPC3 but not yet for TRPC2 (Kiselyov et al. 2010). The interaction between RTP1 and TRPC2 appears to be important in cell-surface expression of functional TRPC2, whereas the role of the interaction between Homer and TRPC2 is less well characterized (Mast et al. 2010). Rat TRPC2 is highly localized to VNO sensory microvilli. The absence of Ca^{2+} stores in sensory microvilli and the observation that agents which deplete Ca^{2+} stores do not activate TRPC2 support the conclusion that gating of TRPC2 in the VNO is largely independent of internal Ca^{2+} stores (Liman et al. 1999). Ca^{2+}-bound calmodulin but not Ca^{2+} or calmodulin alone have been shown to inhibit pheromone-sensitive gating of TRPC2. This is thought to represent a direct effect of Ca^{2+}-calmodulin on channel gating, because it occurs in the absence of added ATP or GTP, is abolished by calmodulin inhibitors, and does not require calmodulin kinase activity (Spehr et al. 2009). Phosphorylation and endocytosis are negative regulators of many DAG-sensitive TRPC channels, and because of homology, regulation through this mechanism has been proposed for TRPC2 but not well characterized (Kiselyov et al. 2010).

TRPC2 gating has also been shown to be activated by erythropoietin (Epo) (Chu et al. 2002, 2004). Epo-induced intracellular calcium increase was inhibited with PLCγ inhibitors and by downregulation of PLCγ with RNA interference, demonstrating the requirement PLC activity in Epo-modulated calcium influx (Tong et al. 2004). Epo failed to simulate significant calcium influx through TRPC2 mutants with deletions of IP_3R-binding sites. These data support the conclusion that after Epo stimulation, PLCγ activation produces IP_3, which activates IP_3R. IP_3R interaction with TRPC2 contributes to and is required for channel opening by Epo. Whereas TRPC2 coassociated with TRPC6 in erythroid cells, TRPC6 is not activated by erythropoietin, and coexpression of TRPC6 blocked erythropoietin-stimulated calcium influx through TRPC2 (Chu et al. 2004).

TRPC2 has also been shown to be activated by stimulation of purinergic receptors with ATP (Jungnickel et al. 2001). In addition to receptor-operated calcium entry, in sperm, an antibody directed to TRPC2 decreased both thapsigargin-induced calcium entry, suggesting involvement in store-operated calcium entry, and that induced by zona pellucida 3 (Jungnickel et al. 2001). Recently, TRPC2 was shown to be a major regulator of calcium signaling in rat thyroid cells, and the functional TRPC2 channel was a short TRPC2 splice variant (Sukumaran et al. 2012). TRPC2 was thought to regulate basal calcium entry by regulating the calcium content of the endoplasmic reticulum, and TRPC2 regulated calcium influx following stimulation with ATP (Sukumaran et al. 2012). Thus, modes of TRPC2

activation appear to be cell-specific and require different interactions and activators in different cell types.

6 Physiological Functions in Native Cells, Organs, and Organ Systems

Several physiological functions for TRPC2 have been identified. A key role for TRPC2 is its function in the primary conductance pathway of pheromone-stimulated signaling in the rodent vomeronasal system (Liman et al. 1999; Liman and Innan 2003; Kiselyov et al. 2010; Lucas et al. 2003). TRPC2 is activated in the VNO by DAG and the function is best characterized in TRPC2-depleted mice (see below) (Liman et al. 1999; Stowers et al. 2002; Leypold et al. 2002).

Another important role has been demonstrated in murine erythroblasts. Erythroblasts have been isolated from the spleens of phenylhydrazine-treated mice, and erythropoietin stimulation resulted in an increase in intracellular calcium concentration through TRPC2 (Chu et al. 2002, 2004; Tong et al. 2004). This increase in calcium stimulated by erythropoietin required PLCγ activation by the erythropoietin receptor, production of IP_3, and interaction of IP_3R with TRPC2 (Tong et al. 2004). RBC from TRPC2 knockout mice showed significant resistance to oxidative stress-induced hemolysis (Hirschler-Laszkiewicz et al. 2012). A major phenotype was an increase in mean red blood cell corpuscular volume, discussed below. A third key function is in the ZP3-evoked calcium entry into sperm in rodents. The sperm acrosome reaction is required for spermatozoa to pass through the zona pellucida (ZP), fuse with, and penetrate the oocyte during fertilization. TRPC2 expression has been demonstrated on sperm with immunofluorescence. It is an essential component of the ZP3-activated calcium channel of mouse sperm that drives the acrosome reaction (Jungnickel et al. 2001). However, because TRPC2−/− mice have normal fertility and offspring number, the role of TRPC2−/− in sperm physiology has not been conclusively demonstrated. Recently, a role for TRPC2 in regulation of basal calcium levels and ER calcium content in thyroid cells through protein kinase C and STIM2 and in ATP-evoked calcium entry has also been demonstrated (Sukumaran et al. 2012).

7 Lessons from Knockouts

The best described function of TRPC2 is its unequivocal role in pheromone sensing in mice, which has been extensively reported and reviewed previously (Stowers et al. 2002; Kiselyov et al. 2010; Lucas et al. 2003; Zufall et al. 2005; Zufall 2005; Zhang et al. 2010; Kim et al. 2012; Hasen and Gammie 2009). TRPC2 knockout mice have been generated by several laboratories (Stowers et al. 2002; Leypold et al. 2002). TRPC2−/− mice are similar to wild type in general fitness, age at sexual maturity, litter size, and longevity (Stowers et al. 2002). TRPC2−/− male mice do mate normally with females. Terrestrial vertebrates have two anatomically

and functionally distinct olfactory organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The VNO mediates some social behaviors and neuroendocrine responses to pheromones. While no defects in MOE function were detected, TRPC2 deficiency eliminated the sensory activation of VNO neurons in mice, and the absence of VNO function had pronounced behavioral effects (Stowers et al. 2002; Leypold et al. 2002). Male mice deficient in TRPC2 expression failed to display pheromone-evoked aggression toward male intruders usually seen in wild-type mice (Stowers et al. 2002; Leypold et al. 2002). TRPC2−/− males displayed urine-marking behavior typical of subordinate males (Leypold et al. 2002). Experiments showed that TRPC2−/− males were capable of displaying aggressive behavior, but were not triggered to do so by pheromones. TRPC2−/− males initiated sexual mounting and courtship behavior that was indistinguishable from wild type, but they did not indiscriminate male from female mice (Stowers et al. 2002). TRPC2−/− female mice displayed normal maternal behaviors including crouching and nursing. However, aggressive behavior was also reduced in lactating TRPC2−/− females confronted with a male intruder, suggesting that the VNO has a role in initiating aggressive behavior in both males and females (Leypold et al. 2002). TRPC2-deficient females were also deficient in nest-building skills (Hasen and Gammie 2009). No other TRP family channels have been identified in the VNO. Comprehensive reviews have been written focusing on the behavioral analysis of TRPC2 knockout mice thought to be mediated through TRPC2 deficiency in the VNO (Zufall et al. 2005; Venkatachalam and Montell 2007; Kato and Touhara 2009; Yildirim and Birnbaumer 2007).

TRPC2 and TRPC3 expression has been demonstrated on primary erythroblasts, and both TRPC2 and TRPC3 are activated by erythropoietin stimulation (Chu et al. 2002; Tong et al. 2004, 2008). Erythropoietin receptor expression on erythroblasts is critical for RBC production (Lin et al. 1996; Kieran et al. 1996), and modulation of intracellular calcium is an important signaling pathway (Miller et al. 1989; Misiti and Spivak 1979; Hensold et al. 1991; Gillo et al. 1993). Ca^{2+} also has a key role in mature red cell function and survival (Bogdanova et al. 2013). The importance of TRPC2 and TRPC3 in erythropoiesis was examined in TRPC2, TRPC3, and TRPC2/TRPC3 double knockout mice (Hirschler-Laszkiewicz et al. 2012). None of these mice were anemic, and all had normal red cell blood counts, hemoglobins, and reticulocyte counts compared to wild-type control mice. Despite a significant decrease in the rise in intracellular calcium in the erythroblasts of all three knockouts in response to erythropoietin, no defects in red cell production were observed. The major phenotypic abnormality observed in TRPC2 and TRPC2/TRPC3 knockout mice, but not in TRPC3 knockout mice, was that the mean red cell corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and hematocrits were significantly greater and the mean corpuscular hemoglobin concentration (MCHC) was significantly reduced compared to controls. In TRPC3 knockout mice, all hematologic parameters were similar to wild-type controls. In addition to these differences, red cells from TRPC2 and TRPC3/TRPC2 double knockout mice showed significant resistance to phenylhydrazine-induced hemolysis. These data suggest that TRPC2 depletion and reduced increase in intracellular

calcium could protect murine RBC in red cell disorders in which hemolysis is exacerbated by oxidative stress, such as thalassemia and sickle cell anemia. An interaction of TRPC2 and the Gardos channel was demonstrated in vitro with immunoprecipitation, and colocalization was shown in the erythroid cell line HCD-57 with confocal microscopy (Hirschler-Laszkiewicz et al. 2012). The Gardos channel plays an important role in volume regulation of murine red cells. Activation of the Gardos channel can lead to erythrocyte dehydration, increased concentration of intracellular hemoglobin S, and decreased deformability, which is directly related to increased MCHC (Clark et al. 1980). This work suggested the possibility that TRPC2 may mediate its effects on the red cell through activation of the Ca^{2+}-sensitive Gardos. In the red cells of the TRPC2 knockout mouse, the Ca^{2+}-sensitive K^+ channel Gardos cannot be activated by calcium influx through TRPC2, resulting in retention of intracellular potassium and water, an increase in the MCV, and decrease in the MCHC.

8 Role in Hereditary and Acquired Diseases

At the present, it is unlikely that a human disease will be identified involving TRPC2, because TRPC2 is a pseudogene in humans. The function of TRPC2 is best described in the VNO, but this organ is vestigial in humans and Old World primates.

A number of red cell disorders in humans are mediated through increased calcium influx (Foller et al. 2008; Lang et al. 2003) and exacerbated by oxidative stress (Sangokoya et al. 2010), including thalassemia and sickle cell anemia. Sickle red cells are particularly susceptible to oxidative stress-induced hemolysis (Steinberg and Brugnara 2003; Amer et al. 2006). This raises the possibility that TRPC2 depletion or inhibition in mice could protect RBC from hemolysis associated with thalassemia or sickle cell anemia both by protecting cells from oxidative stress and reducing cellular rigidity and hemolysis through decreased MCHC (Hirschler-Laszkiewicz et al. 2012). However, because TRPCs is a pseudogene in humans, application of this approach would require identification of a functional homolog of TRPC2 in humans.

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TRPC3: A Multifunctional Signaling Molecule

Michaela Lichtenegger and Klaus Groschner

Contents

Abstract

TRPC3 represents one of the first identified mammalian relative of the *Drosophila trp* gene product. Despite extensive biochemical and biophysical characterization as well as ambitious attempts to uncover its physiological role in native cell systems, the channel protein still represents a rather enigmatic member of the TRP superfamily. TRPC3 is significantly expressed in the brain and heart and appears of (patho)physiological importance in both non-excitable and excitable cells, being potentially involved in a wide spectrum of Ca^{2+} signaling mechanisms. TRPC3 cation channels display unique gating and regulatory properties that allow for recognition and integration of multiple input stimuli

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including lipid mediators, cellular Ca^{2+} gradients, as well as redox signals. Physiological/pathophysiological functions of this highly versatile cation channel protein are as yet incompletely understood. Its ability to associate in a dynamic manner with a variety of partner proteins enables TRPC3 to serve coordination of multiple downstream signaling pathways and control of divergent cellular functions. Here, we summarize current knowledge on ion channel features as well as possible signaling functions of TRPC3 and discuss the potential biological relevance of this signaling molecule.

Keywords
TRPC3 • Nonselective cation channel • Cellular regulation • Ca^{2+} signaling • Lipid sensor

1 Gene Products and Expression Pattern

Human TRPC3 (hTRPC3) cDNA was originally cloned using a library derived from HEK293 cells and the EST sequence R34716 as probe (Zhu et al. 1996). The human TRPC3 gene consists of 11 exons located on chromosome 4. TRPC3 mRNA comprises ~4 kb and was found abundantly expressed in the brain (Riccio et al. 2002). In peripheral tissues, substantial expression of TRPC3 mRNA has been detected in pituitary gland and somewhat lower levels in the heart and lungs (Riccio et al. 2002). Notably, TRPC3 appears expressed predominantly in embryonic and developing tissues (Strubing et al. 2003). The human TRPC3 protein comprises either 836 amino acids (aa) (isoform 1, UniProtKB-ID: Q13507-1), 921aa (isoform 2, UniProtKB-ID: Q13507-2), or 848aa (isoform 3, UniProtKB-ID: Q13507-3), of which isoform 1 has been designated as the "canonical" sequence. Isoform 3 shares 96.41 % homology with mouse TRPC3 (mTRPC3; 836aa) and 94 % with rat TRPC3 (rTRPC3; 828aa) (Preuss et al. 1997). Human, mouse, and rat genomes contain one additional exon that gives rise to the expression of an N-terminally extended splice variant of TRPC3 [(Yildirim et al. 2005); hTRPC3a, 921aa; mTRPC3a, 911aa; rTRPC3a, 910aa]. A short splice variant of TRPC3, termed Trp3sv, has been isolated from a rat heart cDNA library encoding a 736aa protein with a truncated N-terminus (Ohki et al. 2000).

2 Molecular Structure

2.1 Domain Structure and Membrane Topology

TRPC3 has been characterized as an integral membrane protein with seven predicted hydrophobic domains (H1–H7), of which six helical stretches form the transmembrane core domain (TM1–TM6), which is flanked by an intracellular

N- and C-terminal domain (Zhu et al. 1996; Vannier et al. 1998). As shown in Fig. 1a, the first hydrophobic region H1 has been proposed as an intracellular, membrane-associated segment, and based on the similarity of the TRPC membrane topology to that of voltage-gated K^+ channels (K_V), transmembrane segments TM5, TM6, and their connecting loop including the pore helix were designated as the putative pore region (Vannier et al. 1998).

2.2 Subunit Assembly and Multimerization

According to the general concept of a tetrameric pore structure in cation channels with a 6TM membrane topology, TRPC3 is assumed to form tetrameric channel complexes as illustrated in Fig. 1b,i. It is important to note that the existence of native homotetrameric TRPC3 channels has not definitely been proven and the subunit composition and stoichiometry of native TRPC3 heterotetramers remain elusive. Nonetheless, heterologous overexpression of TRPC3 is likely to generate homomultimeric channels. The stoichiometry of pore complexes may be tested in cells expressing defined mixtures of blocking sensitive mutants and wild-type proteins (Kosari et al. 1998). Characterization of a TRPC3 mutant that contains an exo-HA tag, which confers sensitivity to block by anti-HA antibody (Poteser et al. 2006a), substantiated the concept of TRPC3 being able to assemble in homotetramers. Analysis of the ability of other TRPC isoforms to associate with TRPC3 upon heterologous overexpression suggested a preference of TRPC3 to associate with its closer relatives TRPC6 and TRPC7 (Hofmann et al. 2002), while its ability to associate with more distant relatives was controversially interpreted (Lintschinger et al. 2000; Liu et al. 2005). In HEK293 cells, TRPC3 has been demonstrated to interact with TRPC6 and 7 to form receptor-operated channels. Co-transfection of a dominant negative mutant of TRPC6 (dnTRPC) and TRPC3 resulted in a decreased receptor-activated Mn^{2+} influx. Moreover, association of TRPC3 and TRPC6 fusion proteins was clearly demonstrated by FRET experiments, while minimal FRET signals were detected in cells co-expressing fluorescent protein fusions of TRPC3 and TRPC1, 4, or 5 (Hofmann et al. 2002). In epithelial cells, the existence of TRPC3/6 complexes associated with Ca^{2+} signaling proteins of the G_q-PI-PLC pathway has been demonstrated (Bandyopadhyay et al. 2005). Similarly, in brain synaptosomes, TRPC3 has been shown to interact with TRPC6 and 7, but not with other members of the TRPC family (Goel et al. 2002). Lintschinger et al. (2000) were the first to report functional interactions between TRPC1 and TRPC3 in the HEK293 expression system, and such interactions have subsequently been confirmed in hippocampal neurons (Wu et al. 2004) as well as in human salivary gland cells in which TRPC3 and TRPC1 were shown to associate via N-terminal interactions to form heteromeric store-operated channels (Liu et al. 2005). Importantly, a tight physical interaction between TRPC3 and TRPC1 was also suggested by a heteromerization-dependent regulatory interaction between TRPC3 and the ER-resident Ca^{2+} sensor STIM1 [(Yuan et al. 2007), see also below] that is suggested as the basis of store-operated

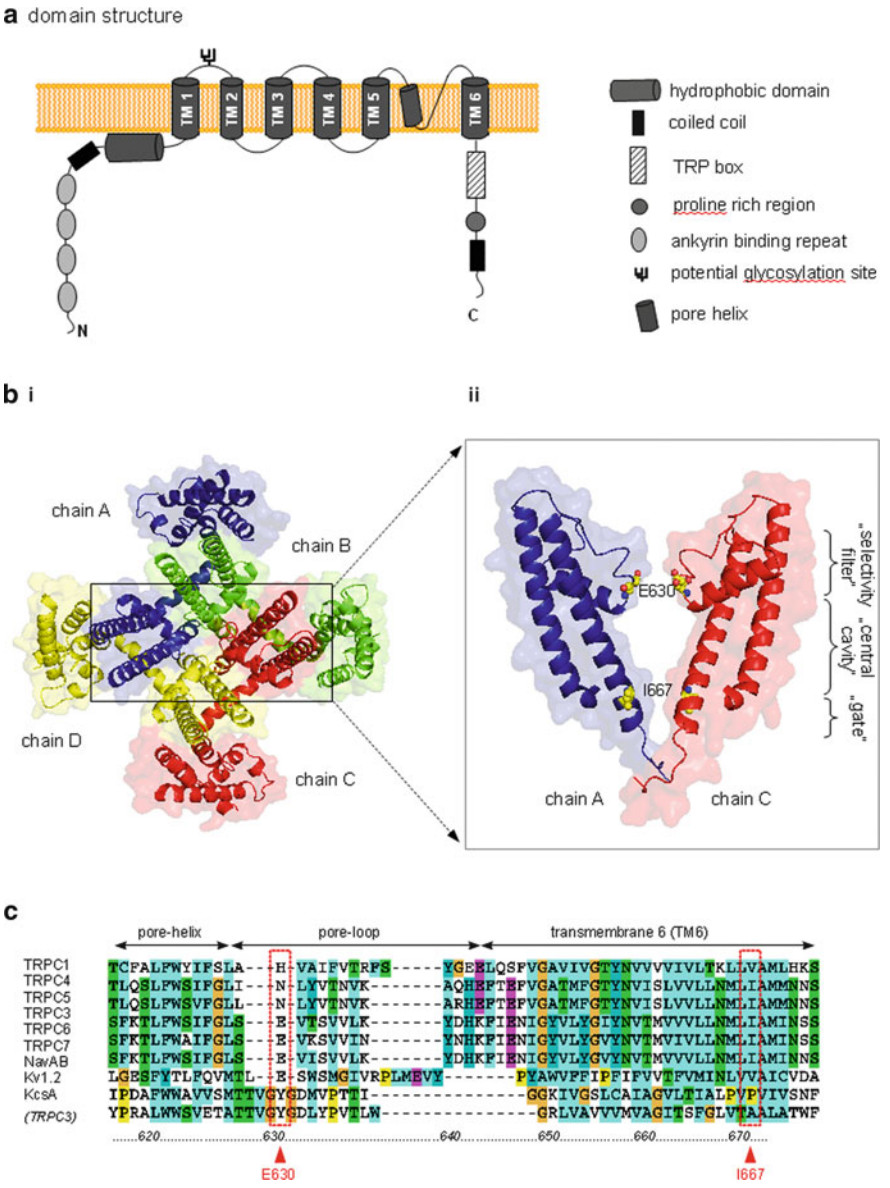

Fig. 1 (**a**) *Proposed membrane topology and domain structure of TRPC3*. The N-terminus of TRPC3 contains 4 ankyrin domains, a coiled-coil domain and a hydrophobic domain preceding the first transmembrane segment. A second coiled-coil domain is located in the C-terminus downstream of the TRP box and the proline-rich region. A putative glycosylation site is located in the first extracellular loop. TM5, TM6, and the connecting pore loop are proposed to form the central cation-conducting pore. (**b**) *Molecular model of human TRPC3 based on the structure of NaVAB*. (i) *Top* view of the four subunits (chain A–D) forming the complete TRPC3 tetramer. (ii) Excerpt shows the TM5–6 regions (residues 570–680) of two opposing subunits A and C. Amino acid residues with potential significance for ion selectivity (E630) and gating (I667) are highlighted in *yellow*. Amino acid numbering is valid for hTRPC3 isoform 3. (**c**) Sequence alignment of the

TRPC3 activation. Association of TRPC3 with TRPC4 was demonstrated in HEK293 as well as native vascular endothelial cells (Poteser et al. 2006a) and was found to generate a redox-sensitive channel complex.

In summary, TRPC3 displays a remarkable potential to form divergent types of cation channels by multimerization with other TRPC proteins. Assembly of TRP pore complexes via N-terminal interactions was suggested by demonstration of the ability of N-terminal domains to associate as well as by marked dominant negative effects of N-terminal fragments (Liu et al. 2005; Engelke et al. 2002; Groschner et al. 1998). Nonetheless, stable assembly of tetramers is likely to involve additional interactions between other domains in hydrophobic segments (Xu et al. 1997) as well as the C-terminus (Poteser et al. 2006a).

3 Channel Features

3.1 The Selectivity Filter

TRPC3 has been characterized as a nonselective cation channel displaying moderate selectivity for Ca^{2+} over monovalent cations with a permeability ratio of $P_{Ca}/P_{Na} = 1.6$ (Kamouchi et al. 1999a). In analogy to the better investigated superfamily of potassium channels, ion selectivity in TRPC3 is attributed to the loop connecting the pore-forming transmembrane segments 5 and 6 (TM5–TM6), which reenters the transmembrane region to line the outer permeation path (Vannier et al. 1998). Recent molecular modeling attempts provided first insights into the architecture of this region. Guided by a novel homology model using the voltage-gated sodium channels Na_VAB as template, site-directed mutagenesis experiments identified a non-helical stretch of 13 amino acids (629–641 in isoform 3) within the putative pore loop as the actual selectivity filter (Lichtenegger et al. 2013). A single glutamate residue (E630, Fig. 1b, ii) within this stretch was recognized as the key determinant of ion selectivity (Poteser et al. 2011). It is important to note that E630 is conserved in the closely related isoforms 3/6/7 (Fig. 1c). Neutralization of this negative charge completely disrupts Ca^{2+} permeation. Systematic scanning of the pore loop region using the engineered disulfide approach localized the narrowest region of the open TRPC3 permeation path to residues E630 and V631 centrally within the putative selectivity filter. Introduction of cysteines at each of three adjacent positions (629–631) within the pore loop was found to form subunit cross-linking disulphide bridges, implying a non-helical selectivity filter defined

Fig. 1 (continued) putative pore region of Na_VAB, KcsA, $K_V1.2$, and mammalian TRPC isoforms. E630 as the key element of the TRPC3 selectivity filter is conserved among close related isoforms TRPC3/6/7. An isoleucine residue at the cytosolic end of TM6 (I667 in TRPC3) is conserved in TRPC channels and may contribute to the physical channel gate. Amino acid numbering refers to hTRPC3 isoform 3

by amino acid side chains that project toward the permeation path to govern selectivity mainly via the negative charge in position 630. Beyond that, a conserved LFW motif within the pore loop was identified as essential for channel function. Mutations in the LFW motif completely disrupted channel function in a dominant negative manner (Hofmann et al. 2002; Liu et al. 2003). To date, the significance of this motif for proper channel function is unknown.

Monovalent permeation through human TRPC3 expressed in HEK293 was shown to follow the Eisenman III permeability sequence ($P_{Li+} < P_{Na+} < P_{Cs+} < P_{K+} < P_{Rb+}$), which is indicative of a weak field-strength binding site for monovalent cations (Lichtenegger et al. 2013). Interestingly, the Ca^{2+}-impermeable E630Q mutant as well exhibited the Eisenman III permeation profile, suggesting fairly separate mechanisms and distinct structures underlying divalent and monovalent permeation in TRPC3. It is of note that an earlier study classified TRPC3 expressed in bovine pulmonary artery endothelial cells with a strong field-strength interaction site for monovalent cations ($P_{Na+} > P_{Cs+} = P_{K+}$) (Kamouchi et al. 1999b). Nonetheless, since expression levels of endogenous TRPC proteins are expected to differ between endothelial and HEK293 cells, a certain amount of heteromerization may explain the observed discrepancy in selectivity behavior.

Pore-size estimation from the relative permeability for differently sized alkylammonium ions suggested a limiting pore diameter of <5.8 Å for TRPC3 homomeric complexes. Notably, the E630Q mutant exhibited a smaller diameter of <5.2 Å, implying a stabilizing role of E630 within the pore complex (Lichtenegger et al. 2013).

3.2 The Channel Gate

Structure-guided mutagenesis based on the Na_VAB template shed recently some light on the TRPC channel gating machinery. The Na_VAB-based homology modeling approach identified molecular key elements of the physical channel gate (Lichtenegger et al. 2013). The pore-forming TM6 helices were predicted to narrow toward the cytosol to form the channel gate at a helix-bundle crossing structure. Exchange of a bulky, hydrophobic residue within this region (I667, Fig. 1b, ii) by either the small alanine or the charged glutamate residue indeed destabilized the hydrophobic seal and rendered the channel constitutively active. The large basal currents recorded immediately after whole-cell break-in were further enhanced by channel activation via carbachol, indicating that the I667A mutation produces a gain in function without generating a maximum TRPC3 conductance. Carbachol-evoked I667A currents did not display the transient nature of a classical TRPC3 response, indicating that the gate mutant is unable to enter a certain de- or inactivated closed state after channel activation. The I667A mutant generated an unusual sensitivity of outward currents to the pore blocker RR, caused changes in disulfide bond formation of cysteines introduced in the pore loop, and furthermore reduced the Ca^{2+}/Cs^+ permeability ratio from 3.7 to approximately 0.1 in the mutant. These findings point toward an impact of reduced gating stability on

the outer pore structure including the selectivity filter. Consequently, allosteric linkage between the TRPC3 main occluding gate at the TM6 helix-bundle crossing and the pore loop as a secondary gate has been proposed.

Hitherto, no crystal structure is available for any TRP channel, but substantial first insights into 3D structure of TRPC3 at 15 Å resolution were obtained with cryo-electron microscopy (Mio et al. 2007). This study suggested the TRPC3 channel gate to be located at the level of the cytosolic face of the inner leaflet of the membrane bilayer, which is in accordance with the results of our recent mutagenesis study.

3.3 Mechanisms of Activation

Several studies suggest that TRPC3 channels display significant constitutive activity (Dietrich et al. 2003; Hurst et al. 1998) exceeding that of other related TRPC species such as TRPC6. Basal activity of TRPC channels, specifically of TRPC3/6/7 channels, has been related to the glycosylation status. TRPC3 mutants mimicking the higher glycosylation state of TRPC6 displayed reduced constitutive activity (Dietrich et al. 2003). Typically, TRPC3 currents display only little voltage dependence as illustrated in Fig. 2b. There is general agreement in that cellular TRPC3 activity is enhanced in response to stimulation of cellular phospholipase C (PLC) activity (Fig. 2b), and solid evidence has been presented for direct activation of TRPC3 currents by the lipid mediator diacylglycerol [DAG; (Lintschinger et al. 2000; Hofmann et al. 1999; McKay et al. 2000)]. Hence, TRPC3 as well as its closest relatives TRPC6 and TRPC7 are considered as a unique family of lipid-sensitive cation channels. Besides direct activation of TRPC3 by PLC-derived DAG, control of TRPC3 channels by the filling state of intracellular Ca^{2+} stores has repeatedly been suggested (Kiselyov et al. 1998; Vazquez et al. 2003). Channels generated by the long TRPC3 splice variant TRPC3a were reported sensitive to both activation via the G_q-PLC pathway and to direct store depletion (Yildirim et al. 2005). The ability of TRPC3 to contribute to store-operated Ca^{2+} entry is likely dependent on the expression pattern of Ca^{2+} signaling molecules such as TRPC heteromerization partners or regulatory channel subunits. TRPC1 has been identified as one potential heteromerization partner that enables store depletion-dependent activation of TRPC3 cation channels (Liu et al. 2005). Several potential mechanisms for store operation of TRPC3 have been proposed. More recently a direct interaction with the ER Ca^{2+} sensor STIM1, based on electrostatic interactions involving critical negative charges in the C-terminus of TRPC3 near the TRP box, was identified as crucial for store operation (Zeng et al. 2008). The interaction between TRPC3 and STIM1 is, in a complex manner, dependent on heteromerization with TRPC1 (Yuan et al. 2007). Alternatively, activation of TRPC channels in terms of store-operated activation may involve Ca^{2+} entry through Orai1 channels as a trigger for membrane recruitment of TRPC channels and increased conductance in response to store depletion (Cheng et al. 2011). Further signaling molecules already earlier implicated in gating of TRPC3 channels

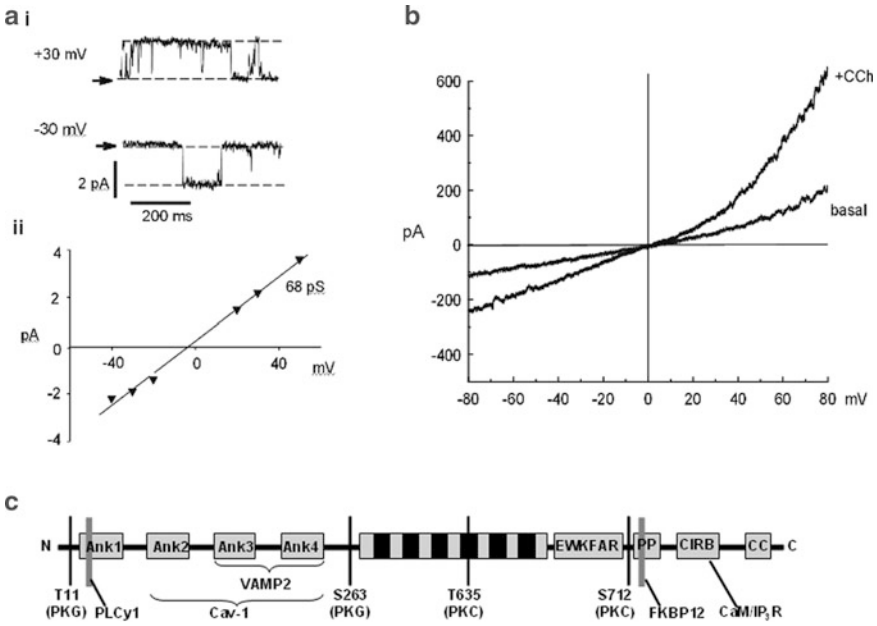

Fig. 2 (**a**) *Single-channel properties of TRPC3*. (i) Typical single-channel activity recorded at +30 mV and −30 mV in cell-attached patches of TRPC3 expressing HEK293 cells subsequently to incubation with cholesterol oxidase (Poteser et al. 2006b). *Arrows* indicate closed state. Pipette solution contained 137 mm NaCl, 5 mm KCl, 2 mm $CaCl_2$, 2 mm $MgCl_2$, and 10 mm HEPES; the bath solution contained 145 mm potassium gluconate, 5.3 mm KCl, 3 mm $MgCl_2$, and 15 mm HEPES. The pH of all solutions was adjusted to 7.4. Currents were filtered at 1 kHz (low pass, −3 dB) and digitized at 5 kHz. Experiments were performed at room temperature. (**b**) Whole-cell properties of TRPC3. Current–voltage relation derived from whole-cell voltage-clamp recordings of TRPC3 expressed in HEK293 cells (T3–9) under basal conditions and after challenge with carbachol (CCh, 200 μM) is shown. Bath solution contained 137 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 10 mM glucose, and 1 mM $MgCl_2$ (nominally Ca^{2+}-free). Intracellular (pipette) solution contained 120 mM Cs-methanesulfonate, 20 mM CsCl, 10 mM HEPES, 1 mM $MgCl_2$, and 1 mM EGTA (pH adjusted to 7.4). Voltage-clamp protocols: depolarizing ramps from −100 to +80 mV/0.6 V/s, 0.2 Hz, holding potential −70 mV. Experiments were performed at room temperature. (**c**) Putative sites relevant for regulatory phosphorylation and protein–protein interactions. Sites relevant for regulatory phosphorylation by cGMP-dependent kinases PKG and protein kinase C (PKC) are indicated along with domains involved in the interaction of TRPC3 with phospholipase Cγ1 (PLCγ1), caveolin-1 (Cav-1), vesicle-associated membrane protein (VAMP), FK506 binding protein 12 (FKBP12), calmodulin (CaM), and inositol-*tris*-phosphate receptor (IP_3R). Domain for mutual exclusive binding of CaM or the IP_3R designated as CIRB is shown. Amino acid numbering refers to hTRPC3 isoform 3

are the IP_3 receptor (IP_3R) and calmodulin (CaM) (Zhang et al. 2001), which bind to a combined interaction domain in the C-terminus of TRPC3 that has been termed CIRB (CaM-IP_3R-binding site; Fig. 2c; (Tang et al. 2001). IP_3R-mediated gating of TRPC3 channels was suggested as a mechanism that confers sensitivity of TRPC3 channels to the filling state of intracellular Ca^{2+} stores in terms of a "conformational coupling model" (Berridge 1995; Irvine 1990). This concept was subsequently put

forward by the identification of another integral membrane protein resident in the membrane of the endoplasmic reticulum, junctate, which was found to associate with both TRPC3 and the IP_3R (Treves et al. 2004). However, a key role of IP_3Rs was questioned by other studies demonstrating the presence of PLC-dependent as well as store-operated function of TRPC3 in expression systems lacking all three isoforms of the IP_3R (Wedel et al. 2003). Ca^{2+}-dependent binding of CaM to the CIRB region was shown to interfere with the IP_3R-TRPC3 interaction and to inhibit TRPC channel activity (Zhang et al. 2001). Intracellular Ca^{2+} has repeatedly been demonstrated as a key regulator of TRPC3 channels, which governs TRPC3 conductances in a rather complex manner as both permissive (Zitt et al. 1997) and inhibitory effects of intracellular Ca^{2+} have been demonstrated (Lintschinger et al. 2000).

Both classical gating mechanisms, which determines open probability, and processes that control of plasma membrane recruitment of channels are considered essential for cellular control of TRPC3 conductances. Rapid enhancement of TRPC3 channel density in the plasma membrane may confer increases in TRPC3 conductance concomitant with or even without a classical gating process. As recently reported for TRPC5 (Bezzerides et al. 2004), a substantial fraction of TRPC3 may be targeted to a pool of highly mobile, plasma membrane-associated vesicles as evident from high-resolution fluorescence microscopy experiments. Rapid insertion and retrieval of TRPC3 channels via regulated exo- and endocytosis may therefore represent a potential mechanism to govern cellular TRPC3 conductances. However, this concept was questioned by other experiments suggesting activation of TRPC3 channels via the G_q-PLC pathway involves a process independent of membrane recruitment (Smyth et al. 2006). Further analysis of the contribution of gating mechanisms and mechanisms that merely govern membrane insertion/retrieval of TRPC3 channels in conductance activation will require a thorough analysis at the single-channel level. Representative single-channel recordings in cell-attached mode from hTRPC3 expressing HEK293 are shown in Fig. 2a [from Poteser et al. (2006a)]. Incubation with cholesterol oxidase, which affects the molecular composition of lipid rafts, was found to activate TRPC3 currents with a unitary conductance of 68 pS. Overall, TRPC3 unitary conductance is insufficiently defined, even for the homotetrameric channel, with estimates ranging from 20 pS (based on noise analysis) to 65 pS, a phenomenon that may in part be based on the existence of subconductance states.

In essence, both a direct binding of lipid mediators to TRPC3 and incompletely defined stimuli generated by reduction of the filling state of intracellular Ca^{2+} stores are considered as gating mechanisms for TRPC3 channels. Activation of TRPC3 conductances is likely to involve both increases in open probability and a regulated increase in functional channels within the plasma membrane.

3.4 Regulatory Phosphorylation

TRPC3 displays several potential sites for regulatory phosphorylation in both the N- and C-terminal cytoplasmic domains (Fig. 2c). Protein kinase Cγ (PKCγ) is involved in the inhibitory phosphorylation of TRPC3 channels, with T635 (murine protein; corresponding to T573 in hTRPC3) within the highly conserved TM4/TM5-linker region as a regulatory site (Becker et al. 2009). Threonine-to-alanine amino acid change at this position accounted for the murine *moonwalker* (*Mwk*) phenotype that is characterized by a pathophysiologically relevant gain in TRPC3 basal activity causing presumably Ca^{2+}-mediated neuronal damage, loss of Purkinje cells, and cerebellar ataxia. Moreover, PKC has been implicated in downregulation of TRPC3 activity via phosphorylation of S712 (Fig. 2c) (Trebak et al. 2005), and cyclic GMP-dependent phosphorylation has been shown to suppress TRPC3-mediated store-operated Ca^{2+} entry HEK293 cells mediated by phosphorylation of TRPC3 at positions T11 and S263 (Kwan et al. 2004). Notably, suppression of TRPC3 activity in response to PKC activation was suggested to involve PKG and a cross talk between these phosphorylation pathways in PKG-expressing cell systems such as in endothelial cells (Kwan et al. 2006). Moreover, phospholipase C-dependent activation of TRPC3 has been demonstrated to require regulatory phosphorylation involving the non-receptor tyrosine kinase Src. Pharmacological inhibition of Src, as well as a dominant negative Src, was found to suppress TRPC3 activation (Vazquez et al. 2004). More recently, another pathophysiologically relevant regulatory phosphorylation was attributed to serine threonine kinase with-no-lysine 4 [WNLK4; (Park et al. 2011)]. Evidence has been provided that impairment of this pathway, which suppresses TRPC3 activation, leads to vascular smooth muscle hypertrophy, enhanced vasotone and hypertension (Park et al. 2011).

3.5 Cellular Localization

TRPC3 has been suggested as part of a multimolecular signaling complex containing proteins of the Gq-PLC pathway, proteins of the ER membrane, as well as scaffolds and adaptor proteins such as ezrin and caveolin-1 (Cav-1) (Lockwich et al. 2001). Interactions between TRPC3 and a variety of proteins have been identified that are apparently essential for correct targeting or activation of TRPC3 conductances, such as immunophilins, which interact with TRPC3 via the C-terminal proline-rich region (Sinkins et al. 2004) including P704 (see Fig. 2c) as a critical residue. Appropriate assembly of TRPC3 pore complexes with scaffolds and adaptor proteins is likely to enable particular mechanisms of cellular regulation of TRPC3 conductances but also its linkage to downstream signaling pathways (see below). Structural motifs in TRPC3 that are involved in such interactions are highlighted in Fig. 2c. Cytoskeletal rearrangements were found to trigger internalization of TRPC3 complexes, reminiscent of the internalization of caveolae, and Cav-1 was demonstrated to co-localize and associate with TRPC3

(Lockwich et al. 2001). Consequently, a caveolin-binding motif has been identified between aa 324 and 351 (Brazer et al. 2003) in the N-terminus of TRPC3. It remains to be clarified if targeting of TRPC3 to the specific membrane environment of caveolae is essential for channel gating by lipids or if caveolin-TRPC3 association is involved in cellular trafficking of TRPC3 complexes. Oxidative modification of membrane lipids, specifically of cholesterol, was found to promote TRPC3 activity, a mechanism that may enable TRPC3 to serve as a sensors for cellular redox state as proposed for channel in vascular endothelial cells (Groschner et al. 2004; Balzer et al. 1999). Such redox-dependent lipid regulation of TRPC3 may well be related to targeting of TRPC3 channels to the cholesterol-rich environment of caveolae. Consistently, cholesterol has recently been found to promote membrane presentation of TRPC3 (Graziani et al. 2006).

The cytoplasmic N-terminus of TRPC3 (aa 123–221) contains a site for interaction with a protein that most likely governs vesicular trafficking of TRPC3, the vesicle-associated membrane protein VAMP2 (Singh et al. 2004). Moreover, correct plasma membrane targeting of TRPC3 has been shown to involve the ankyrin domains in the N-terminus of TRPC3 (Wedel et al. 2003), as well as on a unique interaction between TRPC3 and PLCγ1, which generates a composite PH (pleckstrin homology) domain (van Rossum et al. 2005). This bimolecular domain comprised of two incomplete lipid-binding structures represented by the very N-terminus of TRPC3, and PH-c of PLCγ1 was found to bind PIP_2 as well as sphingosine-1-phosphate (van Rossum et al. 2005) and has been suggested as a structure essential for plasma membrane recruitment of TRPC3 channel complexes into the plasma membrane. Plasma membrane targeting appears to be governed in addition by the CIRB (calmodulin/IP_3 receptor binding site; 761–795aa) region in the C-terminus of TRPC3 (Wedel et al. 2003). As outlined above, dynamic changes in TRPC3 channel localization within the cell are likely to TRPC3 cellular conductance(s) side by side with lipid gating. Importantly, a recent study demonstrated mitochondria as an alternative, functionally relevant localization of TRPC3 channels. A subfraction of TRPC3 may serve Ca^{2+} uptake into mitochondria (Feng et al. 2013). Regulation of mitochondrial TRPC3 channels and its functional consequences remain to be clarified.

4 Pharmacology

A variety of nonselective Ca^{2+} entry blockers were identified as inhibitors of TRPC3 channel activity. 2-APB (2-aminoethoxydiphenyl-borate) was initially recognized as a common inhibitor of IP_3Rs and store-operated Ca^{2+} channels (Orai) (Hagenston et al. 2009; Bogeski et al. 2010). However, a direct inhibitory effect of 2-APB on TRPC3 channels was early on recognized. IP_3-independent, DAG-triggered TRPC3 activity was inhibited by about 50 % with 2APB (30 μM) in HEK293 cells expressing TRPC3 (Lievremont et al. 2005).

The lanthanides Gd^{3+} and La^{3+} are widely used as rather unspecific Ca^{2+} entry blockers, inhibiting TRP subtypes as well as Orai channels and voltage-gated

calcium ion channels (Putney 2010). However, block of TRP3 channels in different expression systems was reported to require divergent concentrations of lanthanides. When expressed in HEK293 cells, TRPC3 channels were completely inhibited at 150 μM La^{3+} (Zhu et al. 1998), while for channels expressed in COS-1 cells, maximum block required 10 μM of La^{3+} (Preuss et al. 1997). Gd^{3+} was found to inhibit receptor/PLC-regulated TRPC3 in HEK293 cells completely at 200 μM (Zhu et al. 1998). Interestingly, TRPC3 expressed in DT40 chicken B lymphocytes at limited expression levels was reported to generate a store-operated calcium Ca^{2+} entry pathway that is sensitive to Gd^{3+}, whereas the receptor-activated channels formed upon expression of higher levels of TRPC3 were insensitive to gadolinium (Vazquez et al. 2003). This indicates the lanthanides may discriminate different TRPC3 containing pore structures. Lanthanides have been shown to block TRPC3 currents in CHO cells at rather low levels from the cytoplasmic side (EC_{50} 0.02 μM) (Halaszovich et al. 2000). Thus intracellular accumulation of lanthanides may contribute at a variable degree to block of channels depending on the experimental conditions.

SKF96365 (1-(beta-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride) and flufenamate represent inhibitors that have been widely employed for inhibition of TRPC cation channels. These compounds effectively suppress membrane transport pathways in a rather nonselective manner with remarkable inhibitory potency for channels outside the TRP superfamily such as voltage-gated Ca^{2+} channels (Merritt et al. 1990) as well as Cl^- channels (Sergeant et al. 2001) .

The polycationic dye ruthenium red (RR) acts as a pan inhibitor on a wide range of cation channels including ryanodine receptors, mitochondrial calcium transporters, CatSper1, TASK, and moreover all members of the TRPV family as well as TRPM6 and TRPA1 (Vincent and Duncton 2011; Clapham 2007; Voets et al. 2004; Garcia-Martinez et al. 2000; Watanabe et al. 2002). Recent evidence reported a substantial voltage-dependent blocking action of 3 μM extracellularly applied RR on TRPC3 expressed in HEK293 (Lichtenegger et al. 2013). Notably, the "selectivity-filter glutamate" E630 was identified as the key determinant of RR sensitivity in TRPC3. Moreover, 10 mM RR was shown to inhibit a nonselective cation current in rat dorsal root ganglion neurons that was accounted to endogenously expressed TRPC3 (Qu et al. 2012).

The pyrazole derivative Pyr3 (ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate) has been presented as a first selective blocker of TRPC3 channels (Kiyonaka et al. 2009). TRPC3-dependent Ca^{2+} entry in HEK293 cells was inhibited by Pyr3 with the IC_{50} value of 0.7 μM. Subtype specificity was attributed to the trichloroacrylic amide group, and electron drawing side groups in C3 position of the pyrazole ring were proposed as key determinants of the inhibitory effect (Law et al. 2011). Studies reporting on a potent blocking effect of Pyr3 on store-operated calcium entry via Orai channels (Salmon and Ahluwalia 2010; Kim et al. 2011) raised doubts about the specificity for TRPC3. However, a novel pyrazole compound (Pyr10) was shown to exhibit substantial selectivity for TRPC3 (Schleifer et al. 2012). Pyr10 inhibited TRPC3 with an IC_{50}

of 0.72 μM while affecting Orai1 and other TRPC isoforms such as TRPC4, 5, and 6 to a much lower extent ($IC_{50} > 10$ μM). The class of pyrazole blockers provides with Pyr2 (BTP2) and Pyr6 further, but less selective TRPC3 blocker (Schleifer et al. 2012). Pyr2, for example, shows no subtype specificity among TRPC subtypes, blocking both TRPC5 and TRPC3 (He et al. 2005) as well as store-operated Ca^{2+} entry via Orai channels (Bogeski et al. 2010). It is of note that a recent in vitro study provided first evidence for suitability of Pyr3 as inhibitor of in-stent restenosis when applied locally in human blood vessels in terms of a drug-eluting stent strategy (Koenig et al. 2013). In view of the potential therapeutic significance (see below), efforts are currently made to develop small molecule inhibitors and modulators of TRPC3 with a potency exceeding that of pyrazoles. The selectivity and pharmacological value of such small molecule blocker and modulator awaits pharmacological characterization.

5 Downstream Signaling and (Patho)Physiological Role

TRPC3 is most prominently expressed in specific regions of the brain, in the heart and in the lungs along with particular high expression in embryonic tissues (Strubing et al. 2003), implying a function of the channel in development of neuronal and cardiac tissue, including a possible role as a determinant of cell proliferation and differentiation. In general, TRPC3 is likely to control cellular phenotype transitions and fate associated with tissue remodeling or degeneration. Ca^{2+} transport by TRPC3 appears to govern Ca^{2+} transcription coupling (Nakayama et al. 2006; Poteser et al. 2011) and was suggested to mediate Ca^{2+}-dependent tissue damage (Kim et al. 2011). Recent evidence suggests a specific and dynamic linkage of TRPC3 to downstream Ca^{2+} signaling cascades (Poteser et al. 2011) as well as a tight functional interaction of cardiovascular TRPC channels with signaling partners to enable spatially restricted and well-segregated cellular Ca^{2+} signals (Poburko et al. 2007, 2008). Moreover, TRPC3 was recently identified to control not only cytosolic Ca^{2+} gradients but also mitochondrial Ca^{2+} and membrane potential, as a subpopulation of the channel protein was found to localize to mitochondria and to determine mitochondrial Ca^{2+} handling and membrane potential (Feng et al. 2013). This peculiar aspect of TRPC3 channel function may well contribute to its impact on apoptosis and role in degenerative processes.

The function of TRPC3 channels in excitable cells may critically depend on the link of TRPC3 to other prominent Ca^{2+} transport systems in these cells. One example for such a signaling partnership is the observed association of TRPC3 channels to the cardiac-type Na^{+}/Ca^{2+} exchanger NCX1 (Rosker et al. 2004). It is of note that this close interaction between TRPC3 and NCX1 may be of significance as a determinant of cardiac excitability and susceptibility to arrhythmogenic stimuli. Similarly, functional cross talk may exist between TRPC3 and voltage-gated Ca^{2+} channels as reported for pulmonary artery smooth muscle dysfunction (Yu et al. 2004). Enhanced expression of TRPC3 is expected to cause membrane depolarization of vascular smooth muscle due to enhanced constitutive TRPC3

activity, leading to increased, pathophysiologically relevant Ca^{2+} entry through $Ca_V1.2$ (L-type) Ca^{2+} channels.

Recent evidence established TRPC3 as a novel postsynaptic channel critically involved in motor coordination by mediating mGluR-dependent synaptic transmission in cerebellar Purkinje cells. TRPC3 double knockout mice completely lack slow synaptic potentials and mGluR-mediated inward currents and display disturbed walking behavior (Hartmann et al. 2008). Consistently, a gain-of-function mutation (T635A) in TRPC3, referred to as moonwalker (Mwk) mutation, was shown to cause degeneration of Purkinje cells and cerebellar ataxia in these animals. Since growth and differentiation of Purkinje cells was dramatically impaired in Mwk mice, the authors proposed a critical role of TRPC3 in dendritogenesis and survival of Purkinje cells in the cerebellum (Becker et al. 2009). Thus, TRPC3 may be essential in development and function of certain brain regions.

TRPC homologs are widely expressed in human vessels of all calibers, with subtype TRPC3 showing the most abundant expression in coronary and cerebral arteries (Yip et al. 2004). Direct evidence for implication of TRPC3 in vasoregulation has been initially suggested by the finding of a key role of TRPC3 in endothelial Ca^{2+} signaling (Groschner et al. 1998; Kamouchi et al. 1999b) and thus in the formation of vasoactive mediators. The vascular role of TRPC3 was later extended for vascular smooth muscle. In human cerebellar artery myocytes, IP_3 was suggested to induce vasoconstriction by IP_3R-mediated TRPC3 activation, independently of Ca^{2+} release from intracellular stores (Xi et al. 2008). In those cells, vasoconstriction was finally shown to involve direct coupling between IP_3R1 and membrane-resident TRPC3 channels (Adebiyi et al. 2010). These studies support a role of TRPC3 channels in regulating the myogenic tone of resistance arteries and in controlling arterial blood pressure.

Beyond control of smooth muscle contractility, TRPC3 was identified as a determinant of phenotype switching in vascular smooth muscle and a key player in pathological remodeling such as restenosis in response to angioplasty. Impairment of inhibitory regulation of TRPC3 via serine threonine kinase with-no-lysine was found associated with both enhanced vascular tone and vascular smooth muscle hypertrophy (Park et al. 2011). Consistently, block of TRPC3 by Pyr3 was demonstrated as an efficient strategy for the prevention of in-stent restenosis in human arteries (Koenig et al. 2013). In airway smooth muscle cells, siRNA-mediated gene silencing and antibodies against TRPC3 were shown to block endogenous nonselective cation currents. Ovalbumin-sensitized cells exhibited increased TRPC3 protein expression and greatly augmented nonselective currents, which could be reversed by application of TRPC3 antibodies. Hence, TRPC3 likely constitutes an important molecular component of native nonselective cation channels regulating airway smooth muscle function as well as asthma-evoked airway hyper-responsiveness (Xiao et al. 2010).

In classical non-excitable tissues such as epi- and endothelial cells as well as immune cells (Liu et al. 2005; Bandyopadhyay et al. 2005; Groschner et al. 1998; Philipp et al. 2003), a physiological role of TRPC3 channels was suggested by

various approaches including dominant negative suppression, siRNA-mediated knockdown of expression, or selective block of channels by isoform specific antibodies. Similarly as in smooth muscle, TRPC3 may control both acute functions such as secretion of mediators and phenotype transitions. In immune cells, full understanding of the physiological function of TRPC3 needs a more complete understanding of the interaction of this channel subunit with the STIM/Orai Ca^{2+} entry machinery as a pivotal Ca^{2+} signaling mechanism in this system.

With respect to the potential (patho)physiological role of TRPC3, we face growing evidence for its involvement in the pathogenesis of cardiovascular diseases such as hypertension (Thilo et al. 2008, 2009) and cardiac maladaptive remodeling and hypertrophy (Nakayama et al. 2006; Wu et al. 2010; Onohara et al. 2006). A recent study uncovered a direct link between TRPC3 activity and pro-hypertrophic [nuclear factor of activated T cells (NFAT)] signaling pathway (Poteser et al. 2011). In HL1 atrial myocytes, nuclear translocation of NFAT was shown to require TRPC3-dependent microdomain Ca^{2+} signaling.

In summary, our current knowledge suggests TRPC3 as a multifunctional cellular sensor molecule with a wide range of (patho)physiological functions. TRPC3 channels give rise to cellular Ca^{2+} signals that are linked to divergent array of downstream signaling cascades including mechanism that control phenotype transitions and cell fate. A crucial role of this signaling molecule in cardiovascular and neuronal pathologies as well as its significance as a therapeutic target is likely.

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TRPC4- and TRPC4-Containing Channels

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Contents

Abstract

TRPC4 proteins comprise six transmembrane domains, a putative pore-forming region, and an intracellularly located amino- and carboxy-terminus. Among eleven splice variants identified so far, TRPC4α and TRPC4β are the most abundantly expressed and functionally characterized. TRPC4 is expressed in various organs and cell types including the soma and dendrites of numerous types of neurons; the cardiovascular system including endothelial, smooth muscle, and cardiac cells; myometrial and skeletal muscle cells; kidney; and immune cells such as mast cells. Both recombinant and native TRPC4-containing channels differ tremendously in their permeability and other biophysical properties, pharmacological modulation, and mode of activation depending on the cellular environment. They vary from inwardly rectifying store-operated channels with a high Ca^{2+} selectivity to non-store-operated channels predominantly carrying Na^{+} and activated by $G\alpha_q$- and/or $G\alpha_i$-coupled receptors with a complex U-shaped current–voltage relationship. Thus, individual TRPC4-containing channels contribute to agonist-induced Ca^{2+} entry directly or indirectly via depolarization and activation of voltage-gated Ca^{2+} channels. The

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differences in channel properties may arise from variations in the composition of the channel complexes, in the specific regulatory pathways in the corresponding cell system, and/or in the expression pattern of interaction partners which comprise other TRPC proteins to form heteromultimeric channels. Additional interaction partners of TRPC4 that can mediate the activity of TRPC4-containing channels include (1) scaffolding proteins (e.g., NHERF) that may mediate interactions with signaling molecules in or in close vicinity to the plasma membrane such as Gα proteins or phospholipase C and with the cytoskeleton, (2) proteins in specific membrane microdomains (e.g., caveolin-1), or (3) proteins on cellular organelles (e.g., Stim1). The diversity of TRPC4-containing channels hampers the development of specific agonists or antagonists, but recently, ML204 was identified as a blocker of both recombinant and endogenous TRPC4-containing channels with an IC_{50} in the lower micromolar range that lacks activity on most voltage-gated channels and other TRPs except TRPC5 and TRPC3. Lanthanides are specific activators of heterologously expressed TRPC4- and TRPC5-containing channels but can block individual native TRPC4-containing channels. The biological relevance of TRPC4-containing channels was demonstrated by knockdown of TRPC4 expression in numerous native systems including gene expression, cell differentiation and proliferation, formation of myotubes, and axonal regeneration. Studies of TRPC4 single and TRPC compound knockout mice uncovered their role for the regulation of vascular tone, endothelial permeability, gastrointestinal contractility and motility, neurotransmitter release, and social exploratory behavior as well as for excitotoxicity and epileptogenesis. Recently, a single-nucleotide polymorphism (SNP) in the *Trpc4* gene was associated with a reduced risk for experience of myocardial infarction.

Keywords

TRPC4-containing cation channels • Receptor- and store-operated activation • Ca^{2+} signaling • Biological functions

1 Molecular Properties, Expression, and Interaction Sites

The murine (chromosome 3) and human (chromosome 13) *Trpc4* gene exhibits 13 exons with genome loci spanning >250 kb (Birnbaumer et al. 2003). A full-length TRPC4 complementary DNA (cDNA) was initially cloned from bovine adrenal gland (Philipp et al. 1996). Subsequently, orthologue genes from mouse, rat, and human were cloned (McKay et al. 2000; Mizuno et al. 1999; Mori et al. 1998). The encoded proteins include TRPC4α splice variants (bovineTRPC4α, mouseTRPC4α, ratTRPC4α, humanTRPC4α) that comprise 974–981 amino acids. Besides TRPC4α, ten different splice variants of TRPC4 have been reported among which TRPC4α and TRPC4β are most abundantly

Table 1 Reported expression pattern of TRPC4 using different methodologies

Type of expression analysis	RT-PCR	Northern blot	In situ hybridization	Western blot	ICC	IHC	Other
Organ/tissue/cell							
Nervous system							
Brain	(Petersen et al. 1995)-m (McKay et al. 2000)-h (Walker et al. 2001)-m, -d (Facemire et al. 2004)-r (Fowler et al. 2007)-r (Liu et al. 2008b)-gp (Alvarez et al. 2008)-r	(McKay et al. 2000)-h (Freichel et al. 2001)-m (Gilliam and Wensel 2011)-m		(Freichel et al. 2001)-m (C-KO (Crousillac et al. 2003)-c/r (C-pab) (Liu et al. 2008b)-gp (C-pab) (Phelan et al. 2012)-m (C-KO)			
Fetal/embryonic brain	(McKay et al. 2000)-h (Boisseau et al. 2009)-m		(Zechel et al. 2007)-m				
Forebrain	(Kunert-Keil et al. 2006)-m						
Amygdala	(Riccio et al. 2002)-h (Faber et al. 2006)-r		(Fowler et al. 2007)-r-m	(Faber et al. 2006)-r (C-nd)			
Astrocytes	(Song et al. 2005)-h			(Song et al. 2005)-h (C-pab)	(Song et al. 2005)-h (C-ns)		(Song et al. 2005)-h (IEM) (C-ns)
Caudate nucleus (Basal ganglia)	(Meis et al. 2007)-m						
Caudate putamen (Basal ganglia)						(Chung et al. 2007)-r (C-pcp)	
Cerebrum	(Garcia and Schilling 1997)-r (Kunert-Keil et al. 2006)-m	(Mori et al. 1998)-m					
Cerebellum	(Garcia and Schilling 1997)-r (Riccio et al. 2002)-h (Kunert-Keil et al. 2006)-m (Huang et al. 2007)-r (Zechel et al. 2007)-m	(Philipp et al. 1996)-b (Mori et al. 1998)-m (Otsuka et al. 1998)-m	(Zechel et al. 2007)-m (Fowler et al. 2007)-r-m	(Zechel et al. 2007)-m (C-ns)		(Huang et al. 2007)-r (C-nd)	
Cingulate gyrus	(Riccio et al. 2002)-h						
Cortex	(Zechel et al. 2007)-m (Boisseau et al. 2009)-m (Embryo)			(Zechel et al. 2007)-m (C-ns)			

(continued)

Table 1 (continued)

Type of expression analysis	RT-PCR	Northern blot	In situ hybridization	Western blot	ICC	IHC	Other
Entorhinal cortex			(Fowler et al. 2007)-r-m				
Frontal cortex			(Fowler et al. 2007)-r				
Globus pallidus	(Riccio et al. 2002)-h					(Chung et al. 2007)-r (C-pcp)	
Habenula			(Fowler et al. 2007)-r-m				
Hippocampus	(Riccio et al. 2002)-h (Kunert-Keil et al. 2006)-m (Zechel et al. 2007)-m (Phelan et al. 2012)-m	(Philipp et al. 1998)-m (Mori et al. 1998)-m (Otsuka et al. 1998)-m	(Zechel et al. 2007)-m (Fowler et al. 2007)-r-m	(Zechel et al. 2007)-m (C-ns) (Wang et al. 2007a)-m (C-nd)		(Chung et al. 2006)-r (C-pcp) (Zechel et al. 2007)-m (C-ns)	
Hypothalamus	(Riccio et al. 2002)-h (Qiu et al. 2010)-m		(Fowler et al. 2007)-r-m				
Lateral geniculate nucleus	(Munsch et al. 2003)-r						
Lateral septum	(Phelan et al. 2012)-m		(Fowler et al. 2007)-r-m	(Fowler et al. 2007)-r (C-nd) (Phelan et al. 2012)-m (Control: KO)			
Locus coeruleus	(Riccio et al. 2002)-h						
Microglia	(Ohana et al. 2009)-r						
Middle frontal gyrus	(Riccio et al. 2002)-h						
Medulla oblongata	(Riccio et al. 2002)-h						
Motor cortex			(Fowler et al. 2007)-r-m				
Nucleus accumbens	(Riccio et al. 2002)-h						
Orbitofrontal cortex			(Fowler et al. 2007)-r-m				
Parahippocampus	(Riccio et al. 2002)-h						
Piriform cortex			(Fowler et al. 2007)-r-m				

Prefrontal cortex			(Fowler et al. 2007)-r-m	(Fowler et al. 2007)-r (C-nd)		
Putamen	(Riccio et al. 2002)-h					
Somatosensory cortex			(Fowler et al. 2007)-r-m			
Striatum	(Riccio et al. 2002)-h					
Subiculum			(Fowler et al. 2007)-r-m			
Substantia nigra	(Riccio et al. 2002)-h		(Fowler et al. 2007)-r-m		(Chung et al. 2007)-r (C-pcp)	
Subthalamic nucleus (Basal ganglia)	(Kunert-Keil et al. 2006)-m				(Chung et al. 2007)-r (C-pcp)	
Superior frontal gyrus	(Riccio et al. 2002)-h					
Tenia tecta			(Fowler et al. 2007)-r-m			
Thalamus	(Riccio et al. 2002)-h (Zechel et al. 2007)-m					
Tuberomammillary nucleus	(Sergeeva et al. 2003)-r					
Ventral tegmental area	(Sergeeva et al. 2003)-r					
Trigeminal ganglia	(Vandewauw et al. 2013)-m					
Spinal cord	(Riccio et al. 2002)-h			(Zechel et al. 2007)-m (C-ns)		
Nodose ganglion	(Garcia and Schilling 1997)-r (Elg et al. 2007)-m					
Dorsal root ganglion	(Elg et al. 2007)-m (Wu et al. 2008)-r (Staaf et al. 2009)-r-m (Vandewauw et al. 2013)-m				(Wu et al. 2008)-r (C-pcp)	
Olfactory bulb	(Zechel et al. 2007)-m	(Philipp et al. 1998)-m (Mori et al. 1998)-m (Otsuka et al. 1998)-m			(Stroh et al. 2012)-m (C-KO)	(Stroh et al. 2012)-m (IEM) (C-KO)

(continued)

Table 1 (continued)

Type of expression analysis	RT-PCR	Northern blot	In situ hybridization	Western blot	ICC	IHC	Other
Carotid body						(Buniel et al. 2003)-r (C-pab)	
Petrosal ganglion						(Buniel et al. 2003)-r (C-pab)	
Gonadotropin-releasing hormone neurons	(Zhang et al. 2013, 2008)-m (Bosch et al. 2013)-m						
Eye							
Retina	(Wimmers and Strauss 2007)-h (Gilliam and Wensel 2011)-m	(Philipp et al. 1996)-b (Gilliam and Wensel 2011)-m	(Gilliam and Wensel 2011)-m	(Gilliam and Wensel 2011)-m (C-nd)		(Crousillac et al. 2003)-c (C-pab (Gilliam and Wensel 2011)-m (C-nd)	
Ciliary muscle	(Takai et al. 2004)-b				(Sugawara et al. 2006)-b (C-nd)		
Corneal endothelial cells	(Xie et al. 2005)-b (Yang et al. 2005)-h			(Xie et al. 2005)-b (C-pab) (Yang et al. 2005)-h (C-pab)	(Xie et al. 2005)-b (C-pab)		
Pituitary gland	(Riccio et al. 2002)-h						
Thyroid gland	(McKay et al. 2000)-h						
Parathyroid gland	(Lu et al. 2010)-h			(Lu et al. 2010)-h (C-pcp)			
Adrenal gland	(Garcia and Schilling 1997)-r (McKay et al. 2000)-h	(Philipp et al. 1996)-b (Philipp et al. 2000)-b (cortex)	(Philipp et al. 2000)-b (cortex)				
Heart	(Garcia and Schilling 1997)-r (McKay et al. 2000)-h (Riccio et al. 2002)-h (Kuwahara et al. 2006)-m (Nakayama et al. 2006)-m (Alvarez et al. 2008)-r (Seth et al. 2009)-m	(Philipp et al. 1996)-b (McKay et al. 2000)-h		(Wu et al. 2010)-m (C-nd)			

Left ventricle	(Liu et al. 2010)-r		(Liu et al. 2010)-r (C-nd)		(Gronich et al. 2010)-h (MA)
Sinoatrial node	(Ju et al. 2007)-m		(Ju et al. 2007)-m (C-pcp)		
Pacemaker cells (SAN)				(Ju et al. 2007)-m (C-pcp)	
Ventricular myocytes				(Kojima et al. 2010)-m (C-nd)	
Cardiac fibroblasts	(Rose et al. 2007)-r (Chen et al. 2010)-h (Du et al. 2010)-h, -m				
Aorta	(Facemire et al. 2004)-r		(Facemire et al. 2004)-r (C-pcp)		
Pulmonary vein	(Peng et al. 2010)-r		(Peng et al. 2010)-r (C-nd)		
Endothelial cells	(Fantozzi et al. 2003)-h (PAEC)	(Freichel et al. 2001)-m (MAEC)	(Freichel et al. 2001)-m (C-KO) (Fantozzi et al. 2003)-h (C-KO) (C-pab) (Zhang et al. 2004)-h (C-nd)		
Lungs	(Garcia and Schilling 1997)-r (Riccio et al. 2002)-h				
Lung endothelial cells	(Tiruppathi et al. 2002)-m (Sundivakkam et al. 2012)-m		(Sundivakkam et al. 2012)-m (C-KO)		
Spleen	(McKay et al. 2000)-h (Riccio et al. 2002)-h (Kunert-Keil et al. 2006)-m				
Peripheral blood lymphocytes	(McKay et al. 2000)-h				
Bone marrow	(McKay et al. 2000)-h				
Megakaryocytes	(den Dekker et al. 2001)-h				
Platelets	(den Dekker et al. 2001)-h		(Brownlow and Sage 2005)-h (C-nd) (Wakabayashi et al. 2006)-h (C-nd)	(Liu et al. 2008a)-h (C-nd)	

(continued)

Table 1 (continued)

Type of expression analysis	RT-PCR	Northern blot	In situ hybridization	Western blot	ICC	IHC	Other
Neutrophils	(Itagaki et al. 2004)-h			(Itagaki et al. 2004)-h (C-ns)	(Itagaki et al. 2004)-h (C-ns)		
Kidney	(Garcia and Schilling 1997)-r (McKay et al. 2000)-h (Walker et al. 2001)-m/d (Riccio et al. 2002)-h (Kunert-Keil et al. 2006)-m	(McKay et al. 2000)-h		(Zechel et al. 2007)-m (C-ns)		(Wang et al. 2004)-m (C-nd) (Sours et al. 2006)-h (C-nd)	
Cortex				(Lee-Kwon et al. 2005)-r (C-ns)			
Medulla				(Lee-Kwon et al. 2005)-r (C-pcp)		(Lee-Kwon et al. 2005)-r (C-ns)	
Mesangial cells	(Wang et al. 2004)-m (Sours-Brothers et al. 2009)-h			(Wang et al. 2004)-m (C-ns) (Sours et al. 2006)-h (C-ns) (Du et al. 2008)-h (C-ns (Sours-Brothers et al. 2009)-h (C-nd)	(Wang et al. 2007b; Wang et al. 2004)-m, -h (C-pab) (Sours et al. 2006)-h (C-nd)	(Wang et al. 2004)-m (C-nd) (Sours et al. 2006)-r (C-nd)	
Renal epithelial cells	(Veliceasa et al. 2007)-h						
Preglomerular resistance vessels	(Facemire et al. 2004)-r						
Vasa recta	(Lee-Kwon et al. 2005)-r					(Lee-Kwon et al. 2005)-r (C-ns)	
Bladder	(Yu et al. 2011)-m					(Yu et al. 2011)-m (C-ns)	
Urothelium	(Yu et al. 2011)-m			(Yu et al. 2011)-m (C-ns)		(Yu et al. 2011)-m (C-ns)	
Liver	(Riccio et al. 2002)-h			(Zechel et al. 2007)-m (C-ns)			
Fetal liver	(McKay et al. 2000)-h						
Digestive system							
Stomach	(McKay et al. 2000)-(Walker et al. 2001)-m, -d (Riccio et al. 2002)-h						

Intestine	(Riccio et al. 2002)-h					
Small intestine	(McKay et al. 2000)-h		(Lee et al. 2005)-m (C-ns)			
Submucosal plexus	(Liu et al. 2008b)-gp		(Liu et al. 2008b)-gp (C-pab)		(Liu et al. 2008b)-gp (C-pab)	
Longitudinal muscle myenteric plexus	(Liu et al. 2008b)-gp		(Liu et al. 2008b)-gp (C-pab)		(Liu et al. 2008b)-gp (C-pab)	
Myenteric ganglia	(Liu et al. 2008b)-gp		(Liu et al. 2008b)-gp (C-pab)			
Ileum smooth muscle cells			(Tsvilovskyy et al. 2009)-m (C-KO)			
Jejunum	(Walker et al. 2001)-m, -d					
Colon	(Walker et al. 2001)-m, -d		(Torihashi et al. 2002)-m (C-pab)		(Torihashi et al. 2002)-m (C-pab)	(Torihashi et al. 2002)-m (IEM) (C-nd)
Mesenteric arterioles			(Chen et al. 2010)-r (C-ns)			
Pancreas	(McKay et al. 2000)- (Riccio et al. 2002)-h	(McKay et al. 2000)-h				
Adipose tissue	(Riccio et al. 2002)-h					
Prostate	(McKay et al. 2000)-h (Riccio et al. 2002)-h					
Testis	(Garcia and Schilling 1997)-r (McKay et al. 2000)-h	(Philipp et al. 1996)-b	(Zechel et al. 2007)-m (C-ns)			
Sperm				(Castellano et al. 2003)-h (C-nd)		
Ovary	(Garcia and Schilling 1997)-r (McKay et al. 2000)-h					
Uterus	(McKay et al. 2000)-h	(Mori et al. 1998)-m				
Myometrium (no pregnancy)	(Dalrymple et al. 2004)-h (Ulloa et al. 2009)-h		(Ulloa et al. 2009)-h (C-nd)			
Myometrium (pregnancy)	(Dalrymple et al. 2002, 2004)-h (Ku et al. 2006)-h		(Dalrymple et al. 2002, 2004)-h (C-pab) (Ku et al. 2006)-h (C-ns)		(Dalrymple et al. 2002)-h (C-pab) (Ku et al. 2006)-h (C:PBS)	

(continued)

Table 1 (continued)

Type of expression analysis	RT-PCR	Northern blot	In situ hybridization	Western blot	ICC	IHC	Other
Myometrial SMC	(Dalrymple et al. 2007, 2002, 2004)-h (Ku et al. 2006)-h			(Dalrymple et al. 2007, 2002)-h (C-pab)	(Dalrymple et al. 2002)-h (C-pab)		
Placenta	(McKay et al. 2000)-h (Riccio et al. 2002)-h (Clarson et al. 2003)-h	(McKay et al. 2000)-h				(Clarson et al. 2003)-h (C-pab)	
Skeletal muscle	(McKay et al. 2000)-h (Riccio et al. 2002)-h (Kunert-Keil et al. 2006)-m			(Sabourin et al. 2009)-m (C-nd) (Antigny et al. 2013)-h (C-nd)			
Skin	(McKay et al. 2000)-h						
Keratinocytes	(Fatherazi et al. 2007)-h (Beck et al. 2008)-h			(Fatherazi et al. 2007)-h (C-pab[a]) (Beck et al. 2008)-h (C-nd)	(Beck et al. 2008)-h (C-nd)		
Cartilage	(Riccio et al. 2002)-h						
Bone	(Riccio et al. 2002)-h						
Osteoblasts (-like cells)	(Abed et al. 2009)-h-m						

For those expression analyses using anti-TRPC4 antibodies, the type of the corresponding control experiment(s) or a comment regarding the validation of the antibodies is included. *ICC* immunocytochemistry, *IEM* immunoelectron microscopy, *IHC* immunohistochemistry, *ab* antibody, *C-KO* control used corresponding knockout mouse specimen, *C-nd* control not defined, *C-pab* control used pre-absorbed antibody, *C-pcp* control preincubation with control peptide/antigen, *C-ns* control not shown, *MA* microarray, *SMC* smooth muscle cells, *MAEC* mouse aortic endothelial cells, *PAEC* pulmonary artery endothelial cells, *SAN* sinoatrial node, *h* human, *m* mouse, *r* rat, *gp* guinea pig, *d* dog, *b* bovine, *c* chicken

[a]In this case, the antibody was validated previously using different tissues from KO mice

expressed and functionally characterized (Cavalie 2007; Plant and Schaefer 2003, 2005). The TRPC4β variant lacks a domain of 84 amino acids in the C-terminal region containing a putative binding site for calmodulin (CaM) binding and inositol 1, 4, 5-trisphosphate (IP_3) receptors present in TRPC4α (see below). This 84 aa domain is also critical for the regulation of TRPC4 channel activity by PI(4, 5)P_2 (Sect. 2). TRPC4 is expressed in numerous cell types and organs. Table 1 gives a comprehensive survey of the expression pattern of TRPC4 transcripts and detection of TRPC4 proteins including detailed information about the methodology to allow interpretation of the reported findings. In addition to the expression pattern summarized in Table 1, TRPC4 is expressed in mast cells (Freichel et al. 2012) and various types of endothelial (Wong and Yao 2011; Yao and Garland 2005) and vascular smooth muscles cells (Beech et al. 2004; Dietrich et al. 2007; Gonzalez-Cobos and Trebak 2010; Inoue et al. 2006, 2009) which is summarized together with expression of other TRPs in detail elsewhere.

The concept of the makeup of TRPC4-containing channel complexes is based on the analysis of the TRPC4 amino acid sequence and the identification of interaction with other ion channel-forming membrane proteins suggesting the formation of heteromultimeric channels as well as with regulatory interaction partners located in the plasma membrane, the cytosol, or on cellular organelles. A summary of the corresponding structural domains is given in Fig. 1. Based on computational sequence analysis, TRPC4α and TRPC4β comprise 6 transmembrane segments (TM1–TM6) with a putative pore-forming region between TM5 and TM6 very similar to other TRPCs with the amino- and carboxy-terminus located intracellularly. Mutations in the pore-forming region that lead to changes in the permeability of channels evoked by expression of *Trpc4* DNA were not reported; however, mutations in glutamic acid E_{559} or E_{598} of TRPC1 which are conserved in TRPC4 evoked a decrease in store-operated Ca^{2+}- but not Na^+ currents and a left shift in the reversal potential (Liu et al. 2003). Another mutation in the pore region in the L_{575}WF motif in TRPC5 (to AAA, Fig. 1) which is conserved in TRPC4 and TRPC1 resulted in dominant-negative channel proteins in TRPC5 without affecting plasma membrane targeting (Strubing et al. 2003). Yoshida et al. (2006) showed that C_{553} and C_{558} located at the N-terminal side of the putative pore in TRPC5 are nitrosylated upon stimulation with NO donors which could explain the NO-induced activation of TRP channels in which these cysteins are conserved such as in TRPC4. TRPC4 contains also the four short amino acid sequence motifs, M1–M4, that represent typical features of TRP proteins. Motif M1 is located upstream of TM1 and is conserved in this form throughout the TRPC subfamily. Motif M2 resides within the cytosolic loop between TM 4 and TM 5. Motif M3 (VVLLNMLIAMM) is part of TM6 of all TRPCs, TRPVs, and TRPMs. Motif M4 is a highly conserved TRP protein-specific domain containing a "TRP box" (consensus sequence **WK**X**Q**R throughout the whole TRP family and WKFAR in the TRPC subfamily) immediately downstream of TM 6. $E_{648}E_{649}$ located within this M4 motif are conserved as $D_{652}E_{653}$ in TRPC5 (Fig. 1) and $D_{639}D_{640}$ in TRPC1 (not shown in Fig. 1). These negatively charged residues are critical for electrostatic interaction with the positively charged $K_{684}K_{685}$ in Stim1 proteins which are located in the membrane of the endoplasmic reticulum and can contribute to

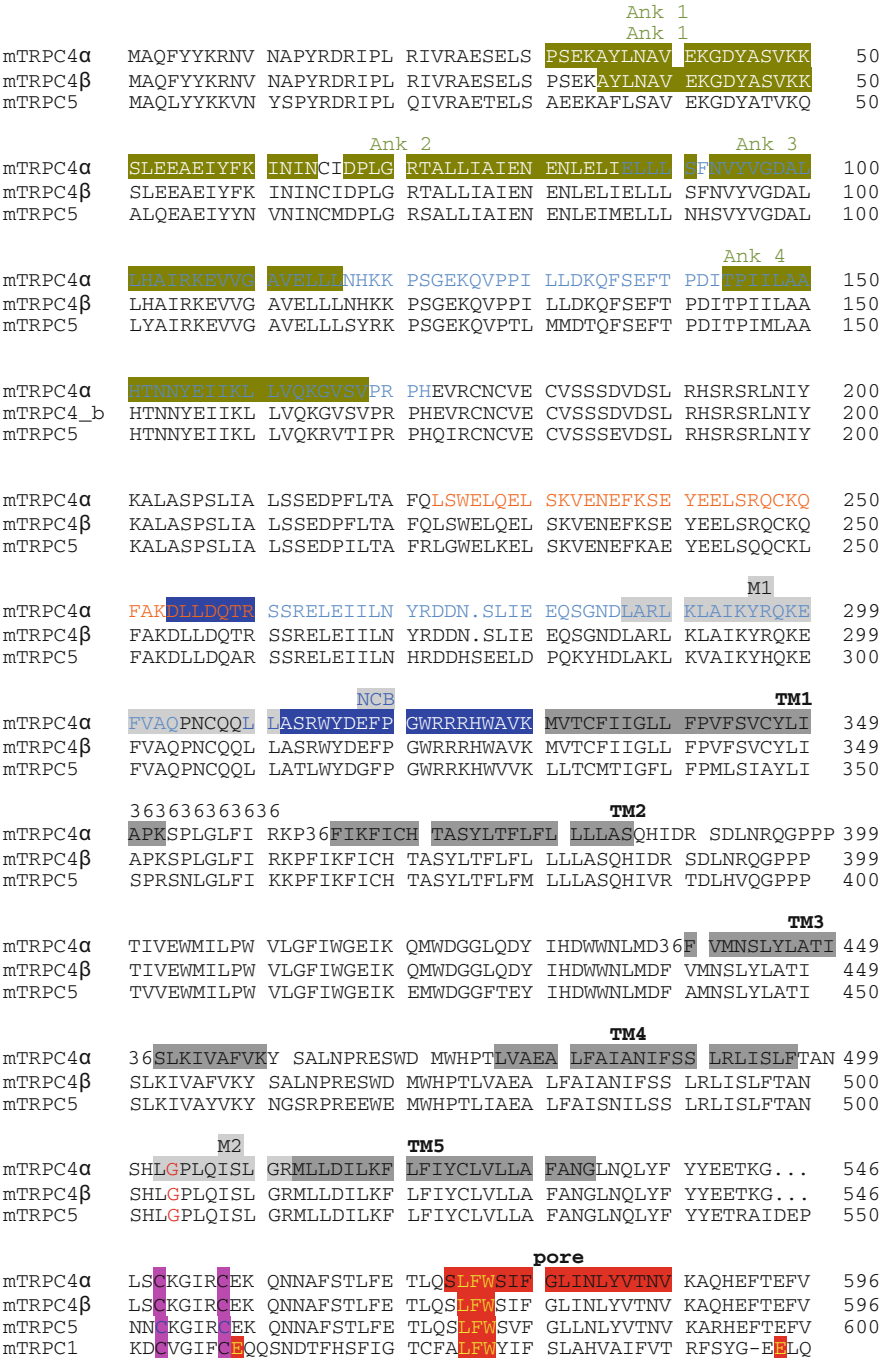

Fig. 1 (continued)

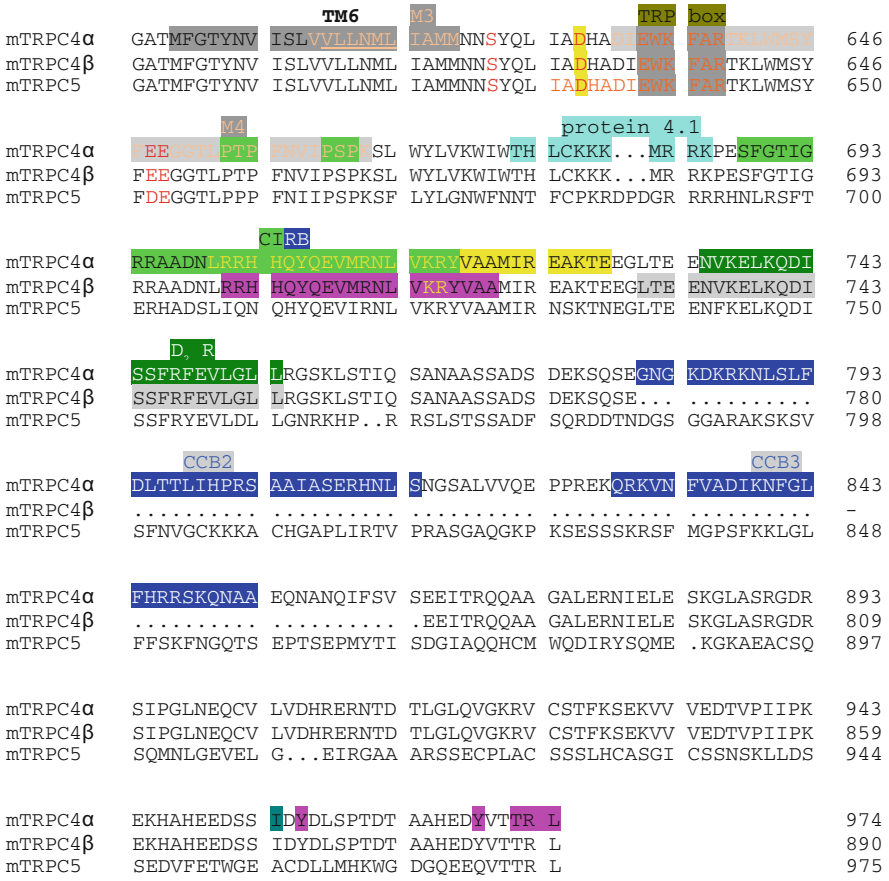

Fig. 1 Amino acid sequence alignment of TRPC4α and TRPC4β and TRPC5 highlighting functional domains and interaction sites. The sequence of mTRPC1 is also given in the region of the putative pore. TM1–6, transmembrane domains 1–6; pore, putative pore-forming region; and Ank 1–4, ankyrin-like repeats. Ank 1 was not identified with the algorithms used in Schindl et al. (2008). E_{87} to H_{172} and D_{254}–Q_{304} are predicted as multimerization domains (http://www.nextprot.org); L_{223}–R_{260} represent a coiled-coil domain. Motifs M1–M4 representing TRP protein characteristic features are indicated as X or X. The proline-rich region within M4 is indicated by X. G_{503} (and corresponding G_{504} in TRPC5) were identified as part of a channel-gating element that could interact with S_{623} at the end of TM 6. NCB: N-terminal calmodulin-binding site. Carboxy-terminal calmodulin-binding site 1 (CCB1), IP_3 receptor-binding site 1, the $G_{\alpha i2}$ interaction site, and the SSTDE1-binding site (L_{706} to E_{728}) overlap; $G_{\alpha i2}$ interaction site (R_{701}-A_{720}) is highlighted in the TRPC4β sequence where it was identified; K_{715} and R_{716} are most critical for activation of TRPC4β by co-expressed $G_{\alpha i2}$ proteins. The second IP_3R-binding site in the C-terminus of TRPC4α and TRPC4β (between aa 733 and 974) could not be confined to a short sequence like CIRB and is not labeled. A protein 4.1 interaction site was predicted based on consensus sequences. N_{735}-L_{754} identified as a binding site for proteins of the spectrin family (spectrin αII and βV) overlaps almost completely with the fragment (L_{731}-L_{754}) that was mapped as interaction site with D2 dopamine receptors. I957V (SNP A3104G in hTRPC4α) is a gain-of-function mutation associated with protection against myocardial infarction. Y at position 956 and 969 are phosphorylated by Src family tyrosine kinases following EGF receptor stimulation. Accession number of mTRPC4α is NM_016984; of mTRPC4β, NM_001253682; of mTRPC5, NM_009428; and of mTRPC1, NM_011643

activation of cation currents evoked by these TRPCs (Lee et al. 2010b; Zeng et al. 2008). D_{633} in TRPC5 which is also conserved in TRPC4 is located just upstream of the TRP box and resides in the cytosol according to the assumed topology of TRPC4 and TRPC5. A negatively charged residue at this position of TRPC5 was shown to be critical for the amplitude of inward currents and Mg^{2+}-dependent block of outward currents suggesting an important role of D_{633} for electrostatic attraction of cation (Obukhov and Nowycky 2005). Recently, a conserved structural element was identified in Motif M2 in TRPC4 and TRPC5. Mutation of the nonpolar G_{503} in TRPC4 to a polar serine resulted in spontaneous activity channels that could not be further increased by receptor stimulation as well as in intracellular Ca^{2+} overload and cell death. Thus, this conserved glycine residue within the cytosolic S4–S5 linker is a critical constituent of the gating site of TRPC4 and TRPC5 channels, and structural modeling and mutagenesis experiments suggest that S_{623} in TM6 is the corresponding interaction site (Beck et al. 2013).

TRPC4 contains four ankyrin-like repeats (Ank 1–Ank 4, Fig. 1), a coiled-coil domain, and two predicted multimerization domains (http://www.nextprot.org) in the N-terminus (Fig. 1) as potential protein–protein interaction motifs. Lepage et al. used chimeric proteins containing these domains for immunoprecipitation experiments and found that all ankyrin repeats and the coiled-coil domain of TRPC4 are required for interaction and that also the C-terminal tail is involved in oligomerization of TRPC4 (Lepage et al. 2006). In addition, this N-terminal fragment comprising Ank 1 to Ank 4 was shown to interact with MxA, an interferon-induced 76-kDa GTPase that is also associated with other TRPC proteins (Lussier et al. 2005). Later two inter-subunit interaction domains were narrowed down within the TRPC4 N-terminus, one in the third and fourth ankyrin repeat (E_{87}–H_{172}) and one downstream from the predicted coiled-coil domain (D_{254}–P_{304}) (Lepage et al. 2009). More recently, Schindl et al. showed that Ank 1 is sufficient to mediate homo- and heteromeric interaction of heterologously expressed TRPC4 and TRPC5 protein fragments using FRET microscopy (Schindl et al. 2008).

TRPC4α contains four calmodulin-binding sites as a structural correlate for the regulation of TRPC4 channel activity by changes in the cytosolic Ca^{2+} concentration: one in the N-terminus (NCB) immediately before TM1 which is not well characterized functionally and three in the C-terminus (CCB1–CCB3) as summarized in detail (Cavalie 2007; Zhu 2005). CCB1 and CCB2 (Tang et al. 2001) and CCB1 and CCB3 (Trost et al. 2001) were found independently using TRPC4–GST fusion proteins retained by calmodulin-bound matrices. CCB2 and CCB3 reside within the 84 aa stretch of TRPC4α that is lacking in TRPC4β. Interestingly, CCB1 overlaps with one of two IP_3 receptor-binding sites found in TRPC4 and the interaction of TRPC4 and IP_3 receptor fragments is inhibited by calmodulin (Mery et al. 2001; Tang et al. 2001). Therefore, this domain is called a dual CaM- and IP_3 receptor-binding site (CIRB). A second IP_3R-binding site found in the C-terminus of TRPC4α and TRPC4β (between aa 733–974) was found, but it could not be confined to a short sequence like CIRB (Mery et al. 2001; Tang et al. 2001). The finding of TRPC4–IP_3 receptor interaction fits to the concept that some TRPC4-containing channels may be activated following IP_3 receptor

activation and subsequent Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores. Local Ca^{2+} elevation enhances the affinity of CaM to the TRPC4 CIRB site and may inhibit TRPC4 channel activity by a displacement mechanism (Zhu 2005). However, the complexity of interactions at the CIRB domain increased recently since the CIRB site overlaps to a large extent with a stretch of amino acids that were identified as the interaction sites of TRPC4 with SSTDE1 (TRPC4 fragment L_{700}–E_{728}) proteins. SSTDE1 was found using a yeast two-hybrid screen and represents a previously uncharacterized protein containing a lipid-binding SEC14-like domain as well as spectrin-type cytoskeleton interaction domains (Miehe et al. 2010). SSTDE1 binds several phospholipids including PI(4, 5)P_2 in a Ca^{2+}-dependent manner, interacts also with TRPC5, and modulates TRPC5-mediated Ca^{2+} entry. In addition, a fragment that encompasses parts of the CIRB and the SSTDE1 binding site was mapped as domain of TRPC4 that interacts with $G_{\alpha i2}$ (TRPC4 fragment R_{701}–A_{720}) (Jeon et al. 2012). Within that domain, K_{715} and R_{716} are most critical for the activation of TRPC4β by co-expressed $G_{\alpha i2}$ proteins.

A few amino acids downstream of the CIRB site, an interaction site between the D_2 dopamine receptor and TRPC1, TRPC4, and TRPC5 was found. GST pull-down assays and co-immunoprecipitation experiments narrowed down residues L_{735}–L_{754} as the interacting amino acids in TRPC4 (Hannan et al. 2008). Similarly, like the CIRB site, this TRPC4 domain may serve as an interaction hot spot since L_{731}–L_{754} was pinpointed as the binding site for proteins of the spectrin family (spectrin αII and βV) which are involved in EGF-induced membrane insertion of TRPC4 and activation of TRPC4-mediated Ca^{2+} entry (Odell et al. 2008).

The absolute C-terminus of TRPC4 (TTRL motif, also present in TRPC5) serves as a PDZ-interacting domain through which TRPC4/TRPC5 associate with NHERF (Na^+/H^+ exchanger regulatory factor). NHERF is a scaffolding protein with two PDZ domains that also associates with phospholipase C–β and interacts with the actin cytoskeleton through binding of members of ezrin/radixin/moesin family (Mery et al. 2002; Tang et al. 2000). Deletion of the carboxy-terminal TRL residues of hTRPC4 dramatically reduced the plasma membrane levels of the channel, which led the authors to suggest that the interaction between hTRPC4 and NHERF is required for the retention and stabilization of hTRPC4 channels in the cell membrane (Mery et al. 2002). However, TRPC4-mediated cation currents were not reduced following deletion of the C-terminal PDZ-binding motif (Otsuguro et al. 2008).

Additional interacting partners of TRPC4 (also summarized at http://trpchannel.org/summaries/TRPC4), whose binding site was not narrowed down by, e.g., GST fusion proteins, include FKBP52, an immunophilin precipitated using an antibody directed against TRPC4 (Sinkins et al. 2004), and FKBP51 which was found as a TRPC4-interaction partner (together with FKBP 52) in endothelial cells (Kadeba et al. 2013), the scaffolding protein zonula occludens 1 (ZO-1) (Song et al. 2005), RNF24 which can reduce TRPC4 plasma membrane insertion (Lussier et al. 2008), and phosphorylated VASP in mesangial cells (Wang et al. 2007b). TRPC4 can also be co-immunoprecipitated with Src family tyrosine kinases Fyn, Lyn, and Src. Tyrosine phosphorylation at positions 956 and 969 by these kinases was implicated

in Ca^{2+} entry evoked following EGF receptor stimulation (Odell et al. 2005). Already earlier, TRPC4-mediated Ca^{2+} entry evoked by EGF was shown to be inhibited by the tyrosine kinase inhibitor genistein (Schaefer et al. 2000). A molecular association was also found between TRPC1 and TRPC4 channels and the α1-syntrophin–dystrophin complex, and both TRPCs contribute to abnormal Ca^{2+} entry when expression of α1-syntrophin is reduced (Sabourin et al. 2009). In murine lung endothelial cells, TRPC4 binds caveolin-1 (Cav-1) which was shown by co-immunoprecipitations using Cav-1-deficient cells as a control (Murata et al. 2007). Results from another study performed in pulmonary endothelial cells also suggested TRPC4 localization in membrane-bound caveolae, and an interaction was demonstrated with protein 4.1 which like ankyrin links transmembrane proteins to the underlying membrane skeleton (Cioffi et al. 2005). A conserved protein 4.1 binding domain was predicted after the proline-rich region in the M4 motif (PTPXXXXPSP, Fig. 1), and deletion of this domain reduced TRPC4-mediated store-operated Ca^{2+} currents in endothelial cells. In a follow-up study, it could be shown that the protein 4.1–TRPC4 complex also comprises TRPC1 and Orai1 proteins (Cioffi et al. 2012). The formation of heteromeric TRPC complexes was already shown in 2002 upon co-expression in HEK 293 cells (FRET-based assay) and Sf9 insect cells (immunoprecipitations using anti-TRPC antibodies) showing interaction of TRPC4 with TRPC1 and TRPC5 but not with members of the TRPC3, TRPC6, and TRPC7 subgroup (Goel et al. 2002; Hofmann et al. 2002); in the latter study, the first co-immunoprecipitation from native tissue using synaptosomal preparations confirmed that such heteromultimers may also occur in vivo in cerebellum and cortex of adult rats. In embryonic rat brain in which additional cofactors crucial for TRPC protein multimerization may be expressed, TRPC1 and TRPC4 could also be co-immunoprecipitated with TRPC3 or TRPC6 (Strubing et al. 2003). In porcine aortic endothelial cells, an interaction of TRPC4 with TRPC3 was shown, and both TRPCs may associate as constituents of a redox-sensitive cation channel (Poteser et al. 2006). Nevertheless, in none of the reported immunoprecipitation studies, preparations from corresponding TRPC-deficient tissues were used as controls. This is of particular importance in this respect since specific antibodies are rare for most TRP proteins (Flockerzi et al. 2005; Meissner et al. 2011), and, accordingly, this impedes investigations of the assembly and composition of TRP channel complexes by proteomic approaches as have been performed for other ion channels (Muller et al. 2010; Schwenk et al. 2012). In addition, it aggravates the analysis of localization of endogenous TRP channel proteins but also the control of the effectiveness of RNAi approaches (see Sect. 3).

2 Biophysical Properties and Regulation of Activity of TRPC4-Containing Channels

Heterologous expression of TRPC4: Functional characterization of TRPC4-dependent cation channel activity is referred to TRPC4α and TRPC4β, but the functional role of the additional splice variants remains unclear until now. The first

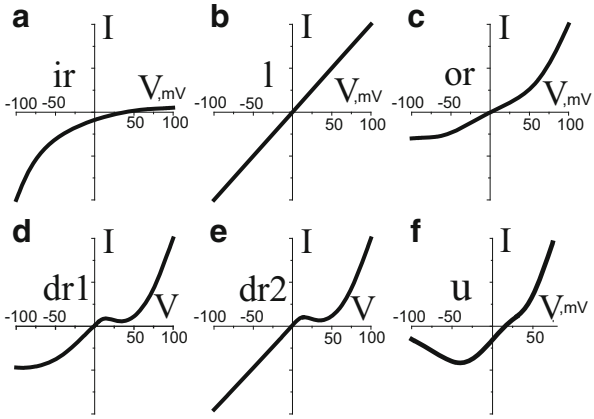

Fig. 2 Typical shapes of current–voltage relationships observed by measurements of TRPC4-containing channels in different cell systems and experimental conditions. (**a**) ir, inwardly rectifying; (**b**) l, linear; (**c**) or, outwardly rectifying; (**d**) dr1, doubly rectifying 1; (**e**) dr2, doubly rectifying 2; and (**f**) u, U-shaped

functional characterization of bovine TRPC4α proteins heterologously overexpressed in HEK 293 cells by Veit Flockerzi's laboratory described it as a calcium entry channel activated by depletion of intracellular Ca^{2+} stores (Philipp et al. 1996) which made it a candidate for a pore-forming subunit of capacitative Ca^{2+} entry (CCE) channel. This Ca^{2+} entry pathway was first described by Jim Putney (1986), and TRPC4 was therefore termed CCE1 originally until a common nomenclature was introduced for all mammalian orthologues of *Drosophila trp* and *trpl* (Montell et al. 2002b). TRPC4α-mediated currents had a reversal potential close to 0 mV in conditions of physiological extracellular cation concentrations (Philipp et al. 1996). The calculated P_{Ca}/P_{Na} was 7. The current–voltage (I–V) relationship of this current was inwardly rectifying (see Fig. 2a). In a consecutive study, overexpression of this protein in CHO and RBL cells confirmed the store-operated activation mode and its high calcium selectivity indicating that expression of TRPC4 proteins enhances the density of endogenous calcium-selective store-operated channels and suggesting that TRPC4 serves as a subunit relevant for the permeability of these channels (Warnat et al. 1999). Also in *Xenopus* oocytes, endogenous store-operated Ca^{2+} currents were enhanced by heterologous expression of rTRPC4; interestingly, these store-operated currents were facilitated by cytosolic calcium ions with a higher sensitivity compared to the oocytes endogenous CCE channels (Kinoshita et al. 2000). The group of James Putney expressed human TRPC4α in CHO cells. However, whole-cell patch clamp experiments revealed a constitutively active nonselective cation current with linear current–voltage relationship (see Fig. 2b) which could not be further increased by phospholipase C-linked receptor activation or by depletion of Ca^{2+} stores (McKay et al. 2000). In another study performed with HEK cells stably expressing murine TRPC4α or TRPC4β proteins, the corresponding currents were activated after chelating intracellular calcium following application of calcium chelators and exhibited properties of nonselective currents with typical outward rectification (Fig. 2c) (Walker et al. 2002). After application of the calmodulin inhibitor calmidazolium, the current amplitude increased and was restored to control levels

by 10 μM La^{3+}. Overall, it was concluded that these TRPC4α- and TRPC4β-evoked Ca^{2+}-inhibited nonselective currents share many characteristics with pacemaker currents present interstitial cells of Cajal (Koh et al. 2002). The group of Guenter Schultz investigated the regulatory and biophysical properties of murine TRPC4β heterologously expressed in HEK cells and found that stimulation of $G_{q/11}$-coupled receptors evoked large, nonselective cation currents ($P_{Ca}/P_{Na} = 1.1$), but no constitutive activity was observed and depletion of intracellular Ca^{2+} stores failed to activate mTRPC4 (Schaefer et al. 2000). Similar currents were recorded when human or rat TRPC4α or TRPC4β were expressed in HEK cells, although TRPC4-α-mediated currents were smaller in amplitude which was explained by autoinhibitory structures in its C-terminal part (Schaefer et al. 2002). From these results, it was concluded that TRPC4 proteins form nonselective cation channels that integrate signaling pathways from G-protein-coupled receptors independently of store depletion (Schaefer et al. 2000, 2002). Common features of TRPC4 currents were a reversal potential close to 0 mV with physiological extracellular cation concentrations and a current–voltage relationship with a complex shape usually described as doubly rectifying (see Fig. 2d, dr1). Inward currents increase at negative potentials, but the slope of the current–voltage relationship decreases at very negative potentials. When inward currents are close to maximum, the inward component of the current–voltage relationship becomes more linear [Fig. 2e, dr2, for review see Plant and Schaefer (2003)]. Therefore, the differences in the shape of the current–voltage relationship were explained by differences in the extent of channel activation. A detailed analysis of a similar phenomenon observed in a native current in ileal smooth muscle cells regarding this process was performed by Alexander Zholos et al. in 1994 on muscarinic cationic current termed mI_{CAT}, of which TRPC4 is an essential constituent (see below), and it was attributed to a shift of current activation curve toward more negative potentials by increasing fractional receptor occupancy (Zholos and Bolton 1994). At potentials above 0 mV (see Fig. 2e, dr2), currents first increase, being followed by a flat region, or a region of negative slope between +20 and +40 mV. Above these values, currents increase again. In currents obtained following heterologous expression of TRPC5, this flat region of the *I–V* at positive potentials has been attributed to a blockade by internal Mg^{2+} (Obukhov and Nowycky 2005). Substitution of the aspartate residue at position 633 by alanine abolished this flat region, making the *I–V* more similar to typical outwardly rectifying currents (see Fig. 2c) and indicating that D633 is a determinant of the blockade of TRPC5 channels by internal Mg^{2+} (Obukhov and Nowycky 2005). It is likely that ionic channels formed by TRPC4 are also blocked by internal Mg^{2+}, but this issue to date has not been addressed experimentally for TRPC4. Following TRPC4 expression, also a "U"-shaped *I–V* relationship of the inward current was observed (see Fig. 2f) (Strubing et al. 2001) which was attributed to low levels of channel activation (Plant and Schaefer 2003; Schaefer et al. 2002) or could be evoked by applying slow voltage ramp protocols (Otsuguro et al. 2008; Phelan et al. 2012). Interestingly, expression of murine TRPC4 in HEK 293 cells leads to an increase in inwardly rectifying K^+ currents suggesting that

expression of TRPC4 most likely upregulates the expression or activity of endogenous inwardly rectifying K^+ channels upon TRPC4 expression (Zhang et al. 2001).

Heterologous expression of TRPC4 with other TRPCs: Within the TRPC subfamily, TRPC4 is most closely related to TRPC5 sharing 65 % identity (Montell et al. 2002a). The TRPC4 and TRPC5 channels share many functional characteristics: both could be potentiated by G-protein-coupled receptors (GPCRs), and similar current–voltage relationships were reported in many studies (Lee et al. 2003; Okada et al. 1998; Schaefer et al. 2000). TRPC4 can associate with TRPC1, TRPC3, TRPC5, and TRPC6 in some cell systems (see above), but functional characterization of TRPC4 expressed with other TRPCs and its comparison with TRPC4 alone have been described in detail for TRPC4 and TRPC1 only (Strubing et al. 2001). It was shown that the *I–V* relationship in cells overexpressing TRPC4 differed markedly from that observed in cells co-transfected with TRPC1 and TRPC4. At membrane potentials more positive than the reversal potential, the outward currents were nearly constant up to +60 mV in cells transfected with TRPC4, but this flat region was absent in cells expressing TRPC1 + TRPC4. Below the reversal potential, the inward current amplitudes were larger at slightly negative membrane potentials than at strongly negative potentials both in cells expressing TRPC4 and TRPC1 + TRPC4 similarly as depicted in Fig. 2f.

TRPC4-containing channels analyzed using knockdown or knockout of TRPC4: After functional characterization of TRPC4 proteins in heterologously expression systems (Philipp et al. 1996; Warnat et al. 1999), the group of Veit Flockerzi expressed *Trpc4* cDNA in antisense orientation in bovine adrenal cells which significantly reduced both the amount of native TRPC4 protein expression and endogenous CRAC-like currents. These currents exhibited properties similar to those previously described by these authors following heterologous expression of TRPC4 such as store-operated mode of activation, high calcium selectivity, and clear inward rectification indicating that TRPC4 contributes essentially to the formation of native CRAC-like channels in these adrenal gland cells (Philipp et al. 2000). In human corneal epithelial cells, a knockdown approach using siRNA demonstrated a contribution of TRPC4 to store-operated channels activated in physiological conditions by epidermal growth factor (Yang et al. 2005). A similar approach revealed a participation of TRPC4 to calcium-selective CRAC-like currents in human gingival keratinocytes (Fatherazi et al. 2007). A strong argument supporting that TRPC4 serves as an essential constituent of highly calcium-selective store-operated channels in primary cells was worked out by the groups of Veit Flockerzi and Bernd Nilius in an analysis of aortic endothelial cells from a *Trpc4*-deficient mouse model. Inwardly, rectifying currents in endothelial cells lacking *Trpc4* were activated by store depletion induced by $InsP_3$ and the SERCA inhibitor tBHQ (tert-butyl-benzohydroquinone). Store-operated currents, which could be blocked by 1 μM La^{3+} to a large extent, displayed reversal potentials above +40 mV and strong inward rectification and had an extremely high calcium selectivity (the calculated P_{Ca}/P_{Na} was 159.7). Receptor-operated TRPC4-dependent currents were not analyzed in these cells.

In smooth muscle cells of the longitudinal layer of the ileum, a cation current activated by stimulation of muscarinic receptors (M_3 and M_2, respectively), the so-called muscarinic cationic current mI_{CAT}, was described more than 25 years ago as a receptor-operated current with a U-shaped *I–V* relationship similar to those currents described by expression of TRPC4 in some reports and co-expression of TRPC4 with TRPC1 (Benham et al. 1985). Activation of this current was coupled via G proteins, since its activation was blocked by pertussis toxin or by intracellular guanosine 5′-*O*-(2-thio-diphosphate) (GDPβS), whereas intracellular guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) activates the channel in the absence of acetylcholine. This current conducts sodium ions and was facilitated by submicromolar calcium ions in the cytosol. TRPC5 was proposed as a potential constituent of the underlying channel (Lee et al. 2003). However, the analysis of different TRPC single and compound knockout mouse models revealed that the current density of mI_{CAT} is reduced by 80 % and 20 % in *Trpc4*$^{-/-}$ and *Trpc6*$^{-/-}$ mice, respectively, and it was suggested that TRPC4 and TRPC6 are constituents of different channel complexes that contribute to mI_{CAT} activity by 80 % and 20 %, respectively (Tsvilovskyy et al. 2009). mI_{CAT} currents that are lacking in ileal smooth muscle cells isolated from *Trpc4*$^{-/-}$ mice show an extremely low permeability for Ca^{2+} [estimated fractional current 0.9 % (Kim et al. 1998)] (Tsvilovskyy et al. 2009). In gastric smooth muscle cells, nonselective cation channels activated by acetylcholine or carbachol ($NSCC_{ACh}$ or $NSCC_{CCh}$) that share many features with mI_{CAT} were described, and the amplitude of these I_{NSCC} inward currents was reduced by more than 90 % in *Trpc4* knockout mice demonstrating that TRPC4 is part of this receptor-operated nonselective cation channel (Lee et al. 2005). Currents activated with the mGluR agonist 1S,3R-ACPD exhibiting a very prominent negative slope region similar to the "U"-shaped currents (Fig. 2f) are lacking in TRPC4/TRPC1 double-deficient neurons of the dorsolateral septum (Phelan et al. 2012) indicating that TRPC4 proteins are also constituents of the channels underlying these receptor-operated currents.

Single-channel properties: Single-channel recordings in cells heterologously expressing TRPC4 proteins were reported in several studies with unitary channel conductance of 41 pS (mTRPC4β) (Schaefer et al. 2000), 30 pS (hTRPC4α) (Schaefer et al. 2002), 28 pS (rTRPC4α) (Schaefer et al. 2002), 30 pS (hTRPC4β) (Schaefer et al. 2002), and 18 pS (mTRPC4β) (Walker et al. 2002). Single-channel studies on mI_{CAT} that critically depends on TRPC4 expression revealed the activity of three types of cationic channels with unitary conductances of 7 pS, 55 pS, and 116 pS in ileal myocytes from wild-type mice. Only the activity of the intermediate 55-pS conductance channel was not detectable in TRPC4-deficient myocytes, indicating that the 55-pS channels are formed by TRPC4 (Tsvilovskyy et al. 2009).

The biophysical properties of TRPC4 homomultimers and heteromultimers as well as TRPC4-containing channels in cell lines and primary cells are summarized in Tables 2, 3, and 4. The channels that arise from heterologous expression of TRPC4 may represent TRPC4 homomers and the majority of our knowledge regarding biophysical properties, regulatory mechanisms of channel activity, and pharmacological modulation (see below) is based on this experimental strategy; the

Table 2 Properties of ion channels recorded following heterologous expression of TRPC4 proteins

Cell system	TRPC4 splice variant (species)	IV	Selectivity	Activation	References
HEK 293	α (b)	ir	$P_{Ca}/P_{Na} = 7$	SOC	Philipp et al. (1996)
CHO cells	α (b)	ir	Ca^{2+}-selective	SOC	Warnat et al. (1999)
CHO cells	α (h)	l	$P_{Ca}/P_{Na} = 1.1$	SA	McKay et al. (2000)
Xenopus oocytes	Not reported	ir	Ca^{2+}-selective	SOC	Kinoshita et al. (2000)
HEK 293	β (m, C terminal YFP tag)	dr	$P_{Ca}/P_{Na} = 1.1$	ROC	Schaefer et al. (2000)
HEK 293	α (h, r), β (h, r)	dr	Nonselective	ROC	Schaefer et al. (2002)
HEK 293	α, β (m)	or	Nonselective	$[Ca^{2+}]_i$-inhibited	Walker et al. (2002)
HEK 293	α, β (m)	dr	Nonselective	ROC	Otsuguro et al. (2008)
HEK 293	β (m)	dr	Nonselective	ROC	Jeon et al. (2008)
HEK 293	β (m)	dr	n.d.	ROC	Sung et al. (2009)
HEK 293	α (m)	n.a.	n.a.	No current activation	Sung et al. (2009)
HEK 293	α (h, C terminal YFP tag)	dr	n.d.	ROC	Sung et al. (2009)
HEK 293	β	dr	n.d.	ROC	Blair et al. (2009)
HEK 293	β (m)	dr	Nonselective	ROC	(Jeon et al. 2012)
CHO	β (h)	dr	n.d.	ROC	Jung et al. (2011)

ROC receptor-operated channel, *SOC* store-operated channel, *OAG* 1-oleoyl-2-acetyl-sn-glycerol; species: b, bovine; r, rat; h, human; m, mouse; n.d., not determined; n.a., not applicable; ir, inwardly rectifying; l, linear; or, outwardly rectifying; and dr, doubly rectifying

differences in properties of these channels in different approaches may be explained by variations in the expression levels of TRPC4 and alterations in regulatory pathways in the corresponding cell system and/or in the expression pattern of interaction partners such as TRPC1 which is expressed in many cells including HEK293 cells leading to differences in the composition of the channel complex. The demonstration of interaction of TRPC4 with other TRPCs as well as the changed properties of channels recorded upon co-expression of TRPC4 with, e.g., TRPC1 suggests that TRPC4-containing channels may represent heteromultimers. Some results obtained from cells of TRPC compound knockout mice support this concept, whereas others support that TRPC4- and, e.g., TRPC6-containing channel complexes act independently to generate, e.g., mI_{CAT} currents (Tsvilovskyy et al. 2009). Like in heterologously expressed TRPC4 channels, the biophysical properties and mode of activation differ tremendously between TRPC4-containing channels in primary cell systems, as can be seen from those mediating I_{SOC} in aortic

Table 3 Properties of ion channels recorded following expression of TRPC4 proteins together with other TRPCs

Cell system	TRPC4 splice variant (species)	Coexpression	IV	Selectivity	Activation	References
HEK 293	α (m)	TRPC4/ TRPC1	u	Nonselective	ROC	Strubing et al. (2001)
HEK 293	α (m)	TRPC4/ TRPC3	l	n.d.	OAG	Poteser et al. (2006)

ROC receptor-operated channel, *SOC* store-operated channel, *OAG* 1-oleoyl-2-acetyl-sn-glycerol; species: b, bovine; r, rat; h, human; m, mouse; n.d., not determined; l, linear; and u, U-shaped

Table 4 Properties of TRPC4-containing channels analyzed using knockdown or knockout of TRPC4

Cell system	IV	Selectivity	Activation	References
Bovine adrenocortical cells (KD)	ir	Ca^{2+}-selective	SOC	Philipp et al. (2000)
Aortic endothelial cells (KO)	ir	$P_{Ca}/P_{Na} = 160$	SOC	Freichel et al. (2001)
Human Corneal Epithelial Cells (KD)	ir	Not reported	SOC	Yang et al. (2005)
Human gingival keratinocytes (KD)	ir	Ca^{2+}-selective	SOC	Fatherazi et al. (2007)
Gastric muscle cells (KO)	u	Nonselective	ROC	Lee et al. (2005)
Ileal smooth muscle cells (KO)	u	Nonselective	ROC	Tsvilovskyy et al. (2009)

ROC receptor-operated channel, *SOC* store-operated channel; species: b, bovine; r, rat; h, human; m, mouse; ir, inwardly rectifying; and u, U-shaped

endothelial cells (Freichel et al. 2001) or mI_{CAT} in ileal smooth muscle cells (Tsvilovskyy et al. 2009). It is assumed that TRPC4 proteins form an ion-conducting subunit of TRPC4-containing channels; nevertheless, final proof of this concept is still lacking, and mutations in the putative pore-forming region of TRPC4 proteins leading to changes in the permeability of such channels have not been described. Pure auxiliary subunits modulating the activity and properties of TRPC4-containing channels were also not reported. In addition, studies using ligands or antibodies with sufficient affinity and specificity that allowed purification of the entire TRPC4-containing channel complexes from native cell systems with conventional biochemical methods have not been reported so far. However, the increasing availability and use of highly sensitive mass spectrometry approaches may overcome this obstacle in the near future. Until then, TRPC4-containing channels remain to be defined indirectly as by the analysis of channels whose activity and/or properties are affected by deletion or downregulation of TRPC4 expression or by expression of defined TRPC4 variants in these cells.

2.1 Activation Mechanisms of TRPC4-Containing Channels

ROC, SOC, and G proteins: Activation of G-protein-coupled receptors triggers both receptor- and store-operated Ca^{2+} entry mechanisms. Studies from the last 15 years

demonstrated that TRPCs can contribute to both pathways. Almost all studies with cells overexpressing TRPC4 have shown that the activation of the recombinant channels requires activation of G proteins (Philipp et al. 1996; Schaefer et al. 2000, 2002; Strubing et al. 2001). mI_{CAT} is a native TRPC4-containing receptor-operated channel, and the importance of activation of M_3-muscarinic receptors and corresponding $G_{q/11}$-proteins for generation of mI_{CAT} was already shown in 1997 (Zholos and Bolton 1997), although $G\alpha_i$-coupled M_2 receptors are activated simultaneously in these cells and M_2 receptors are also required for mI_{CAT} activation (Sakamoto et al. 2007). Nevertheless, activation of recombinant channels formed by TRPC4 also required activation of PLCβ or/and PLCγ (Schaefer et al. 2000), and mI_{CAT} is effectively blocked by the PLC blocker U-73122 indicating the involvement of $G\alpha_q$–PLC pathway in the activation on this native TRPC4-containing channel (Zholos et al. 2004). There are multiple mechanisms downstream of PLC activation coupled to phosphoinositide signaling that may lead to activation of TRPC4-containing channels and endogenous cation currents that depend on TRPC4 expression in mouse aortic endothelial cells (MAEC), and adrenal cells appear to be activated by depletion of internal Ca^{2+} stores (Freichel et al. 2001; Philipp et al. 2000). The store-operated activation mode of TRPC4-containing channels was demonstrated also in corneal epithelial cells (Yang et al. 2005) and keratinocytes (Fatherazi et al. 2007). Surprisingly, knockdown of either TRPC1 or TRPC4 proteins had no effect on store-operated Ca^{2+} entry and CRAC currents in HUVECs (human umbilical vein endothelial cells) (Abdullaev et al. 2008). There are a large number of reports showing that Orai1 proteins are ion-conducting subunits of store-operated CRAC currents, especially in mast cells and lymphocytes (Hogan et al. 2010). However, Orai1 can also interact with, e.g., TRPC3 to form cation channels whose regulation depends on multiple factors (Liao et al. 2007, 2008, 2009), and in rat pulmonary endothelial cells, TRPC4 and Orai1 were found to interact constitutively with protein 4.1, and together with TRPC1 following depletion of intracellular Ca^{2+} stores with thapsigargin (Cioffi et al. 2012). Although downregulation of Orai1 proteins affected activity and permeability of channels mediating store-operated currents that were shown to depend on TRPC1 and TRPC4 expression in earlier studies (Brough et al. 2001; Cioffi et al. 2005), it still remains unresolved whether TRPC4 forms heteromeric channels with Orai1 or whether they form independent channels that may be linked indirectly by protein 4.1. The first possibility is favored by this study and supported also by other groups [for a review, see Lee et al. (2010a)], and the latter possibility is supported by a study in human umbilical vein endothelial cells (HUVEC) in which store-operated Ca^{2+} entry and CRAC currents were unchanged following downregulation of TRPC4 (or TRPC1) (Abdullaev et al. 2008). In aortic endothelial cells from *Trpc4*$^{-/-}$ mice, store-operated cation currents and Ca^{2+} entry were largely reduced (Freichel et al. 2001) similarly like in TRPC4-deficient lung endothelial cells and could not be enhanced by overexpression of Orai1 (Sundivakkam et al. 2012). Recently, a TRPC4-mediated Ca^{2+} signaling pathway evoked by EGF was identified specifically in sub-confluent, proliferating clusters of human microvascular endothelial cells (but not, e.g., in single migrating endothelial cells).

Additional results in that study supported the concept that the abundance of TRPC4 in the plasma membrane and its contribution to Ca^{2+} entry depend on the proliferation state, and its activity is regulated by cell–cell contact formation in a β-catenin-dependent manner (Graziani et al. 2010). Obviously, the constituents of store-operated channels and the contribution of TRPC4 proteins differ between cell types and particularly between endothelial cells in different vascular beds and may depend on other environmental factors such as the proliferation state of these cells and the extent of cell–cell contact formation.

In addition to the $G_{q/11}$/phospholipase C pathway, TRPC4-containing channels can also be activated via $G\alpha_i$ proteins. It could be shown that constitutively active variants of $G\alpha_{i,}$, $G\alpha_{i3}$, and $G\alpha_o$ mimicked the activation of TRPC4 by GTPγS (Jeon et al. 2008, 2012). Constitutively active $G\alpha_{12}$ and $G\alpha_{13}$ proteins did not show any effect on TRPC4 (Jeon et al. 2008). Jeon et al. (2012) found that $G\alpha_i$ subunits, rather than $G\alpha_q$, are the predominant and direct activators of TRPC4 β channels. The activation of mI_{CAT} by pertussis toxin (PTX)-sensitive $G\alpha_{i/o}$ proteins was already shown in 1990 (Inoue and Isenberg 1990) and follow-up studies revealed that intracellular application of antibodies directed against $G\alpha_{(i3)}$/$G\alpha_{(o)}$ or $G\alpha_{(o)}$ via the pipette solution resulted in about a 70 % decrease of the mI_{CAT} (Yan et al. 2003). In contrast to G_q and $G\alpha_i$ subunits, co-expression of a constitutively active form of $G\alpha_s$, $G\alpha_{sQ227L}$, inhibited GTPγS-activated TRPC4 and TRPC5 current (Sung et al. 2011).

PI(4, 5)P_2: Otsuguro et al. have shown that PI(4, 5)P_2 inhibited TRPC4α but not TRPC4β (Otsuguro et al. 2008). Additionally they demonstrated that deletion of the PDZ-binding domain (ΔTTRL) prevented PIP_2-dependent TRPC4α inhibition. The authors also suggested a novel mechanistic model of TRPC4 gating, whereby TRPC4 interaction via its C-terminal PDZ-binding domain with the adaptor NHERF, ERM proteins, and cortical actin is necessary for keeping the 84aa stretch missing in TRPC4β close to the inner surface of the plasma membrane, thus stabilizing its binding with PI(4, 5)P_2. PI(4, 5)P_2 inhibits also mI_{CAT} (Tsvilovskyy et al. 2009), but another group concluded from their experiments that PI(4, 5)P_2 is required for TRPC4β channel activation (Jeon et al. 2012).

$[Ca^{2+}]_i$ and Calmodulin (*CaM*): TRPC4 channel activity clearly depends on $[Ca^{2+}]_i$ in many reports, but Ca^{2+}-dependent regulation of TRPC4-containing channels seems to be complex. Activation of TRPC4-containing channels is largely reduced upon strong buffering of $[Ca^{2+}]_i$ and requires concentrations $\geq$100 nM in many cases (Inoue and Isenberg 1990; Kim et al. 2012; Plant and Schaefer 2005). In cells overexpressing TRPC4, it has been shown that both external and internal Ca^{2+} ions potentiate the activation of ionic currents induced by stimulation of G proteins (Blair et al. 2009; Schaefer et al. 2000). Similar to TRPC5 (see review by A. Zholos in this compendium), in inside-out patches formed from plasma membrane of TRPC4-expressing HEK cells, Schaefer et al. (Schaefer et al. 2000) observed strong channel activation when $[Ca^{2+}]_i$ was raised from 100 nM to 10 μM at the internal side of the membrane, even in the absence of GTPγS. Similar activation of TRPC4-containing channels was observed in whole-cell experiments and perfusion with 10 μM Ca^{2+} in the pipette solution. Other researchers also have shown in *Xenopus*

oocytes that TRPC4-mediated store-operated channels are positively regulated by cytosolic Ca^{2+} with higher sensitivity than the endogenous store-operated channels (Kinoshita et al. 2000). However, four CaM-binding sites exist in TRPC4α, and CaM binds TRPC4 in a Ca^{2+}-dependent manner (Tang et al. 2001; Trost et al. 2001). As CaM inhibitors such as calmidazolium activate TRPC4-mediated currents (Tang et al. 2001; Walker et al. 2002), it was suggested that Ca^{2+}–CaM binding mediates inhibition at higher intracellular Ca^{2+}- concentrations.

Taken together, there are multiple signaling mechanisms downstream of receptor stimulation that mediate activation of TRPC4-containing channels. TRPC4 activation following stimulation of G-protein-coupled receptors can occur via direct interaction with $G\alpha_{i/o}$ subunits and/or indirectly by signals generated by the $G\alpha_q$–PLC cascade including IP_3-receptor stimulation followed by store depletion and $[Ca^{2+}]_i$ rise and $PI(4, 5)P_2$ breakdown. Their proportional relevance may differ between individual types of TRPC4-containing channels, and additional signaling mechanisms cannot be excluded.

Additional mechanisms: Wegierski et al. showed that ubiquitination represents an important mechanism to control the presence of TRPC4 at the plasma membrane. The HECT ubiquitin ligase AIP4 promotes the endocytosis of TRPC4 and decreases its amount at the plasma membrane, leading to the increased intracellular localization of TRPC4 and the reduction of its basal (i.e., constitutive) activity (Wegierski et al. 2006). Mechanisms regulating the activity of TRPC4-containing channels by direct interaction or modification of TRPC4 proteins such as NO or tyrosine phosphorylation were already addressed in Sect. 1.

3 Pharmacology

Several blockers of nonselective cation currents including SKF96365 or 2-APB can block TRPC4-containing channels, but they act on a number of other channels with comparable potency (Birnbaumer 2009) and are not discussed here in more detail. Lanthanides such as La^{3+} and Gd^{3+} are of particular interest for TRPC4 (and TRPC5) as Schäfer et al. worked out that La^{3+} (10–100 μM) potentiates the ionic currents through TRPC4 and TRPC5 but not cation currents evoked by heterologous expression of other TRPCs (Schaefer et al. 2000; Strubing et al. 2001), and 100 μM La^{3+} potentiates TRPC4α and TRPC4β by three- to fourfold. In a single-channel study of TRPC5, Jung et al. (2003) showed that lanthanides (La^{3+}, Gd^{3+}) enhance the open probability of channels and reduce the single-channel conductance in a dose-dependent manner. At higher concentrations (5 mM), La^3 but not Gd^{3+} inhibited TRPC5 currents. Neutralization of three glutamates residues (E_{543}, E_{595}, and E_{598}), which are very close to the transmembrane segments TM5 and TM6 of TRPC5 and are also conserved in TRPC4, results not only in a loss of the La^{3+} potentiation but instead in inhibition of ionic currents by La^{3+} (Jung et al. 2003). The effect of Gd^{3+} on TRPC4 currents was not analyzed in detail so far to our knowledge. Interestingly, the TRPC4-mediated current activated by the calmodulin inhibitor calmidazolium was inhibited by 10 μM La^{3+} (Walker

et al. 2002). La^{3+} may help to identify TRPC4/C5-mediated Ca^{2+} entry pathways in native cells, but examples of La^{3+}-evoked Ca^{2+} entry that is missing in TRPC4-deficient cells has not been reported so far, and it has to be emphasized that not all native channels formed by TRPC4 are potentiated by La^{3+}. For instance, the ionic currents missing in aortic endothelial cells of *Trpc4*$^{-/-}$ mice are not potentiated but inhibited by lanthanides in the micromolar range (Freichel et al. 2001). Probably, the molecular composition of the corresponding TRPC4-containing channel complexes mediating receptor-operated, nonselective cation (Schaefer et al. 2000) and store-operated currents with fairly high Ca^{2+} selectivity (Freichel et al. 2001) differs significantly and may explain their specific pharmacological properties.

Another strategy employed to develop TRPC4-specific blockers was to screen for inhibitory compounds on cation channels following heterologous expression of TRPC4. Recently, Miller and coworkers (Miller et al. 2010, 2011) performed a fluorescence-based high-throughput screening testing 305,000 compounds on an mTRPC4β-expressing HEK293 cell line and identified ML204 that blocked mouse and guinea pig TRPC4β. ML204 inhibited (IC_{50} value 0.96 μM) the increase in $[Ca^{2+}]_i$ mediated by TRPC4β after activation of μ-opioid receptors. In electrophysiological recordings with the same cell line, ML204 blocked TRPC4β channels with an IC_{50} between 2.6 and 2.9 μM. In addition, mI_{CAT} are inhibited by 86 ± 2 % using ML204 (10 μM) which corresponds to the reduction in current amplitude observed in TRPC4-deficient ileal cells (Tsvilovskyy et al. 2009). In fluorescence-based assays, ML204 (0.3–22.2 μM) did not inhibit Ca^{2+} increase mediated by mTRPM8, mTRPV3, rTRPV1, or hTRPA1 (at high concentration there was a weak and slow activation of hTRPA1), but it significantly inhibited mTRPC5 channels (IC_{50} 9.2 μM). In electrophysiological measurements, 10 μM ML204 showed the following properties: (1) it did not affect the direct activation of mTRPC6 channels by OAG, but it blocked TRPC6 channels activated by 100 μM carbachol by ~40 %; (2) it inhibited (~80 %) TRPC3 channels activated by 100 μM carbachol; and (3) it blocked (~60 %) TRPC5 channel activity by μ-opioid (0.1 μM DAMGO) and muscarinic acetylcholine receptor costimulation. Finally, ML204 (10 mM) did not significantly inhibit currents of voltage-gated sodium, potassium, and calcium channels in mouse dorsal root ganglion neurons (Miller et al. 2011). Taken together, ML204 from this compound could represent an excellent novel tool for investigation of TRPC4 channel and its function in native conditions. Unfortunately, due to the pharmacokinetics of ML204, effective plasma concentrations for in vivo studies are not expected after systemic application due to extensive metabolization in liver microsomes in vitro, but this may be overcome by derivatization of the compound (Miller et al. 2010).

Xu et al. studied the effect of mercurial compounds (Xu et al. 2012) and nonsteroidal anti-inflammatory drugs including fenamate analogues (Jiang et al. 2012) on TRPC4 and TRPC5 channels. However, the agonistic effect of Hg^{2+} on TRPC4α was reported only in the presence of Gd^{3+}, leading to the question if Hg^{2+} alone provokes also such activation. Nevertheless, these results raise the possibility that TRPC4 and TRPC5 may be involved in mercury-induced toxicity.

The inhibitory effects of fenamates on TRPC4 channels were tested on HEK-293 cells stably transfected with hTRPC4α variant (Jiang et al. 2012). Flufenamic acid (FFA) was the most potent substance from that class in inhibiting TRPC4 currents (induced by Gd^{3+} 100 μM) with an IC_{50} value of 55 ± 5 μM, but perfusion times longer than 5 min potentiated TRPC4 currents. Also, FFA inhibits TRPM2, TRPM4, TRPM5, TRPC3, and TRPC5, but activated TRPC6 and TRPA1 make it difficult for the analysis of TRPC4-containing channel in native cells. A recent study analyzed the effect of synthetic and natural steroids, and it was shown that the trypsin-induced calcium entry in TRPC4-expressing HEK-293 cells was reduced by 30 μM progesterone (IC_{50} 6.2 μM); however, a similar effect was observed on TRPC3, TRPC5, and TRPC6 channels (Miehe et al. 2012).

An alternative approach to inhibit the function of TRPC4 channels is the use of antibodies that can specifically bind to TRPC4-containing channels and displace the channel pore. For example, using basilar artery smooth muscle cells from an animal model of cerebral vasospasm, ET-1 (10 nM) induced a nonselective cation current which was blocked with anti-TRPC4 antibodies applied intracellularly as well as with anti-TRPC1 applied extracellularly (Xie et al. 2007). In another work, it was shown that anti-TRPC4 antibodies reduced GTPγs-induced currents in mTRPC4-expressing HEK293 cells, and they were able to reduce the carbachol-induced nonselective cation currents in mouse antral myocytes (Lee et al. 2005). In the same study, it could be shown that these currents are almost completely lacking in corresponding cells from TRPC4-deficient mice. These results show that the use of blocking antibodies can be very useful, but this approach has also been a controversial issue in several cases because the specificity of their action in such experiments is difficult to assess like for the analysis of the expression or subcellular localization (Meissner et al. 2011).

All together, the known agonists, antagonist, and modulators of TRPC4-containing channels give valuable hints for the development of future compounds to understand the biophysical properties and functional relevance of these channels in native cells and possibly open the panorama for such compounds as therapeutic strategies for disease mediated by these channels. However, based on fact that the makeup of TRPC4-containing channels may differ significantly between different native cell types, it is well possible that a substance that specifically inhibits a TRPC4-containing channel in one cell type may not work in the same way in other native cells. Development of drugs that block all TRPC4-containing channels may therefore be very difficult, but this could turn out as an advantage since the obtained compounds may affect not all TRPC4-associated cell and organ functions. Based on this concept, it will be even more important to figure out the contribution of TRPC4 to physiological and pathophysiological processes in individual primary cell systems and the composition of the corresponding TRPC4-containing channel complexes.

4 Relevance for Ca^{2+} Signaling and Biological Functions

The analysis of TRP proteins in cell systems that most perfectly resemble the environment of their native occurrence is essential to unravel their functional relevance. Many studies report expression of TRPCs, and also TRPC4 in particular, in a given cell type (see Table 1) and correlate this with Ca^{2+} entry pathways and cation currents that are similar to those that are recorded upon heterologous expression of the corresponding TRPC protein; based on these experiments, TRPC4-mediated cation entry may be among others involved in the downstream signaling of the G_q-coupled GPR54 receptor after its activation by Kisspeptin in mouse gonadotropin-releasing hormone (GnRH) neurons (Zhang et al. 2008, 2013), the sensitivity of capacitative Ca^{2+} entry to intracellular alkalosis in platelets (Wakabayashi et al. 2006), the bradykinin-induced Ca^{2+} entry in human osteoblasts (Suzuki et al. 2011), the depolarizing effects of leptin in activation of hypothalamic proopiomelanocortin neurons (Qiu et al. 2010), or the plasmalemmal pacemaker current in murine small intestine (Walker et al. 2001, 2002). However, a causal involvement of TRPC4 proteins to such functions cannot be derived from these approaches. Because of the lack of specific pharmacology for TRPC and most other TRP channels, genetic approaches are still required to advance a causal understanding of their physiological functions in primary cells, in organs, for systemic functions of organisms and for disease states. These approaches include overexpression of dominant-negative variants, antisense oligonucleotides, and RNAi as well as targeted deletion of the gene of interest using homologous recombination. Technical aspects, advantages, and drawbacks of these approaches were summarized recently (Freichel et al. 2011).

Expression of dominant-negative TRPC4 variants: The use of overexpressing dominant-negative variants including domains of these proteins that interact with other TRP proteins or pore mutants (if known for a given TRP channel protein) was shown to suppress TRP channel functions to various degrees. Wu and coworkers (2010) expressed an N-terminal fragment of TRPC4 known to disturb the function of heterologously expressed TRPC4 homomeric and TRPC4/TRPC5 heteromeric channels (Schindl et al. 2008) under a cardiac αMHC promoter in mice. The authors showed a reduction of store-operated Ca^{2+} entry and the development cardiac hypertrophy after aortic banding similarly like following expression of dominant-negative fragments of TRPC3 and TRPC6 (Wu et al., 2010). These results suggested a relevant role of TRPC3, TRPC4, and TRPC6 for the development of this pathological cardiac remodeling. This approach can perturb homomeric and heteromeric TRPC4-containing complexes but may also interact with constituents of channel complexes that do not contain TRPC4 proteins naturally and do not unambiguously answer the question whether TRPC4-containing channels themselves are causally involved.

Knockdown of TRPC4 expression: In murine mesangial cells of the glomerulus expressing TRPC4 (Table 1), antisense TRPC4 reduced the plasmalemmal expression of TRPC4 and inhibited Thapsigargin-induced store-operated Ca^{2+} entry (SOCE) by 83 % (Wang et al. 2004). Similarly, in human corneal epithelial cells,

the knockdown of TRPC4 by siRNA reduced cell proliferation and SOCE evoked by the epidermal growth factor (Yang et al. 2005). However, TRPC4 siRNA did not affect SOCE in cultured bovine corneal endothelial cells, and Ca^{2+} entry induced by ATP was increased by TRPC4 siRNA treatment, suggesting that in this cell type, TRPC4 is a negative regulator of ROC (Xie et al. 2005). In contrast, in human pulmonary artery smooth muscle cells (HPASMC), ATP-induced SOCE was reduced after knockdown of TRPC4 with siRNA which negatively affected cell proliferation; long-term stimulation with low concentration of ATP suggested that part of its mitogenic effect in human PASMC occurs via CREB-mediated upregulation of TRPC4 channel expression and activity (Zhang et al. 2004). ATP evokes a calcium entry in human myometrial cells that is mediated by TRPC4 as observed from cells treated with shRNA against TRPC4; this shRNA also reduced the calcium entry after oxytocin or PGF2 stimulation, but not after Thapsigargin or OAG (Ulloa et al. 2009). Silencing of TRPC4 or TRPC1 expression using siRNAs in a mouse muscle cell line showed that cation influx (SOCE) is regulated by the expression level of α1-syntrophin and is mediated by both channel proteins (Sabourin et al. 2009). Beyond that, knockdown of TRPC4 in human myoblasts using siRNA strategy and dominant-negative TRPC4 ($TRPC4^{EE647/648KK}$) overexpression showed that TRPC4 and TRPC1 regulate SOCE which is necessary for myocyte enhancer factor 2 (MEF2) expression and allow the fusion process to generate normal myotubes (Antigny et al. 2013). In the same line of findings, using an siRNA knockdown strategy in endothelial cell line EA.hy926, it was shown that TRPC4 among other TRPCs is important for histamine-induced Ca^{2+} oscillations and Ca^{2+} entry and the formation of tubular structures by these endothelial cells in Matrigel. However, in this case, siRNA against TRPC4 did not affect cell proliferation (Antigny et al. 2012). The role of TRPC4 in calcium entry into the cell was also evident in a human cell line of keratinocytes (HaCaT), where siRNA against TRPC4 (also TRPC1) reduced cation currents evoked by IP_3 or Thapsigargin, and this was associated with Ca^{2+}-induced differentiation pointing out the importance of TRPC4 in keratinocyte Ca^{2+} homeostasis and differentiation (Beck et al. 2008). TRPC4 silencing in human normal kidney epithelial cells caused the retention and impaired secretion of the angiogenesis inhibitor thrombopsondin-1 (TSP1) (Veliceasa et al. 2007). In cultured rat DRG cells, where TRPC4 expression was increased in a model of sciatic nerve injury, the suppression of TRPC4 by corresponding siRNA or antisense treatment reduced the length of neurites; similar results were observed in differentiated ND7/23 (mouse neuroblastoma) cells using an shRNA approach, and the reduction in neurite lengths in ND7/23 cells was rescued by overexpression of human TRPC4. These findings suggest that TRPC4 contributes to axonal regeneration after nerve injury (Wu et al. 2008).

TRPC4-deficient mouse models: Studies published so far comprise the role of TRPC4 in vascular endothelial cells, in smooth muscle cells of the gastrointestinal tract, and in various neuronal cells and networks. $Trpc4^{-/-}$ mice are fertile and exhibit no obvious signs of disease (Freichel et al. 2001). Store-operated calcium entry in MAEC from $Trpc4^{-/-}$ studied by electrophysiology and calcium imaging approaches and receptor-operated Ca^{2+} entry evoked by stimulation with

acetylcholine or ATP are markedly reduced but are not due to differences in membrane potential, which are the driving force of Ca^{2+} entry (Freichel et al. 2001). In addition, in MAEC from *Trpc4*-deficient mice, the activation of a low-conductance, cyclic nucleotide-regulated Cl− channel (the cystic fibrosis transmembrane conductance regulator or CFTR) is absent (Wei et al. 2001). TRPC4 transcripts and proteins are also detected in lung endothelial cells (LECs), and, similarly to MAEC, store- and receptor-operated calcium entry induced by thrombin or PAR-1 agonist peptide is reduced in *Trpc4*$^{-/-}$ LECs (Sundivakkam et al. 2012; Tiruppathi et al. 2002); similar results were observed by knockdown of TRPC4 in LECs using treatment with siRNAs directed against TRPC4 or by expression of a TRPC4 dominant-negative variant that is not able to interact with Stim1, and reduced Ca^{2+} entry could be rescued by transfection of mTRPC4β, but not by overexpression of STIM1 or Orai1 (Sundivakkam et al. 2012). These results show the importance and specific role of TRPC4 for the thrombin-induced calcium entry in lung endothelial cells of the mouse. The reduced calcium entry in TRPC4-deficient LECs is associated with reduced thrombin-induced formation of actin stress fibers, reduced endothelial cell retraction, and reduced microvascular endothelial permeability evoked by a PAR-1 agonist peptide in isolated lungs (Tiruppathi et al. 2002). PAR-1-mediated increase in microvascular permeability from isolated lungs induced by agonist peptide could be reduced by La^{3+} (1 μM). In addition, it was found that in LECs or isolated lungs from *Trpc4*$^{-/-}$ mice thrombin-induced activation of the nuclear factor-kB (NF-κB) is impaired following treatment with thrombin but not with TNFα (Bair et al. 2009).

TRPC4 proteins are expressed in various cells of the nervous system (Table 1), and the analysis of *Trpc4* deficient mice and rats depicted already several functions of this channel in neurons including the regulation of synaptic transmission at several neural networks. In a first study, it was shown that inactivation of TRPC4 leads to an increase in dendritic γ-aminobutyric acid (GABA) release from thalamic interneurons evoked by 5-hydroxytryptamine. GABA release at this synaptic terminal requires Ca^{2+} entry, is not mediated by store-operated or voltage-gated Ca^{2+} channels, but critically depends on TRPC4 proteins (Munsch et al. 2003). TRPC4 proteins are expressed in granule cells of the mouse olfactory bulb (Stroh et al. 2012); granule cells form a reciprocal dendrodendritic synapse with the mitral/tufted cells which release glutamate onto the granule cells. Upon suprathreshold excitation, granule cells respond with long-lasting depolarizations associated with slow global Ca^{2+} entry and GABA release which in turn inhibits other mitral/tufted cells. For this process, a Ca^{2+}-activated nonselective cation current (I_{CAN}) from the granule cells is important; it could be shown that long-lasting depolarizations (LLDs) and associated slow global Ca^{2+} elevations depend on NMDA receptors whereas group I mGluRs are not essential as observed using electrophysiological recordings and two photon imaging in olfactory bulb brain slices. LLDs are partially mediated by TRPC4 channels (Stroh et al. 2012). The other molecular component in this excitation process is TRPC1 as observed from the systematic analysis of *Trpc1*- and *Trpc1*/*Trpc4*-deficient mice. Global Ca^{2+} elevations are also absent in granule cells of *Trpc1*/*Trpc4*-deficient mice.

Measurements of inhibitory postsynaptic currents (IPSC) in mitral/tufted cells demonstrated that GABA release from granule cells is indeed reduced indicating that TRPC1/TRPC4 essentially contribute to glutamate-induced Ca^{2+} elevation in granule cells and dendritic GABA release and thus mediate recurrent inhibition of mitral/tufted cells. Interestingly, TRPC1/TRPC4 are activated downstream of NMDA receptor activation in these cells (Stroh et al. 2012).

Another example depicting the concomitant function of TRPC4 channels comes from the study investigating epileptogenesis and excitotoxicity using several TRPC-deficient knockout mouse lines (Phelan et al. 2012, 2013). TRPC4 proteins are found in the plasma membrane of soma and proximal dendrites of lateral septal neurons colocalizing with mGlu1 receptors from wild-type mice but not from *Trpc4*$^{-/-}$ mice; in addition, a prominent expression of TRPC4 transcripts (together with TRPC1) was found in hippocampal neurons (Phelan et al. 2012). The stimulation of mGlu1 receptors produces epileptiform burst firing in septal neurons with an underlying plateau potential that is completely abolished in *Trpc1*/*Trpc4*-deficient mice, reduced in about 74 % of the cells from *Trpc1*$^{-/-}$ mice, and is lacking in septal neurons from *Trpc4*-deficient rats (Phelan et al. 2012). Epileptiform burst firing was unchanged in mice lacking either TRPC3, TRPC5, or TRPC6. Deletion of TRPC1/TRPC4 proteins also abolished the burst firing induced by mGlu receptor activity in CA1 pyramidal neurons of the hippocampus and protected the mice from the mortality in a model of pilocarpine-induced status epilepticus; seizure-associated cell death in the dorsolateral septal nucleus and the hippocampus was also reduced in *Trpc1*/*Trpc4*-deficient mice (Phelan et al. 2012, 2013). TRPC1 and TRPC4 are expressed in bladder-innervating neurons from mice and rats (Boudes et al. 2013). Using a model of cyclophosphamide-induced cystitis, it was shown that in *Trpc1*/*Trpc4*-deficient mice, but not in *Trpc1*- or *Trpc4*-single-deficient mice, the neuronal sprouting in the inflamed bladder is abolished, and the bladder overactivity is reduced pointing for the role of both TRPC proteins in this pathological process (Boudes et al. 2013). *Trpc4*-deficient rats had been generated by the Sleeping Beauty gene-trap transposon method (Illig et al. 2011; Rasmus et al. 2011) and used as control to show that TRPC4 is expressed in cells from the ventral tegmental area. This area is a brain region with extensive input from dopamine neurons important in regulating several animal behaviors (Illig et al. 2011). *Trpc4*$^{-/-}$ rats present a significantly reduced social exploratory behavior (Rasmus et al. 2011); however, in simple and complex learning tests *Trpc4*$^{-/-}$ rats respond similar to control rats as well as in another test on motivation for water reinforcement (Klipec et al. 2011a, b). Together these observations show the importance of TRPC4-containing channels in various functions of the central nervous system making them an interesting molecular target especially for neurological diseases associated with excitotoxicity.

As described above, TRPC4 channels determine about 80 % of the muscarinic receptor-induced cation current (mI_{CAT}) in ileal smooth muscle cells. In *Trpc4*-deficient cells, these current and carbachol-induced membrane depolarizations are greatly diminished. Consequently, the activation of voltage-dependent Ca^{2+}-channels and associated Ca^{2+} entry as well as atropine-sensitive neurogenic

contraction elicited by electrical field stimulation, which critically depends on activation of voltage-gated L-type Ca^{2+} channels, is greatly reduced demonstrating a decisive role for TRPC4 channels in mediating the signaling cascade evoked by muscarinic receptors to voltage-activated Ca^{2+}-influx and smooth muscle contraction. Accordingly, it could be shown that intestinal transit is slowed down in mice lacking *Trpc4* and *Trpc6* (Tsvilovskyy et al. 2009). In addition, the nonselective cationic currents (I_{NSCC}) in the gastric smooth muscle cells of *Trpc4* knockout mice were significantly reduced, suggesting that TRPC4 is also part of the nonselective cation channel activated by muscarinic stimulation in the stomach (Lee et al. 2005). As TRPC4 is a candidate for pacemaker channels in interstitial cells of Cajal (Walker et al. 2001), spontaneous oscillations of the membrane potential in gastric smooth muscle were recorded in murine stomachs lacking *Trpc4*. However, resting membrane potential and the amplitude of the slow waves were not different from the control indicating that TRPC4-containing channels mediating I_{NSCC} currents are not involved in the generation of slow waves, which are considered to be initiated by ICCs, in the murine stomach (Lee et al. 2005).

TRPC4 and diseases: There are no hereditary diseases described until now with gain- or loss-of-function mutations in the *Trpc4* gene leading to alterations in the activity of TRPC4-containing channels or to channel-independent pathology. Nevertheless, in two recent reports, single-nucleotide polymorphisms (SNPs) in the *Trpc4* gene are associated with human diseases. For example, the missense SNP (TRPC4-I957V) in the *Trpc4* human gene is associated with a reduced risk of myocardial infarction. The I957V mutation leads to an increase in receptor-operated cation currents and Ca^{2+} entry which is explained by more efficient phosphorylation of the adjacent tyrosine 959 and subsequent plasma membrane insertion of TRPC4 since the less bulkier valine 957 permits firmer interaction with the catalytic site of the tyrosine kinase. The proposed mechanism underlying protection is an improved endothelial function, but this needs to be confirmed experimentally (Jung et al. 2011). In a second study, photoparoxysmal responses (PPR) characterized by abnormal visual sensitivity of the brain to photic stimulation which is frequently associated with idiopathic generalized epilepsies (IGE) are associated with various SNPs in the *Trpc4* gene (von Spiczak et al. 2010); despite that the association was not significant after corresponding corrections, this trend toward association of TRPC4 variants and PPR/IGE is of particular interest and deserves further investigation since it could be shown that TRPC4 is involved in several neuronal functions related with epilepsy in mouse models.

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TRPC5

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Contents

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Abstract

Human canonical transient receptor potential channel 5 (TRPC5) has been cloned from the Xq23 region on chromosome X as a suspect in nonsyndromic mental retardation. TRPC5 is a Ca^{2+}-permeable cation channel predominantly expressed in the CNS, including the hippocampus, cerebellum, amygdala, sensory neurons, and retina. It also shows more restricted expression in the periphery, notably in the kidney and cardiovascular system. Homotetrameric TRPC5 channels are primarily activated by receptors coupled to G_q and phospholipase C and/or G_i proteins, but TRPC5 channels may also gate in a store-dependent manner, which requires other partner proteins such TRPC1, STIM1, and Orai1. There is an impressive array of other activators of TRPC5 channels, such as nitric oxide, lysophospholipids, sphingosine-1-phosphate, reduced thioredoxin, protons, lanthanides, and calcium, and many can cause its direct activation. Moreover, TRPC5 shows constitutive activity, and it is responsive to membrane stretch and cold. Thus, TRPC5 channels have significant potential for synergistic activation and may serve as an important focal point in Ca^{2+} signalling and electrogenesis. Moreover, TRPC5 functions in partnership with about 60 proteins, including TRPC1, TRPC4, calmodulin, IP_3 receptors, NHERF, NCS-1, junctate, stathmin 2, Ca^{2+}-binding protein 1, caveolin, and SESTD1, while its desensitisation is mediated by both protein kinases A and C. TRPC5 has a distinct voltage dependence shared only with its closest relative, TRPC4. Its unique N-shaped activation curve underlined by intracellular Mg^{2+} block seems to be perfectly "shaped" to trigger action potential discharge, but not to grossly interfere with the action potential shape. The range of biological functions of TRPC5 channels is also impressive, from neurotransmission to control of axon guidance and vascular smooth muscle cell migration and contractility. Recent studies of *Trpc5* gene knockouts begin to uncover its roles in fear, anxiety, seizures, and cold sensing.

Keywords

Transient receptor potential canonical 5 channel • TRPC5 expression • Receptor-operated channel • TRPC5 regulation • TRPC5 function • Axon guidance • Brain development • *Trpc5*$^{-/-}$ mouse

1 Gene and Protein General Structure

1.1 Trpc5 Cloning

Following cloning of the *Trpc4* gene from bovine retina and adrenal gland by Flockerzi and co-workers (Philipp et al. 1996), the same group 2 years later cloned from rabbit and mouse brain its structurally related *Trpc5* gene (Philipp et al. 1998), which encoded a protein with 69 % amino acid (aa) identity to the TRPC4 channel.

These channels were initially termed capacitative Ca^{2+} entry channels CCE1 and CCE2, respectively, to reflect their activation by Ca^{2+} store depletion by IP_3 or thapsigargin. Hydropathy profile of the protein indicated that TRPC5 was a membrane protein with a hydrophobic core with six peaks likely representing membrane-spanning helices. The core was flanked by long cytoplasmic N- and C-termini. At around the same time, Mori and co-workers cloned the *Trpc5* gene from the mouse brain, but characterised recombinantly expressed channel as receptor (ATP)-activated Ca^{2+}-selective and $[Ca^{2+}]_i$-dependent channel, which required $[Ca^{2+}]_i$ above 10 nM, but could not be activated by Ca^{2+} store depletion (Okada et al. 1998).

The human homologue of mouse *Trpc5* was cloned from the Xq23 region on chromosome X that contains genes implicated in nonsyndromic mental retardation (Sossey-Alaoui et al. 1999). The human *Trpc5* gene consists of 11 exons that span 308.4 kb pairs. Its transcript was 5,839 bp long predicting a 111.5 kDa protein consisting of 973 aa residues. Figure 1a shows the coding segments of the *Trpc5* transcript with exon borders and the positions of the initiation and stop codons according to Sossey-Alaoui et al. (1999). This study also provides further details of the genomic organisation of the *Trpc5* gene.

Similarly to the previous studies in animal species, Sossey-Alaoui et al.(1999) found predominant expression of TRPC5 in the human brain, with much higher levels in fetal compared to adult brain. One splice variant for human *Trpc5* has been reported, which terminates in the middle of the CIRB site (GenBank accession number AB209258).

1.2 Structural Relatedness of TRPC5

The relatedness between seven mammalian TRPC proteins based on the analysis of mouse isoforms is illustrated in Fig. 1b. For the two most structurally similar to TRPC5 channels, TRPC4 and TRPC1, sequence identities were 65 % and 45 %, respectively. The close homology of TRPC4 and TRPC5 is particularly evident in the N-terminus and the transmembrane domains with about 80 % identity in primary sequence, while there is only 44 % homology in their C-termini. Since these channels are most closely related structurally and functionally and since they are both best characterised as receptor-activated nonselective cation channels (NSCC), it is useful to compare their properties as this may provide cross-insights into their structure–function relations. Thus, their several notable common as well as distinct properties will be highlighted in this review, but the interested reader is also referred to the previous chapter on TRPC4.

Figure 1c further illustrates structural relatedness of mammalian TRPC5 proteins in several species. The TRPC5 sequence is highly conserved in these species, with sequence identities in the range 96–99 % and with exchanges mainly found within the C-terminus corresponding to exon 11 (Fig. 1a, bottom panel), indicating high TRPC5 conservation throughout evolution of mammals.

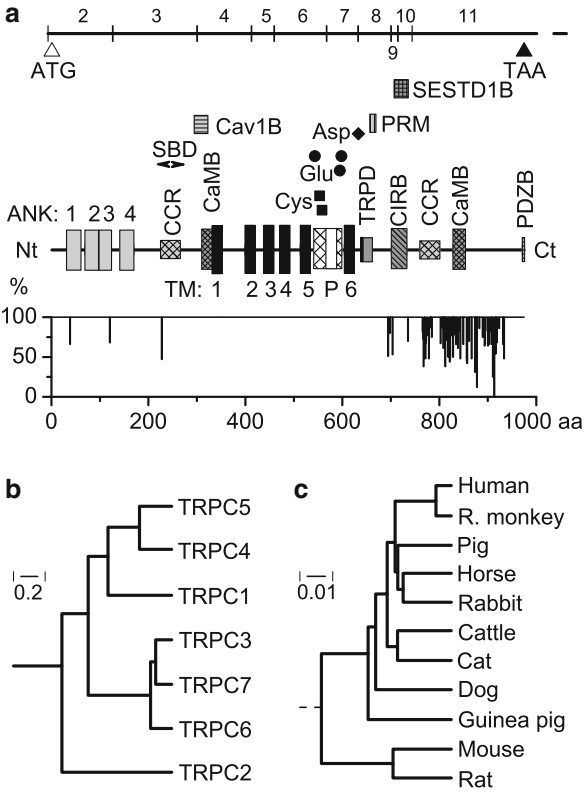

Fig. 1 Structural features of TRPC5. (**a**) *Top*, coding part of the human *Trpc5* 5,839 bp transcript with exon numbers and the positions of the initiation and stop codons indicated. *Middle*, the location of various structural domains and protein interaction sites found in TRPC5: *ANK1–4* ankyrin-like repeats, *CCR* coiled-coil region, *SBD* stathmin 2-binding domain, *Cav1B* caveolin-1-binding domain, *CaMB* calmodulin-binding domain, *TM1–6* transmembrane segments, *P* putative pore region with pore helix shown in *white*; *TRPD* TRP domain with smaller TRP *box* shown in *black*, *PRM* proline-rich motif, *CIRB* calmodulin- and inositol 1,4,5-trisphosphate receptor-binding domain, *SESTD1B* SESTD1-binding domain, *Nt* N-terminus, *Ct* C-terminus. Position of several important amino acid residues is also indicated by different symbols (see text for details). *Bottom*, CLUSTALX 2.1 column scores for aa sequences in 11 mammalian TRPC5s shown in panel (**c**). (**b**) Structural relatedness of mouse TRPC channel proteins (CLUSTAL 2.1 Multiple Sequence Alignments, the phylogenetic tree was rooted to the bacterial NaChBac channel, not shown). Accession numbers of protein sequences used: mTRPC1 (Q61056), mTRPC2 (Q9R244), mTRPC3 (Q9QZC1), mTRPC4 (Q9QUQ5), mTRPC5 (Q9QX29), mTRPC6 (Q61143), mTRPC7 (Q9WVC5), and NaChBac (NP242367). (**c**) Relatedness of mammalian TRPC5 proteins (CLUSTAL 2.1 Multiple Sequence Alignments). Most TRPC5s have 974 aa except for human TRPC5 (hTRPC5, 973 aa) and mouse TRPC5 (mTRPC5, 975 aa). Sequence identities range from 96 % (mouse vs. guinea pig) to 99 % (human vs. Rhesus monkey). Accession numbers of protein sequences used: *Homo sapiens* (NP036603), *Macaca mulatta* (XP001101078), *Sus scrofa* (A1E2E5), *Equus caballus* (F7BXH2), *Oryctolagus cuniculus* (O62852), *Bos taurus* (NP001160046), *Felis catus* (M3WDM1), *Canis lupus familiaris* (XP853241), *Cavia porcellus* (H0VNS6), *Mus musculus* (NP033454), and *Rattus norvegicus* (NP543174). Most sequences have evidence at protein or transcript level, while sequences M3WDM1, F7BXH2, and H0VNS6 are predicted

2 TRPC5 Expression

2.1 TRPC5 Is Predominantly Expressed in the CNS

The two studies that originally reported *Trpc5* cloning examined its tissue distribution using Northern blot analysis and found its highest expression in the brain, particularly high in the forebrain region, and much lower expression in the kidney, liver, testis, and uterus (Okada et al. 1998; Philipp et al. 1998). The TRPC5 mRNA in the human brain was present in the cerebellum and the occipital pole, as well as at a lower level in the medulla and the frontal lobe (Philipp et al. 1998). Depending on the brain area, either overlapping (e.g. CA1 hippocampal pyramidal neurons and the olfactory bulb) or specific TRPC5 and TRPC4 expressions were observed in the mouse brain. Sossey-Alaoui et al. (1999) also determined predominant TRPC5 expression in the human brain, with much higher levels present in the fetal brain, and relatively higher levels of expression in the cerebellum, cerebral cortex, occipital lobe, and frontal lobe compared to the whole brain.

Subsequent studies not only detailed patterns of TRPC5 expression in different brain structures, but also addressed other important aspects, such as TRPC5 subcellular localisation, trafficking, its proximity to other interacting proteins, and mechanisms of plasma membrane insertion and recycling. However, it should be noted that immunofluorescence analysis using TRPC5 antibodies (e.g. for subcellular localisation) relies on antibody specificity, and the use of nonspecific TRPC antibodies has led to a number of disagreements and contradictions regarding the assembly, localisation, and function of these channels (Meissner et al. 2011).

Relative expression level of TRPC5 compared to other TRPC isoforms is also an important issue since TRPC5 interacts with these channels, especially with TRPC1 (see Sect. 4.1), both structurally and functionally. This was systematically investigated by Riccio et al. (2002), who concluded that while all TRPCs were widely expressed in the CNS, significant and distinct patterns of their expression were observed in peripheral tissues. Only TRPC5 and TRPC3 channels demonstrated predominant expression in the CNS. Comparative analysis of tissues distribution of all TRPCs specifically in the developing cortex showed TRPC5 predominance together with TRPC1 and TRPC3 (Boisseau et al. 2009). In all examined tissues and among all TRPC isoforms, only TRPC5 and TRPC7 showed restricted expression (e.g. brain, DRG, and kidney, not observed in the immune system) (Inada et al. 2006). Immunocytochemical analysis of comparative distribution of TRPC isoforms showed that TRPC1, TRPC4, and TRPC5 were highly expressed in the cell bodies of pyramidal cells in the rat hippocampus and basal ganglia suggesting formation of functional heteromultimers (Chung et al. 2006, 2007). Investigation of subcellular localisation of TRPC1 and TRPC5 in the substantia nigra dopamine neurons revealed that these channels were mainly present in dendrites and cell nuclei, respectively, thus suggesting a role for TRPC5 in the nuclear calcium signalling (De March et al. 2006). Similar patterns of expression are present in murine medial temporal lobe structures, where TRPC1 channels are mainly expressed in the soma and dendrites, while TRPC5 channels

are mainly located on cell bodies (von Bohlen und Halbach et al. 2005). Fowler et al. (2007) specifically compared expression of TRPC4 and TRPC5 channels in multiple regions of the rat brain. Their RT-PCR analysis revealed that among TRPC isoforms TRPC4 and TRPC5 were the two predominantly expressed channels comprising 41 % and 24 % of the total TRPC pool, respectively. In the hippocampus, TRPC4/5 expression increased during brain development and peaked at 6–9 weeks postnatally. TRPC5 was highly expressed in the frontal cortex, pyramidal cell layer of the hippocampus, dentate gyrus, hypothalamus, and amygdala (Fowler et al. 2007; Riccio et al. 2009). Taken together with stronger mGluR-mediated electrical activity in brain regions with high TRPC4 and TRPC5 levels and its absence in regions lacking TRPC4 and TRPC5, these results suggested involvement of these channels in learning and memory. In murine dorsal root and nodose ganglia, TRPC4 and TRPC5 isoforms were not as abundant as TRPC1/3/6 in adult mice, but they showed progressively increased expression during embryogenesis. Gomis et al. (2008) and Zimmermann et al. (2011) also found TRPC5 expression in mouse primary sensory neurons (TG and DRG ganglia). These results suggest that TRPC channels, including TRPC5, may play a role in sensory physiology.

Interestingly, although several TRPC isoforms are highly expressed and functional in the retina as part of the CNS (Gilliam and Wensel 2011; Xue et al. 2011), only TRPC5 shows region-specific expression, such as in the inner nuclear layer of the retina, where TRPC5 function may be similar to its function in the brain.

Collectively, these studies show wide expression of all TRPC isoforms in the CNS, as well as tissue- and cell-specific expression of certain isoforms, such as TRPC5, indicating potential for formation of certain preferred heteromeric complexes depending on tissue. It is also evident that TRPC5 is predominantly expressed in the CNS, and especially during brain development. Interestingly, this latter aspect also shows region and age specifics, e.g. cerebral neurons show unaltered TRPC5 expression during postnatal development (Huang et al. 2007).

2.2 TRPC5 Expression in Peripheral Organs

In some of the above studies, relatively low or medium levels of TRPC5 expression have been described in the periphery, most notably in the liver, heart, and kidney, but the vasculature was not examined. However, subsequent studies revealed that multiple TRP isoforms, including several members of the TRPC family, are expressed in the vasculature, where they can be activated by multiplicity of signals (Beech et al. 2004; Beech 2005; Yao and Garland 2005; Dietrich et al. 2006; Watanabe et al. 2008; Albert et al. 2009; Gonzalez-Cobos and Trebak 2010). There is strong evidence for functioning of TRPC1, TRPC4, and TRPC5 and their various complexes as store-operated channels (SOC) (Beech et al. 2004; Beech 2005; Albert and Large 2006; Albert et al. 2007). The molecular composition of native SOC channels present in different blood vessels (coronary and mesenteric arteries, portal vein) appears to be very complex, as TRPC1, TRPC5, TRPC6, and

TRPC7 as well as their various complexes (e.g. TRPC1/5, TRPC1/5/6, TRPC1/5/7) seem to make distinct contributions by forming channels characterised by diverse unitary conductances and gating kinetics (Saleh et al. 2008; Albert et al. 2009).

TRPC5 contribution to SOC in vascular smooth muscle was particularly evident in experiments using custom T5E3 antibody (Xu et al. 2006a). Contributions of STIM1 and Orai to these complexes and their role in "SOC currents" in different cells have not been conclusively established (Gonzalez-Cobos and Trebak 2010; Beech 2012b). Interestingly, receptor-operated currents (ROC) via TRPC5 or its heteromers are apparently not present in vascular myocytes, as vascular receptor-operated NSCC are typically mediated by TRPC1, TRPC3, and TRPC6, which may function as homo- and/or heteromeric channels (Beech et al. 2004; Beech 2005; Albert et al. 2009; Zholos 2011). Within the cardiovascular system, there are also interesting examples of preferential TRPC5 expression in certain regions. Although TRPC5 is not detectable in endothelial cells from most vascular beds, it is expressed in the physiologically and clinically important human cerebral and coronary arteries endothelial cells (Yip et al. 2004; Ahmmed and Malik 2005), where TRPC5 may regulate endothelial permeability and agonist-dependent vasorelaxation similarly to TRPC4 (Freichel et al. 2001; Tiruppathi et al. 2002).

3 Structural Aspects of TRPC5 Channel Protein

TRPC5 shares several common features with other members of the TRP superfamily of ion channels, such as membrane topology and high selectivity to cations over anions (Owsianik et al. 2006; Ramsey et al. 2006; Venkatachalam and Montell 2007; Wu et al. 2010). It also has several characteristics typical for other channels in its own TRPC subfamily. Thus, the channel core consists of six helical transmembrane (TM1–6) segments linked by extracellular and intracellular segments of variable length and flanked by the cytoplasmic N (Nt, 330 aa)- and C (Ct, 351 aa)-termini (Fig. 1a). A putative pore region including pore helix between TM5 and TM6 lines the channel pore when a functional channel is formed by assembling four subunits. This region contains the highly conserved "LWF" motif (Owsianik et al. 2006). Alanine replacement of the "LFW" motif resulted in nonfunctional dominant negative TRPC5 (Strubing et al. 2003).

The N-terminus contains ankyrin repeats (ANK1–4), which form a common protein-binding interface for interaction with various binding partners (Vazquez et al. 2004). There are two predicted coiled-coil regions (CCR), one in the Nt and one in the Ct, which likely control oligomerisation and TRPC5 assembly and may also interact with coiled-coil domains of other proteins (Lepage et al. 2006). This region of TRPC5 is necessary for its interaction with stathmins (Greka et al. 2003). The N-terminus also contains a putative caveolin-binding region (Cav1B), which is conserved in all TRPCs and which is necessary for assembly and plasma membrane targeting to caveolae (at least in case of TRPC1), plasma membrane domains enriched in caveolins (Vazquez et al. 2004; Ambudkar et al. 2006). There is also one of the three calmodulin-binding sites (CaMB) located immediately before

TM1. It has relatively conserved sequence among TRPC isoforms, but it remains less well characterised functionally compared to the C-terminal CaMB sites (Zhu 2005). Two potential sites for cAMP- and cGMP-dependent protein kinase phosphorylation are present in Nt within ANK repeats (Ser^{122} and Thr^{167}) (Okada et al. 1998; Philipp et al. 1998; Sossey-Alaoui et al. 1999). However, one recent study found that TRPC5 is phosphorylated by G_s/cAMP/protein kinase A (PKA) at consensus PKA phosphorylation sites in Ct (S^{794} and S^{796}) causing strong inhibition of TRPC5 current (Sung et al. 2011).

In the C-terminus close to TM6, there is the TRP domain containing the most conserved "EWKFAR" motif, the so-called TRP box (Venkatachalam and Montell 2007). Other features shared with members of the TRPC subfamily include a highly conserved proline-rich "LPPPFNIIPSPK" motif (PRM) (Vazquez et al. 2004). Such motifs typically mediate dynamic protein–protein interactions in signal transduction. Downstream of PRM, there are two more CaMB sites (Ordaz et al. 2005), the first of which also binds to an N-terminal region of IP_3 receptors and therefore was named CaM- and IP_3R-binding site, or CIRB (Tang et al. 2001). Both domains are involved in TRPC5 modulation during receptor agonist responses. Largely overlapping with CIRB, there is SESTD1-binding domain, which is commonly present in TRPC4 and which binds several phospholipids and is required for efficient TRPC5 activation (Miehe et al. 2010).

TRPC5 in common with TRPC4 terminates with a PDZ-binding "TRL" sequence (PDZB), which represents common important protein–protein interaction site (see Sect. 4.3).

Finally, various cofactors of lipid origin are increasingly appreciated as key molecules regulating diverse TRP channels, yet compared to protein-binding sites little is known about lipid-binding domains (Nilius et al. 2007; Rohacs and Nilius 2007; Beech et al. 2009; Beech 2012a). This reflects on important but not completely resolved questions regarding the nature, binding sites, and specificity of regulation of ion channels and ion exchangers by membrane phosphoinositides in general, even in case of the most extensively studied in this respect Kir channels (Gamper and Shapiro 2007; Suh and Hille 2008). Nevertheless, there are several examples of striking ability of TRPM4, TRPM8, and TRPC4α to discriminate between $PI(4,5)P_2$, the substrate for the phospholipase C enzyme (PLC), and related $PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(3,4,5)P_2$ (Nilius et al. 2007; Rohacs and Nilius 2007; Otsuguro et al. 2008), suggesting that TRP–lipid interactions may involve specially structured domains, such as the TRP domain in TRPM8 (Rohacs et al. 2005) or pleckstrin homology-like (PH) domain in TRPM4 (Nilius et al. 2006), rather than simple electrostatic interactions between the negatively charged $PI(4,5)P_2$ and positively charged amino acid residues. One such domain is present in TRPC5 (K^{684}RDPDGRRRR^{693} sequence) in its Ct 15 aa downstream of the TRP domain, but its functional significance for $PI(4,5)P_2$ interaction has not been tested and the site of TRPC5 interaction with $PI(4,5)P_2$ remains unknown (Nilius et al. 2008). This PH-like region in TRPC5 largely overlaps with protein 4.1 binding domain in the corresponding TRPC4 segment (Cioffi et al. 2005).

4 Interacting Proteins

All mammalian TRPC channels can interact with multiple proteins. There is considerable evidence indicating TRPC interactions with proteins involved in vesicle trafficking, plasma membrane expression, cytoskeletal linking, and protein scaffolding, as well as with several key proteins regulating Ca^{2+} signalling. The resulting TRPC "chanellosomes" assembled in microdomains, likely in cell type-specific manner, determine channel localisation, molecular composition, regulation, and distinct functional behaviour in response to receptor agonists and/or Ca^{2+} store depletion (Ambudkar and Ong 2007).

Numerous putative protein interactions have been described for TRPC5, including other members of TRPC family, PDZ-binding proteins such as NHERF and EBP50, calmodulin, $InsP_3R$, stathmins, caveolin, dynamin, clathrin, MxA, Homer, and many other proteins. Currently, about 60 TRPC5 protein partners have been identified (see TRIP Database http://trpchannel.org/summaries/TRPC5 and references therein for a comprehensive list). We will thus focus here only on several interactions with well-characterised structural and functional relations.

4.1 TRPC Channels

Although the rules of formation of heteromeric TRPC channel complexes are not completely understood, their existence is believed to be of major physiological importance since such complexes may account for significant diversity of channel properties in native cells. Clapham and co-workers have identified overlapping distributions of TRPC1 and TRPC5 in rat hippocampus (Strubing et al. 2001). These channels combined to form functional NSCC gated by G_q-coupled receptors, but not by Ca^{2+} store depletion, and carbachol induced channel activity to similar extent in both TRPC5 and TRPC1/5 channels. The current–voltage (I–V) curve of the TRPC1/5 complex was clearly different from that of homomeric TRPC5. Moreover, TRPC1 in TRPC1/5 complexes reduces TRPC5 single-channel conductance from 38 pS to 5 pS (Strubing et al. 2001) and its calcium permeability (Storch et al. 2012). In native vascular myocytes, TRPC1 conferred protein kinase C (PKC) and $PI(4,5)P_2$ and $PI(3,4,5)P_3$ activation on TRPC1/5 complexes, but prevented $[Ca^{2+}]_i$-dependent potentiation of TRPC5 activity (Shi et al. 2012). Taken together, these results show that TRPC1 subunits contribute to the channel pore and determine regulation of TRPC1/5 complexes.

Hofmann et al. (2002) have systematically investigated the subunit composition of TRPC channels using FRET between CFP- and YFP-labelled TRPC subunits and coimmunoprecipitation techniques. They found that all TRPCs were capable of forming homomultimers. TRPC5 could coassemble with TRPC1 and TRPC4, its two most closely structurally related TRPC members (Fig. 1b), but not with other TRPC members.

Similar findings were reported by Goel et al. (2002) for TRPC1/4/5 channels expressed in Sf9 insect cells. Their coimmunoprecipitation analysis using isolated

rat brain synaptosomes revealed that these channels also coassemble in nerve terminals in vivo. Examination of native TRPC complexes in the developing brain has identified channel heteromers composed of TRPC1, TRPC4, or TRPC5, as well as the diacylglycerol-activated TRPC3 or TRPC6 subunits, which were present only in embryonic but not in adult brain (Strubing et al. 2003). In heterologous expression systems, TRPC1 was necessary to reconstitute such TRPC complexes. Individual TRPCs are functionally important in these heteromeric complexes since the *I–V* curves of channels formed by TRPC1/5 and TRPC1/3/5 were markedly different, while coexpression of the nonfunctional DN-TRPC5 mutant strongly reduced channel complex currents (Strubing et al. 2003). FRET analysis showed that the first ankyrin-like domain (ANK1 in Fig. 1a) was the minimal key structure required for the formation of functional homo- and heteromeric TRPC4/5 channels as TRPC5 mutant lacking this domain was completely nonfunctional (Schindl et al. 2008).

4.2 Calmodulin and IP_3R

There are three CaMB in TRPC5 (Fig. 1a), of which CIRB and the second CaMB (CBII) in the C-terminus have been most extensively studied (Ordaz et al. 2005). Disruption of CIRB in TRPC5 rendered the channel nonresponsive to receptor agonists without affecting its membrane expression, while deletion of CBII reduced the rate of channel activation, which is normally accelerated by calmodulin (Ordaz et al. 2005). Zhu and Tang (2008) proposed a general model of TRPC5 regulation, according to which IP_3Rs activate TRPC channels by displacing inhibitory calmodulin from this site. In addition, this competition has complex Ca^{2+} dependence. Compared to other TRPCs, CIRB in TRPC5 requires a much higher Ca^{2+} concentration to be bound to CaM (Zhu 2005). Ca^{2+} affinity for CaM binding to CBII is more than 10 times higher than to the CIRB site of TRPC5, and this site is critical for the Ca^{2+}/CaM-dependent facilitation of agonist-induced activation of TRPC5, although it is not absolutely required for channel activity (Ordaz et al. 2005). Thus, the overall agonist response of TRPC5 requires the CIRB domain, and it is further potentiated by Ct CaMB domain.

Notably, in TRPC4, which is most closely related to TRPC5, there are two CaMB sites downstream from the CIRB site. The second of these coincides with CBII in TRPC5, but the homology between the respective sequences is low (Zhu 2005). This suggests that Ca^{2+}/CaM control of TRPC4 may be different and likely more complex, compared to TRPC5. Interestingly, both CaMB sites in the C-terminal of TRPC4 are missing in its shorter and more active splice variant termed TRPCβ (Schaefer et al. 2002). The C-terminal segment present in the full-length TRPC4α isoform was hence described as an autoinhibitory domain that may require additional regulatory mechanisms (Schaefer et al. 2002). This regulation was more recently attributed to PIP_2-dependent inhibition of the channel (Otsuguro et al. 2008).

4.3 Protein Interactions Involved in Plasma Membrane Localisation, Scaffolding, and Cellular Trafficking

TRPC5, in common with TRPC4, has C-terminal PDZB, which interacts with PDZ-binding domains of scaffolding proteins, such as Na^+/H^+ exchanger regulatory factor (NHERF) and ezrin/moesin/radixin-binding phosphoprotein 50 (EBP50), thus allowing regulation of TRPC5 surface expression and its further interactions with PLCβ and the cytoskeleton (Tang et al. 2000). TRPC4 and TRPC5 mutants lacking the TRL motif showed reduced plasma membrane expression (Mery et al. 2002; Zhu et al. 2005). Although PDZB domain is not absolutely necessary for TRPC5 activation, it is important determinant of the activation kinetics of agonist-induced TRPC5 (Obukhov and Nowycky 2004). Interestingly, mutation of Thr^{972} to Ala in this region completely inhibits PKC-mediated desensitisation of TRPC5 (Zhu et al. 2005).

Stathmin functions in microtubule dynamics and regulates neuronal growth. Stathmin 2 is enriched in the neuronal growth cone. Stathmin 2 complexes with TRPC5 via Ct CCR and specifically targets TRPC5 to newly forming growth cones and synapses, where the channel controls neurite extension and growth cone morphology (Greka et al. 2003). Once in the plasma membrane, interaction of TRPC5 with caveolins via the Cav1B domain in the N-terminus may target it to specific regions, the so-called caveolae, which are membrane microdomains enriched with various signalling proteins that can interact with caveolin (Vazquez et al. 2004; Ambudkar et al. 2006; Eder et al. 2007). Many other proteins have also been found to be involved in TRPC5 cellular vesicular trafficking and targeting, although their binding domains on TRPC5 remain largely unknown. These include SNARE proteins, clathrin, dynamin, and MxA (Ambudkar and Ong 2007; Eder et al. 2007). Cellular trafficking of TRPC5 channels is regulated by growth factor stimulation, which initiates its rapid translocation from vesicle pool close to the membrane and insertion into the plasma membrane (regulated by phosphatidylinositide 3-kinase, Rac1, and phosphatidylinositol 4-phosphate 5-kinase) (Bezzerides et al. 2004), as well as by the G_s/cAMP pathway (Hong et al. 2012).

Intracellular trafficking of TRPCs can also be regulated without affecting their activity. This type of interaction is exemplified by RTNF24, which was shown to cause intracellular retention of TRPC channels by interacting with them in the Golgi complex, thus reducing TRPC5 plasma membrane expression (Lussier et al. 2008).

4.4 Interaction with Calcium Signalling Proteins and Formation of Calcium Signalling Complexes

There is substantial evidence indicating that homomeric TRPC5 functions as ROC, but in complexes with other TRPCs, it can gate in store-dependent manner (see Sect. 6). At low $[Ca^{2+}]_i$, the CIRB site is likely bound to IP_3 receptors, suggesting that the conformational coupling mechanism can contribute to TRPC5 activation

via displacement of the inhibitory calmodulin from this site (Zhu 2005). In addition, Homer proteins may also regulate interaction of TRPC5 with IP_3R with the involvement of Ct CCR and PRM domains (Eder et al. 2007).

Another candidate protein for this role is junctate, an IP_3R-associated protein that interacts with TRPC2 and TRPC5, but not with TRPC1 (Stamboulian et al. 2005). Junctate has recently been "rediscovered" as a Ca^{2+}-sensing component of Orai1 and stromal interaction molecule 1 (STIM1) (Srikanth et al. 2012). There is ongoing debate on the interaction of these two key components of Ca^{2+} release-activated Ca^{2+} (CRAC) channels with TRPC channels in the formation of diverse "SOC channels". STIM1 binds to TRPC1, TRPC4, and TRPC5 channels and directly regulates them. TRPC5 was specifically examined as a "template" ROC to clarify the role of STIM1 in its regulation. Knockdown of STIM1 with siRNA specifically suppressed carbachol-, but not La^{3+}-stimulated TRPC5 current (Yuan et al. 2007). A general STIM-regulated heteromeric Orai/TRPC model for SOCE/I_{CRAC} channels has been proposed (Liao et al. 2008). Orai1 appears to interact with both Nt and Ct of TRPC channels, and although both channel types can function independently of each other (DeHaven et al. 2009), there is growing evidence that in native cells Orai1 and TRPCs exist in the same Ca^{2+} signalling complex and influence the activity of each other (Lee et al. 2010).

In addition to presumably direct regulation of TRPC5 by Ca^{2+}/CaM (Ordaz et al. 2005), upstream effects via Ca^{2+}/CaM-dependent myosin light chain kinase have also been reported, likely due to maintaining plasma membrane localisation of TRPC5 proteins (Kim et al. 2006; Shimizu et al. 2006). Furthermore, TRPC5 activity is negatively regulated by Ca^{2+}-binding protein 1 (CaBP1), which inhibits intracellular Ca^{2+} activation of the channel (see Sect. 5.3) by binding to Ct cites close, but not identical, to CIRB and CaMB (Kinoshita-Kawada et al. 2005). Neuronal Ca^{2+} sensor 1 (NCS-1) has been described as a direct partner of TRPC5, which, by binding to Ct, appears to play an important permissive role in all major modes of TRPC5 activation (e.g. by receptor agonists, store depletion, lanthanides, and elevated $[Ca^{2+}]_i$) (Hui et al. 2006). Thus, TRPC5 is endowed with balanced positive (CaM and NCS-1) and negative (CaBP1) influences from Ca^{2+}-sensing proteins.

Moreover, Ca^{2+} entry via co-expressed CRAC (STIM1 and Orai1) or L-type voltage-gated Ca^{2+} channels (VGCC) was sufficient to activate TRPC5 channels, suggesting functional interaction between TRPC5 and other Ca^{2+}-selective channels (Gross et al. 2009).

5 Biophysical Description of TRPC5 Function, Permeation, and Gating

5.1 TRPC5 Voltage Dependence Uniquely Shared with TRPC4

Among TRP channels, TRPC5 and related TRPC4 display unique voltage dependence. First comprehensive analysis of TRPC4 and TRPC5 proteins activation mechanisms and biophysical properties was carried out by Schaefer et al. (2000).

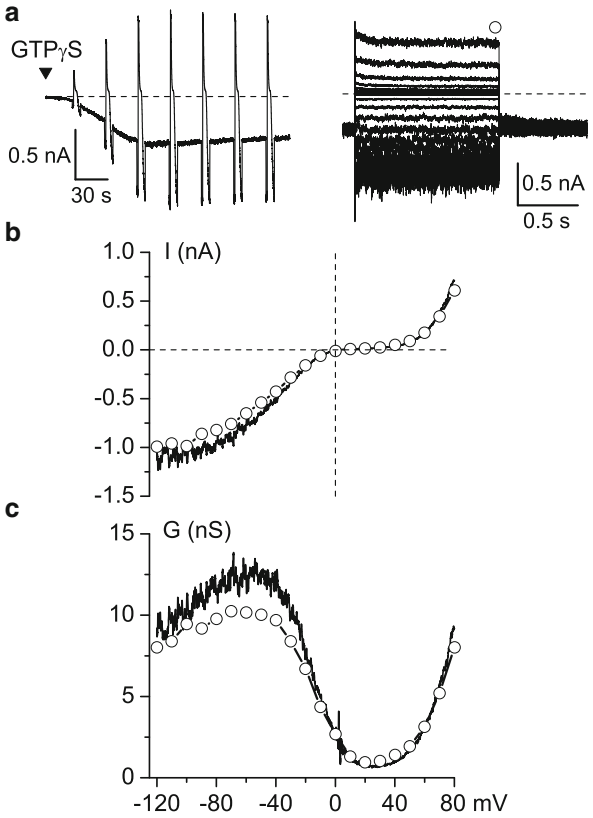

Fig. 2 Voltage dependence of TRPC5. (**a**) Following breakthrough with patch pipette containing 200 μM GTPγS, inward current slowly develops at the holding potential of -40 mV in HEK293 cells expressing TRPC5 channel. The vertical deflections correspond to *I–V* relationship measurements at 30 s intervals by applying slow 6 s voltage ramps from 80 to -120 mV. Recording conditions were as previously described (Otsuguro et al. 2008). (**b**) *I–V* relationship measured at the peak response showing characteristic doubly rectifying TRPC5 shape. *Open circles* show *I–V* relationship measured at the end of each voltage step applied from -40 mV to test potentials from -120 mV to 80 mV with a 10 mV increment, as indicated in (**a**), superimposed current traces on the right. (**c**) TRPC5 activation curve calculated from the *I–V* relationships shown in (**b**) by dividing current amplitude by the driving force (E-Erev) at each potential. Its N-shape is very similar to that of TRPC4 channel except it is shifted towards more negative potentials (compare to Otsuguro et al. 2008). M.V. Kustov, M.X. Zhu and A.V. Zholos, unpublished

They showed that stimulation of $G_{q/11}$-coupled receptors or receptor tyrosine kinases activated these channels expressed in HEK293 cells, while Ca^{2+} store depletion failed in this action. Infusion of GTPγS in the whole-cell configuration readily induced large inward currents carried by Ca^{2+} and Na^{+} (Schaefer et al. 2000 and Fig. 2a). The *I–V* relationship of this current shows characteristic double rectification (e.g. a flat region on either side of the reversal potential) and some decline in current amplitude at very negative potentials, which is especially

pronounced in TRPC4 currents (Fig. 2b). Similar *I–V* curves can be obtained with slow voltage ramps or voltage step protocols (Fig. 2a, b). Upon a voltage step, TRPC5 channel activation and deactivation kinetics are rapid in the whole range of potentials from −120 to 80 mV, and little current relaxations are present during a voltage step (Fig. 2a, right).

This peculiar behaviour can be better understood by constructing conductance–voltage (*G-V*) curves, which show overall N-shaped voltage dependence of TRPC5 activation. Thus, beginning from very negative potentials, channel activity first increases with membrane depolarisation, peaks at about −50 mV, and then starts to decline reaching a minimum at about 20 mV before it again increases at very positive potentials (Fig. 2c). The activation range is about 65 mV less negative in TRPC4, thus explaining why TRPC4 *I–V* relation at negative potentials is much more U-shaped compared to TRPC5 showing half-maximal activation ($V_{1/2}$) at about −120 mV (Fig. 2c and Otsuguro et al. 2008). Interestingly, the $V_{1/2}$ value of spontaneously active TRPC5 is only −62 mV (Yamada et al. 2000) suggesting that channel activation by G-proteins involves a substantial negative shift of the activation similarly to TRPC4 (Zholos et al. 2004). The negative shift of the activation range is an important functional hallmark of activation of many TRP channels, in particular thermosensitive TRPs (Nilius et al. 2005), but this type of analysis in TRPC5 is complicated by the very unusual NP_o-*V* dependence inferred from the macroscopic currents (Obukhov and Nowycky 2008). Clearly more work, and especially at the single-channel level, is needed to understand this phenomenon, if it is indeed present in TRPC5, with focus on the physiologically relevant range of potentials.

5.2 Chanel Pore: Selectivity and Ion Fluxes Inhibited by Intracellular Mg^{2+} and Potentiated by Extracellular La^{3+} and Gd^{3+}

TRPC5 shows some spontaneous activity (Schaefer et al. 2000; Yamada et al. 2000; Jeon et al. 2012). In cell-attached patches, this activity was strongly potentiated by stimulation of H_1 histamine receptors co-expressed with TRPC5. In inside-out patches, application of GTPγS to the internal side of the plasma membrane evoked single-channel current activity characterised by 63 pS conductance at negative potentials, which was reduced at positive potentials suggesting channel flicker block. Thus, aiming to explain the doubly rectifying *I–V* curve, Schaefer et al. (2000) tested the hypothesis of channel voltage-dependent block by intracellular Mg^{2+} and found that in the absence of Mg^{2+} channel conductance indeed increased to 88 pS. This inhibition is mediated by a cytosolic aspartate residue D633 between TM6 and TRPD (Obukhov and Nowycky 2005) (shown Fig. 1a).

One unique property of TRPC4 and TRPC5 channels is potentiation of their activity by micromolar concentrations of La^{3+} or Gd^{3+}, common blockers of other cation channels (Schaefer et al. 2000). This property is retained in TRPC1/5 complexes (Strubing et al. 2001; Jung et al. 2003). This effect is due to a large

increase in channel open probability, as channel conductance is in fact reduced in the presence of La^{3+} (Obukhov and Nowycky 2008). To determine the sites involved in this potentiation, Jung et al. (2003) systematically mutated all negatively charged amino acids (Glu and Asp residues) in the putative extracellular loops of TRPC5. Neutralisation of Glu^{543} and Glu^{595}/Glu^{598} (shown in Fig. 1a), which are located at the start and the end of the putative pore-forming region between TM5 and TM6, resulted in a loss of channel potentiation.

TRPC5 is Ca^{2+}-permeable NSCC, but like many other TRPs, it exhibits little preference for Ca^{2+} over Na^{+}. Estimates of P_{Ca}/P_{Na} ratio calculated from the shifts of the reversal potentials range from 1.8 to 9.5 (Okada et al. 1998; Schaefer et al. 2000; Jung et al. 2003). Nevertheless, at least in heterologous cells, overexpressing TRPC5 receptor agonists can induce robust, up to about 600 nM, $[Ca^{2+}]_i$ rises due to TRPC5 activity (Okada et al. 1998).

5.3 Ca^{2+} Dependence of TRPC5

TRPC5 is tightly regulated by external and internal calcium. HEK293 cells expressing TRPC5 when perfused with abnormally low $[Ca^{2+}]_i$ solution containing 5 mM EGTA failed to produce TRPC5 currents. At least 10 nM intracellular Ca^{2+} was required for TRPC5 activation (Okada et al. 1998). Schaefer et al. (2000) also observed critical dependence of TRPC4 and TRPC5 activity on $[Ca^{2+}]_i$. TRPC5 expressed in *Xenopus* oocytes could be activated at submillimolar $[Ca^{2+}]_o$, but channel activity was abolished by BAPTA injection into the oocytes (Kinoshita-Kawada et al. 2005). In inside-out patches formed from HEK293/TRPC5 cells, Schaefer et al. (2000) observed strong channel activation when $[Ca^{2+}]_i$ was raised from 100 nM to 10 μM at the internal side of the membrane, even in the absence of GTPγS. Even modest elevation of $[Ca^{2+}]_i$ to 200 nM could slowly induce TRPC5 activation, and even in the absence of receptor stimulation, hence this was termed "Ca^{2+} activation" (Zeng et al. 2004; Shi et al. 2012). Thus, intracellular Ca^{2+} plays both permissive and potentiating roles in TRPC5 activity, suggesting involvement of Ca^{2+}-binding proteins, such as calmodulin and Ca^{2+}-dependent enzymes (see Sect. 4.2), and likely direct action as well. Recent direct tests of these possibilities revealed that calmodulin is not involved in the $[Ca^{2+}]_i$ potentiation of TRPC5 channels (Blair et al. 2009).

Okada et al. (1998) observed rapid and dramatic increases of ATP-induced TRPC5 currents upon addition of Ca^{2+} to the extracellular solution that indicated strong dependence of TRPC5 activity on the presence of external Ca^{2+}. In addition, negative time-dependent regulation of TRPC5 by Ca^{2+} was also evident in their experiments. GTPγS-induced and receptor-activated TRPC5 currents were similarly potentiated by external Ca^{2+} (Schaefer et al. 2000; Zeng et al. 2004). This effect was lost in mutants of the already discussed two sites, Glu^{543} and Glu^{595}/Glu^{598}, suggesting that La^{3+} and Ca^{2+} potentiate TRPC5 by a similar extracellular mechanism (Jung et al. 2003). However, it is also possible that Ca^{2+} entering TRPC5 can activate it from the inside. Supporting this possibility, potentiation

was prevented when $[Ca^{2+}]_i$ was tightly buffered, but was promoted in a voltage-dependent manner at higher $[Ca^{2+}]_i$ proving the existence of an intracellular site for Ca^{2+} activation (Blair et al. 2009).

By manipulating $[Ca^{2+}]_i$ levels using Ca^{2+}/EGTA buffer, Gross et al. (2009) convincingly demonstrated that $[Ca^{2+}]_i$ rise is sufficient trigger of TRPC5 channel activity in the absence of other possible channel modulators and agonists. Internal Ca^{2+} induced channel activation with an EC_{50} of about 635 and 360 nM at negative and positive membrane potentials, respectively. Step-like $[Ca^{2+}]_i$ rises by photolysis of caged Ca^{2+} revealed fast channel activation kinetics with time constant of 8.6 ms. Moreover, at high $[Ca^{2+}]_i$ TRPC5 channel current not only decayed consistent with its PKC-mediated mechanism of desensitisation (Zhu et al. 2005), but also changed its gating kinetics. This change in TRPC5 gating resulted in an almost linear *I–V* curve at negative potentials, similar to the earlier described reversible switch between voltage-dependent and voltage-independent gating modes of TRPC5 channels (Obukhov and Nowycky 2008). Thus, internal Ca^{2+} is direct activator of TRPC5 channels likely to be involved in several distinct mechanisms of its regulation.

5.4 Single-Channel Properties

Similarly to whole-cell TRPC5 current, single-channel conductance shows non-linear voltage dependence; hence, its estimates mainly relate to negative potentials where TRPC5 unitary current–voltage relation is linear with slope conductance reportedly 38–63 pS (Schaefer et al. 2000; Yamada et al. 2000; Strubing et al. 2001; Obukhov and Nowycky 2008; Blair et al. 2009). Native TRPC4 shows virtually no spontaneous channel openings, but when activated by G-protein stimulation, it gates in at least 8 kinetically distinct states, 4 open and 4 closed. Its mean open dwell times range from 0.54 to 190 ms (Zholos et al. 2004). In contrast, TRPC5 shows some spontaneous activity in inside-out patches, which is enhanced by internal GTPγS, in part by prolonging 2 open states of the channel (from 0.2 and 1.2 ms before stimulation to 0.4 and 3.8 ms after stimulation). The above described Ca^{2+}-dependent potentiation of TRPC5 involves substantial increase in the frequency of channel openings (e.g. shortening of mean closed time) and about twofold prolongation of mean open time (Blair et al. 2009). Flockerzi and co-workers (Beck et al. 2013) have recently provided new insights into the mechanisms of TRPC4 and TRPC5 activation by showing that replacement of the highly conserved non-polar glycine residue within the linker between TM4 and TM5 by the polar serine rendered both channels fully active, e.g. in case of the $TRPC5_{G503S}$ gain-of-function mutant hardly any additional potentiation by high $[Ca^{2+}]_i$ was observed (Beck et al. 2013).

5.5 Lipid-Sensing Properties

Multiple lipid factors are increasingly recognised as key regulators of TRPC channels. They act on TRPCs both as components of channel microenvironment in specific microdomains and as intermediates linking the activities of various phospholipases (in particular PLCβ, PLCγ, and PLA_2) and TRPCs. This vast area of research revealing close relations between TRPCs and lipids has recently been comprehensively reviewed by Beech (2012a). Thus, in this section, the current knowledge of lipid-sensing properties of TRPC5 will be only briefly outlined.

Sphingosine-1-phosphate (S1P) directly activates TRPC5 and TRPC1/5 complexes when acting intracellularly (e.g. in inside-out patches), but extracellular S1P activates TRPC5 via the S1P receptor pathway and a pertussis toxin-sensitive G-protein (Xu et al. 2006b). Lysophospholipids (LPL) also directly activate TRPC5 (e.g. independent of G-protein signalling) (Flemming et al. 2006), but this action is not specific for TRPC5 as distant TRPM8 is similarly activated by LPL (Abeele et al. 2006). $PI(4,5)P_2$ directly activates TRPC5 channels in isolated inside-out patches, but surprisingly in the whole-cell configuration infusion of $PI(4,5)P_2$, but not $PI(3,4,5)P_3$, inhibited TRPC5 currents (Trebak et al. 2009). $PI(4,5)P_2$ infusion also markedly reduced the rate and extent of desensitisation of agonist-stimulated TRPC5 activity (Kim et al. 2008), while $PI(4,5)P_2$ depletion strongly reduced ATP-induced TRPC5 currents (Gomis et al. 2008). Under the variety of experimental conditions tested, the stimulatory action of $PI(4,5)P_2$ on TRPC5 seems to prevail. Many other lipid factors, including oxidised phospholipids, lysophosphatidic acid, some steroidal derivatives, platelet-activating factor (PAF) and lyso-PAF, and gangliosides also regulate TRPC5 activity, either positively or negatively, highlighting its prominent lipid-sensing properties (Beech 2012a).

5.6 Chemical Activators

Apart from the major mechanism of TRPC5 activation by G-protein-coupled receptors and receptor tyrosine kinases and their downstream components, TRPC5 was found to be activated by a multiplicity of other chemical factors (Fig. 3). Amazingly, most of them appear to be direct activators not requiring any additional mechanisms. On the extracellular side, these include already discussed lanthanides and Ca^{2+}, as well as H^+ and Pb^{2+}, all acting at the same binding sites Glu^{543} and Glu^{595}/Glu^{598} (Semtner et al. 2007; Sukumar and Beech 2010). Reduced thioredoxin (rTRX) which breaks the SS bond Cys^{553}–Cys^{558} in the putative extracellular loop near pore region of TRPC5 (Fig. 1) directly activated TRPC5 and heteromeric TRPC1/5 channels (Xu et al. 2008). Acting at these sites by S-nitrosylation nitric oxide (NO) also directly activates TRPC5 channel, which also turned out to be the only TRPC family member activated by H_2O_2 (Yoshida et al. 2006). Importantly, using this mechanism TRPC5 is essential for Ca^{2+} influx activated by NO via eNOS upon receptor (ATP) stimulation in native endothelial cells.

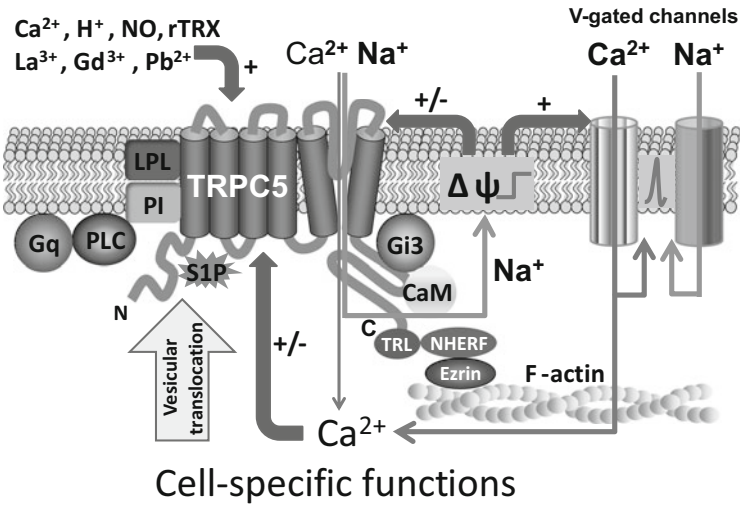

Fig. 3 Schematic illustration of TRPC5 membrane topology and gating mechanisms, as discussed in this review. Ca^{2+} entry through TRPC5 causes an increase in $[Ca^{2+}]_i$ concentration, while Na^+ entry causes membrane depolarisation and opening of voltage-gated Ca^{2+} and Na^+ channels, which in excitable cells induce action potential discharge. Larger Ca^{2+} entry occurs via VGCC compared to TRPC5 as indicated by a *thicker line*. $[Ca^{2+}]_i$ rise underlines cell-specific functions. In case of membrane potential change, the "+/−" refers to channel activation by membrane depolarisation and voltage-dependent inhibition by intracellular Mg^{2+}. In case of intracellular Ca^{2+}, the "+/−" refers to channel activation at lower and inhibition at higher $[Ca^{2+}]_i$ concentration. Various heteromeric TRPC complexes, primarily between TRPC5 and TRPC1/4 channels, can also be formed and regulated in a different manner to mediate store-operated Ca^{2+} entry (not illustrated)

5.7 Physical Factors

TRPC5 can also be activated by mechanical stimulation during hypoosmotic and pressure-induced membrane stretch, and this effect requires external and internal Ca^{2+} and $PI(4,5)P_2$ (Gomis et al. 2008).

Recent search for cold-sensitive TRPCs unexpectedly revealed that TRPC5 channels, but not TRPC1/5 heteromers, are highly cold sensitive between 37 and 25 °C (Zimmermann et al. 2011). Interestingly, cold-induced gating of TRPC5 was potentiated by activation of G_q-coupled receptors, which sensitized TRPC5 to cold.

5.8 Pore Blockers

Although La^{3+} at micromolar concentrations potentiates TRPC5, at millimolar concentrations it inhibits TRPC5 (Jung et al. 2003). In common with many TRPs, 2-APB inhibits TRPC5 (IC_{50} of 20 μM), but in a distinct voltage-dependent manner, suggesting blocker entry into the channel or modification of TRPC5 gating

(Xu et al. 2005). BTP2, a SOC blocker, inhibits TRPC5 channel with an IC_{50} of about 0.3 μM (He et al. 2005). The Na^+/Ca^{2+} exchange inhibitor KB-R7943 inhibits TRPC5 (IC_{50} of 1.4 μM), as well as other TRPCs (Kraft 2007). Intracellular ATP at physiologically relevant concentrations strongly inhibits TRPC5; this effect may provide a link between cellular metabolism and electrical activity and Ca^{2+} entry (Dattilo et al. 2008). Finally, a novel small-molecule selective and potent blocker of TRPC4 and TRPC5 channels, ML204, has recently been introduced (Miller et al. 2011).

6 TRPC5 Physiological Functions in Native Cells, Organs, and Organ Systems

Physiological functions of TRPC5 at the cellular level are much better investigated and understood compared to its wider biological roles in organs and organ systems. As already discussed, homotetrameric assembly of TRPC5 proteins functions as a receptor-activated Ca^{2+}-permeable cation channel, while in complexes with other proteins TRPC5 can mediate SOC. TRPC5 can be directly activated by multiple factors, and it reportedly receives multiple modulatory inputs, as was discussed in previous sections and summarised in Fig. 3 and Table 1. In addition, one recent study has identified G_{i3} subunits as novel direct activators of TRPC5 channels (Jeon et al. 2012). G_{i3} was shown to interact with the SESTD1B domain, which largely overlaps with the CIRB domain (Fig. 1a). Thus, TRPC5 can potentially integrate activation of differentially coupled (G_q and G_i) receptors being a focal point in GPCR signalling. Such integration is prominent in M_2 and M_3 receptor signalling in gastrointestinal smooth muscles mediated by TRPC4 (Tsvilovskyy et al. 2009), but such crosstalk at the level of G-proteins remains to be fully explored in TRPC5.

Cellular functions of Ca^{2+}-permeable ROC channels can be broadly grouped in two main categories: (i) those related to Ca^{2+} influx per se, which are especially important in nonexcitable cells lacking VGCC, and (ii) those related to membrane depolarisation, which may engage other channels, such as voltage-gated Na^+ and/or Ca^{2+} channels, thus inducing secondary and much larger Ca^{2+} influx via VGCC (indicated by a thicker line in Fig. 3). In addition, some TRPs function as intracellular Ca^{2+} release channels, although this third category of TRP functions is less well explored (Gees et al. 2010).

TRPC5 role in electrogenesis is of special interest since it is predominantly expressed in neurons. In this scenario, N-shaped conductance curve of TRPC5 (Fig. 2c) appears to be perfectly shaped to trigger action potential discharge, but not to interfere with the shape and amplitude of the action potential. Thus, when TRPC5 opens its conductance peaks at normal membrane resting potential level ensuring efficiency of membrane depolarisation towards the action potential threshold, but rapid conductance decline occurs over the region of action potential upstroke. If not for this peculiar internal Mg^{2+} channel block, one would envisage an efficient "clamp" of membrane potentials near 0 mV, whereby K^+ efflux begins to dominate in NSCC.

Table 1 TRPC5 at a glance

Gating modes	1. Spontaneously active 2. ROC: Best characterised and widely accepted mode, requires stimulation of GPCR and receptor tyrosine kinases, but signal transduction downstream of PLC activation is incompletely understood 3. SOC: Controversial, apparently requires interaction with TRPC1 and likely STIM1, Orai1, and junctate 4. Directly activated by intracellular Ca^{2+} and S1P, rTRX, NO, LPL. Cold (37-25 °C) sensitises to PLC activation. Mechanical stimulation depends on Ca^{2+} and $PI(4,5)P_2$
Voltage dependence	Doubly rectifying *I–V* curve showing flat region around the reversal potential. N-shaped activation curve
Channel conductance	38–63 pS
P_{Ca}/P_{Na}	1.8–9.5
$[Ca^{2+}]_i$ dependence	Permissive role ($[Ca^{2+}]_i$ >10 nM minimally required) Direct activation (EC_{50} = 635 nM at negative potentials) Inhibition at high $[Ca^{2+}]_i$ levels, likely due to PKC-mediated desensitisation
Interacting proteins	About 60, including TRPC1, TRPC4, CaM, IP_3R, NHERF, PLCβ, NCS-1, junctate, stathmin 2, CaBP1, caveolin, and SESTD1
Desensitisation	PKA and PKC dependent
Activators	Lanthanides (100 μM), Ca^{2+}, Pb^{2+}, H^+, NO, rTRX, LPL
Pore blockers	Lanthanides (>1 mM), 2-APB (IC_{50} = 20 μM), BTP2 (IC_{50} ~0.3 μM), KB-R7943 (IC_{50} = 1.4 μM), ML204, intracellular ATP
Proposed functions	Controls neurite extension and growth cone morphology ROC in glutamatergic synaptic transmission in the amygdala and hippocampus Brain development SOC component in vascular smooth muscle Appears to promote cardiomyocyte hypertrophy
Trpc5$^{-/-}$ phenotype	Diminished innate fear Significantly reduced pilocarpine-induced seizures Modulated cold adaptation

One of the most extensively investigated physiological functions of TRPC5 concerns its key role in axon guidance during brain development. The neuronal growth cone at the end of axon senses gradients of environmental guidance cues and transduces this information into complex Ca^{2+}-regulated cellular signalling, as needed to steer the growth cone (Henley and Poo 2004). Clapham and co-workers (Greka et al. 2003) found that in young rat hippocampal neurons expression of dominant negative TRPC5 enhanced neurite extension, whereas overexpression of wild-type TRPC5 inhibited nerve growth. They attributed this function to regulated delivery of TRPC5 to growth cones (Sect. 4.3), where it may mediate receptor-operated Ca^{2+} entry. Following this seminal discovery, NCS-1 (Sect. 4.4) and TRPC5 were found to interact in retardation of neurite outgrowth (Hui et al. 2006). Other related roles have been uncovered following this seminal discovery. TRPC5 has an essential function in the regulation of dendrite patterning in the mammalian brain through its interaction with Ca^{2+}/CaM-dependent kinase

IIβ (Puram et al. 2011), as well as in NT-3-induced inhibition of the neuronal dendritic growth in the hippocampus through activation of Ca^{2+}/CaM-dependent kinase IIα (He et al. 2012).

One novel mechanism of TRPC5 activation is provided by calpain, which cleaves TRPC5 at Thr^{857}, thus causing its direct activation in excised patches. It is proposed that semaphorin 3A, an axonal guidance peptide, initiates growth cone collapse through activation of calpain resulting in TRPC5 activation (Kaczmarek et al. 2012). In this scenario, TRPC5-mediated electrogenesis may be important in the Ca^{2+} spike activity-dependent competition that regulates motor neuron axon pathfinding via Plexin A3 receptor, which mediates signalling by semaphorins (Plazas et al. 2013). NCS-1 is a direct partner of TRPC5 and NCS-1 is involved in the regulation of both neurite outgrowth and growth cone morphology (Iketani et al. 2009). Also consistent with the above described category (ii) of cellular functions, fibroblast growth factor promotes neurite growth mainly dependent on Ca^{2+} influx in which both TPPCs and VGCCs are involved (Zamburlin et al. 2013).

In non-neuronal tissues, TRPC5 appears to have somewhat related functions in the regulation of actin remodelling and cell motility. Thus, in complex with Rac1, TRPC5 promotes cell migration in fibroblasts and kidney podocytes (Tian et al. 2010), while activated by S1P it stimulates vascular smooth muscle migration (Xu et al. 2006b). In contrast, activation of TRPC5 by LPC inhibits migration of endothelial cells (Chaudhuri et al. 2008).

A classical ROC function of TRPC5 in brain synapses has been revealed in pyramidal neurons of the lateral amygdala and in the hippocampus, whereby glutamate acting at PLC-coupled Group I mGluR1 and mGluR5 receptors activates a NSCC likely mediated, at least in part, by TRPC5 channels (Faber et al. 2006; El-Hassar et al. 2011). TRPC5 was also proposed to be involved in muscarinic receptor-induced slow after depolarisation seen in pyramidal cells of the cerebral cortex and hippocampus (Yan et al. 2009; Tai et al. 2011), although there is one recent conflicting study (Dasari et al. 2013).

As ROC and SOC component, or NSCC that can be directly gated by various factors (rTRX, LPL, NO, H^+, cold, and mechanical forces) TRPC5 has been implicated in many other physiological processes and pathophysiological conditions, including vascular smooth muscle, endothelial and mast cell function, cold sensation, as well as in rheumatoid arthritis and cardiovascular disease (Yip et al. 2004; Zeng et al. 2004; Ahmmed and Malik 2005; Beech 2005; Albert and Large 2006; Bush et al. 2006; Flemming et al. 2006; Xu et al. 2006b, 2008; Yoshida et al. 2006; Gomis et al. 2008; Watanabe et al. 2008; Albert et al. 2009; Gonzalez-Cobos and Trebak 2010; Zimmermann et al. 2011; Zholos and Curtis 2013).

7 Lessons from Knockouts

Trpc5$^{-/-}$ mice did not show any abnormalities in weight, neurological reflexes, sensorimotor responses, or basic motor functions (Riccio et al. 2009). Consistent with previously identified roles of TRPC5 in the glutamatergic synaptic

transmission in the amygdala, this study revealed significantly reduced responses mediated by Group I mGluR and CCK2 receptors in neurons of the amygdala. Accordingly, behavioural tests showed that *Trpc5*$^{-/-}$ mice were significantly less anxious in response to innately aversive stimuli compared to WT mice. In addition, TRPC5 contributes to conditioned (learned) fear (Riccio et al. 2009).

Trpc5$^{-/-}$ mice show significant reduction in pilocarpine-induced seizures and seizure-induced neuronal cell death in the hippocampus, as well as greatly reduced long-term potentiation. Surprisingly, TRPC5 were not involved in the generation of epileptiform bursts with an underlying plateau potential, induced by Group I mGluR (Phelan et al. 2013).

Recent identification of the cold-sensing properties of TRPC5 prompted specific tests of any alteration in cold sensitivity in *Trpc5*$^{-/-}$ mice (Zimmermann et al. 2011). No significant temperature-related behavioural changes were observed in these mice. Rather, plastic changes in detection and regional adaptation to cold occur, which is different from noxious cold sensing.

8 Role in Hereditary and Acquired Diseases

There is no known TRPC5-related channelopathy, yet TRPC5 may be one of the risk factors among many other emerging "risky TRPs" (Nilius and Owsianik 2010).

Since the Xq23 locus contains *Trpc5* gene as well as two genes linked to nonsyndromic mental retardation, Sossey-Alaoui et al. (1999) investigated whether the *Trpc5* gene is involved in these disorders. They identified five single-nucleotide changes in X-linked mental retardation families, but these likely represented polymorphisms rather than disease-causing mutations. Nevertheless, stronger expression of TRPC5 in human fetal brain and its expression pattern during mouse embryogenesis led them to propose an early concept of TRPC5 role in brain development.

Functionally, the gene has been also tested for association with a cardiomegaly (Bush et al. 2006). This study surveyed expression of TRPC1, 3, 4, 5, and 6 in patients with idiopathic dilated cardiomyopathy and found that only TRPC5 was upregulated in failing human heart, likely promoting cardiomyocyte hypertrophy through calcineurin signalling.

Infantile hypertrophic pyloric stenosis (IHPS) is the most common inherited form of stomach obstruction in infancy with male preponderance. Five loci for IHPS have been identified, including chromosomes 11q14–q22 (IHPS3) and Xq23–q24 (IHPS4), which contain *Trpc6* and *Trpc5* genes, respectively. Both channels are potentially involved in smooth muscle function and hypertrophy, making them good positional and functional candidate genes for IHPS. The best evidence was obtained for TRPC6, while TRPC5 re-sequencing identified only rare variants, suggested to increase disease susceptibility (Everett et al. 2009).

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TRPC6: Physiological Function and Pathophysiological Relevance

Alexander Dietrich and Thomas Gudermann

Contents

Abstract

TRPC6 is a non-selective cation channel 6 times more permeable to Ca^{2+} than to Na^{+}. Channel homotetramers heterologously expressed have a characteristic doubly rectifying current-voltage relationship and are directly activated by the second messenger diacylglycerol (DAG). TRPC6 proteins are also regulated by specific tyrosine or serine phosphorylation and phosphoinositides. Given its specific expression pattern, TRPC6 is likely to play a number of physiological roles which are confirmed by the analysis of a *Trpc6*$^{-/-}$ mouse model. In smooth muscle Na^{+} influx through TRPC6 channels and activation of voltage-gated Ca^{2+}

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channels by membrane depolarisation is the driving force for contraction. Permeability of pulmonary endothelial cells depends on TRPC6 and induces ischaemia–reperfusion oedema formation in the lungs. TRPC6 was also identified as an essential component of the slit diaphragm architecture of kidney podocytes and plays an important role in the protection of neurons after cerebral ischaemia. Other functions especially in immune and blood cells remain elusive. Recently identified TRPC6 blockers may be helpful for therapeutic approaches in diseases with highly activated TRPC6 channel activity.

Keywords
Classical transient receptor channel 6 • TRPC6 function • TRPC6 expression • *Trpc6*$^{-/-}$ mice • Lung • Cardiopulmonary vasculature • Kidney • Platelets • Tumour

1 Basic Features of the TRPC6 Gene, Protein and the TRPC6 Expression Pattern

1.1 Gene and Protein

Full-length cDNA of mouse TRPC6 was isolated from the brain (Boulay et al. 1997), while human TRPC6 was cloned from the placenta (Hofmann et al. 1999). The gene for human TRPC6 is localised on chromosome 11q21-q22 and has 13 exons (D'Esposito et al. 1998). Its murine homologue with the same number of exons is located on chromosome 9. Full-length human and mouse proteins consist of 931 and 930 amino acids, respectively.

The putative transmembrane structure is similar to that of other TRP channels with intracellular N- and C-termini, six membrane-spanning helices (S1–S6) and a predicted pore-forming loop (P) between S5 and S6 (Fig. 1a). Four TRPC6 monomers form a homomeric TRPC tetramer with a functional pore domain in the centre (Fig. 1b).

By glycosylation scanning we were able to identify two glycosylation sites (Fig. 1a) in the first and second extracellular loops (Asn^{473}; Asn^{561}) that are important determinants for the tightly receptor-operated behaviour of TRPC6 (Dietrich et al. 2003). Mutation of Asn^{651} to Gln prevents glycosylation and was sufficient to increase basal activity of TRPC6 (Dietrich et al. 2003).

The functional characteristics of TRPC6 tetrahomomers overexpressed in different cell lines may not truly represent their physiological properties in vivo, because they form heteromeric channel complexes in native environments. Different experimental approaches led to the conclusion that TRPC6 is assembled into homo- and heterotetramers within the confines of the TRPC3/6/7 subfamily (Hofmann et al. 2002) (see Fig. 1b). These results were confirmed by systematic coimmunoprecipitation of TRPCs from isolated rat brain synaptosomes (Goel

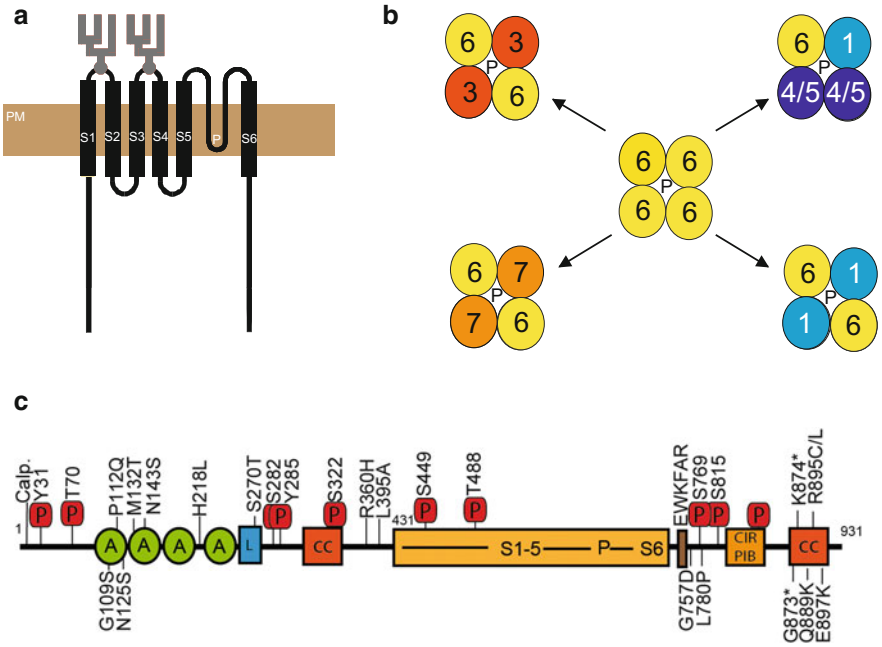

Fig. 1 (**a**–**c**) Structural features of TRPC6. (**a**) Topology of TRPC6 in the plasma membrane (*PM*) indicating transmembrane regions (S1–S6) and the predicted pore domain (*P*). Two glycosylated sites in TRPC6 are indicated by covalently bound carbohydrates (in *grey*). (**b**) Heteromultimerisation potential of TRPC6. TRPC6 can interact with TRPC3 (*3*), TRPC7 (*7*) and TRPC1 (*1*) to form functional tetramers but with TRPC4 (*4*) or TRPC5 (*5*) only in heteromeric complexes including TRPC1. (**c**) Domain structure of TRPC6. *A*, ankyrin repeat; *cc*, coiled coil domain; *CIRPIB*, Ca^{2+}/CaM IP3 receptor phosphoinositide-binding site; *EWKFAR*, conserved TRP-box motif; *L*, DAG sensitive lipid trafficking domain; *P*, phosphorylation sites (e.g. *Y31*); and mutants in patients with proteinuria (e.g. *P112Q*) are indicated. See text for details

et al. 2002). Other combinations of TRPC1–TRPC4/5 together with TRPC6 were identified in HEK293 cells as well as in embryonic brain but not in adult rat tissues (Strubing et al. 2003 and see Fig. 1b). Evidence for novel TRPC1/3, TRPC1/6 as well as TRPC1/7 heteromers was obtained by analysing the respective currents of heteromeric channel complexes in a heterologous expression system (Storch et al. 2012) (see Fig. 1b). In agreement with these combinatorial rules, TRPC4 and TRPC6 appear to be the molecular correlates of a muscarinic receptor-induced cation current in intestinal smooth muscle, but do not form heteromeric channel complexes and function separately from each other (Tsvilovskyy et al. 2009).

In general, TRPC channel complexes might be organised in supramolecular signalling complexes called signalplexes with other adaptor proteins in native tissues. Such complexes were also observed for TRPC6. A multiprotein complex identified in neuronal PC12 cells was centred around TRPC6 channels and contained protein kinase C (PKC), FK506-binding protein 12 kDa (FKBP12) and calcineurin/calmodulin (Kim and Saffen 2005). By a proteomics approach with

anti-TRPC5 and anti-TRPC6 antibodies, signalplexes for TRPC5 and TRPC6 containing several cytoskeletal proteins as well as the plasmalemmal Na^+/K^+-ATPase (NKA) pump were identified from brain lysates, but most surprisingly, this analysis did not identify TRPC5 or TRPC6 as part of these "complexes". In lysates from rat kidney and in a heterologous expression system using HEK293 cells, the TRPC6 interaction with the NKA pump and with some cytoskeletal proteins could be confirmed (Goel et al. 2005).

However, an interaction of TRPCs and Orai channels as well as STIM proteins essential for store-operated calcium entry (SOCE) is still a matter of debate. While TRPC6 is not able to interact with STIM proteins directly, but only indirectly in heteromeric complexes with TRPC1 (Yuan et al. 2007), other reports suggested a TRPC6/Orai interaction in a heterologous expression system in HEK293 cells by observing increased SOCE currents in cells stably expressing TRPC6 (Liao et al. 2008, 2009). The activation of TRPCs by STIM proteins and/or Orai channels has been challenged, however, because STIM proteins did not influence endogenous TRPC6 currents in smooth muscle cells or in heterologous expression systems (DeHaven et al. 2009). Therefore, these authors concluded that TRPC and Orai/STIM signalling occurs in distinct plasma membrane domains (DeHaven et al. 2009). Recent reports however suggest that a store-operated TRPC6/STIM1/Orai complex might exist in human platelets (Jardin et al. 2009; Berna-Erro et al. 2012).

Four ankyrin domains (A in Fig. 1c) are found in the amino terminus of TRPC6 representing common protein-protein interaction platforms. In a yeast two-hybrid screen, the second repeat was identified to interact with MxA, a member of the dynamin superfamily. Moreover, MxA enhanced TRPC6 activity in a GTP-dependent way (Lussier et al. 2005).

The TRPC6 protein contains an EWKFAR TRP box (EWKFAR in Fig. 1c) conserved within the TRPC family and an IP_3 receptor-binding domain (Boulay et al. 1999) that overlaps with a calmodulin (Zhang et al. 2001) and phosphoinositide-binding site (Kwon et al. 2007) (calmodulin/IP3 receptor phosphoinositide-binding domain; CIRPIB in Fig. 1c). An identified lipid domain in TRPC3 is conserved in TRPC6 (L in Fig. 1c) and mediates channel trafficking to the plasma membrane as well as binding to plasma membrane lipids (van Rossum et al. 2008).

1.2 Ion Channel Properties and Expression Pattern

TRPC6 is a Ca^{2+} permeable non-selective cation channel displaying double rectification with a single-channel conductance of 28–37 pS. The ion permeability ratio P_{Ca}/P_{Na} is ~6 (Hofmann et al. 1999; Dietrich et al. 2003; Shi et al. 2004). These features distinguish TRPC6 from the closely related TRPC3 channel which is significantly less Ca^{2+} selective with an ion permeability ratio P_{Ca}/P_{Na} of 1.1 and a higher single-channel conductance of 60–66 pS (reviewed in Owsianik et al. 2006). However, Ca^{2+} ions contribute only a small percentage (~4 %) to

whole-cell currents of HEK293 cells stably expressing TRPC6 in the presence of extracellular Na^+ (Estacion et al. 2006). Along these lines, application of blockers of voltage-gated calcium channels completely abolishes Ca^{2+} influx after stimulation of TRPC6 in A7r5 smooth muscle cells (Soboloff et al. 2005), favouring a model emphasising Na^+ entry through TRPC6 channels resulting in membrane depolarisation and activation of voltage-gated calcium channels that are responsible for the bulk of Ca^{2+} influx (reviewed in Gudermann et al. 2004).

Recently it was demonstrated that TRPC6 also forms Zn^{2+}-conducting channels which favour the intracellular accumulation of Zn^{2+} at least in HEK293 cells (Gibon et al. 2011). Whether the channel contributes to Zn^{2+} homeostasis in the human body, however, is still elusive.

TRPC6 is a tightly receptor-operated channel with little basal activity in contrast to TRPC3 characterised by high basal activity, when heterologously expressed in HEK293 cells. As mentioned above, the dual glycosylation pattern is a critical determinant of the quiescence of TRPC6 channel activity (Dietrich et al. 2003).

While human TRPC6 appears to be expressed ubiquitously with higher expression levels in the lungs, placenta, ovary and spleen (Hofmann et al. 2000), murine TRPC6 was identified in the lungs and brain using multiple tissue Northern blots (Boulay et al. 1997).

In general, TRPC6 is found in numerous tissues harbouring smooth muscle cells like the lungs, stomach, colon, oesophagus and myometrium (reviewed in Beech et al. 2004). TRPC6 expression in the kidney glomerulus became evident by the analysis of families with focal and segmental glomerulosclerosis (FSGS). Several research groups identified gain-of-function mutations in the TRPC6 gene [see below and Fig. 1c (Mottl et al. 2013)]. A detailed description of possible physiological functions of TRPC6 in different tissues can be found in paragraph 3 of this book chapter.

2 Regulation of TRPC6 Channels

2.1 SOC Versus ROC?

TRPs were cloned and identified assuming that they are calcium selective and activated by emptying of internal Ca^{2+} stores (store-operated channels = SOC). After their initial functional characterisation, it turned out that both assumptions do not hold true especially for TRPCs. These ion channels only show a moderate Ca^{2+} selectivity (P_{Ca}/P_{Na} from ~0.5 to 9), and the TRPC3/6/7 subfamily of TRPC channels can be activated by the second messenger diacylglycerol (DAG) produced by receptor-activated phospholipase C without any involvement of internal stores (receptor-operated channels = ROC; see Fig. 2; Hofmann et al. 1999; Okada et al. 1999). Store-operated calcium entry (SOCE), however, occurs when inositol 1,4,5-trisphosphate (IP_3) or some other signal discharges Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER). The subsequent fall in the ER Ca^{2+} concentration then signals to the plasma membrane and activates store-operated

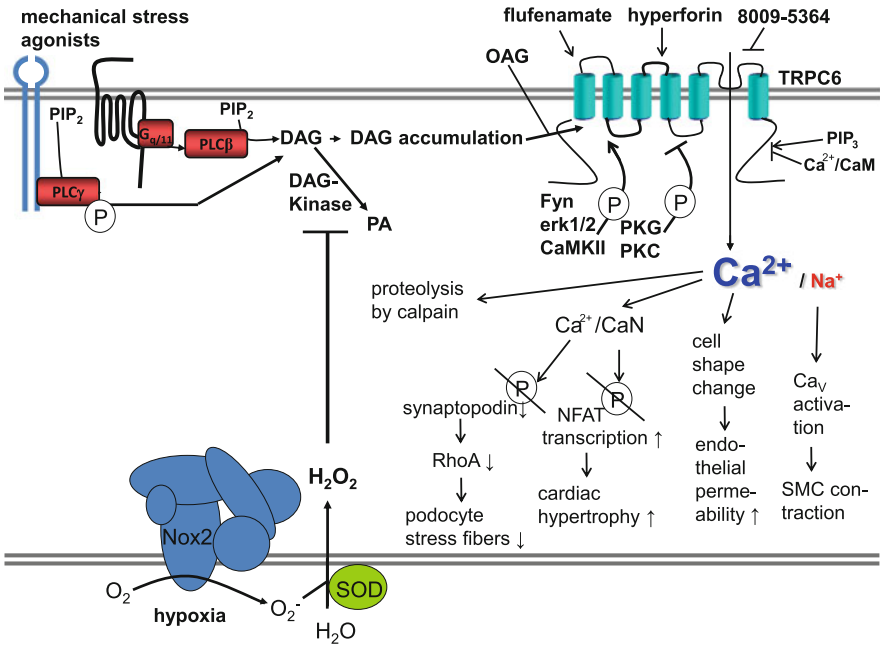

Fig. 2 Pharmacological manipulation and physiological relevance of TRPC6 in different cell types. Activating (*arrow*) and inhibiting (-) acting drugs or intracellular protein interactions are depicted. The activation mechanism of TRPC6 in endothelial cells as well as some of its physiological functions in other cell types is shown. See text for details. Ca_V, voltage-gated Ca^{2+} channel; CaM, calmodulin; CaMK, calmodulin kinase; DAG, diacylglycerol; Nox2, NADPH oxidase 2; OAG, One-oleoyl-1-acetyl-*sn*-glycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; SMC, smooth muscle cell; SOD, superoxide dismutase

channels. A major breakthrough in our understanding of SOCE was the identification of the Ca^{2+} sensor STIM and the Orai channels in 2005 and 2006, respectively (reviewed in Cahalan 2009). STIM1 predominantly located in the ER is a single-transmembrane domain protein containing two N-terminal Ca^{2+}-binding EF hands in the ER lumen, thus ideally located to detect Ca^{2+} levels in the ER. Upon Ca^{2+} release reduced ER Ca^{2+} levels induce STIM redistribution to punctae and opening of store-operated channels in the plasma membrane. The so-called Orai proteins with four predicted transmembrane domains and intracellular C- and N-termini were identified as pore-forming units of store-operated channels by the analysis of T cells from patients with severe combined immunodeficiency (SCID) (reviewed in Cahalan 2009). Although Orai 1 channels can form homomultimers to create pore-forming units, it was also suggested that Orai molecules might interact with TRPC channels heterologously expressed in HEK293 cells to form store-operated channels (Liao et al. 2009). Moreover, evidence for the interaction of the Ca^{2+} sensor STIM1 with TRPC proteins was presented in the same heterologous

expression system (reviewed in Yuan et al. 2009), and recent reports suggest that such a TRPC6/Orai/STIM complex might exist in platelets (see below).

TRPC6 was the first ion channel identified that is activated by DAG in a membrane-delimited fashion, independently of protein kinases C. One-oleoyl-1-acetyl-*sn*-glycerol (OAG), a membrane-permeable analogue of diacylglycerol (DAG), as well as the DAG lipase inhibitor RHC80267 markedly increased TRPC6 activity (Hofmann et al. 1999). The exact location of a putative binding site for diacylglycerol in the TRPC6 protein is still elusive. An OAG-insensitive splice variant of TRPC6 (TRPC6B (Δ 3–56 in Fig. 1c) (Zhang and Saffen 2001)), characterised by fluorometric Ca^{2+} imaging, actually turned out to be activated by DAG when analysed by electrophysiological methods (Jung et al. 2002). Another report suggested that TRPC3 and probably other DAG-sensitive channels like TRPC6 are not activated by DAG per se, but rather by increased fusion of TRPC3/6 containing vesicles with the plasma membrane (van Rossum et al. 2008).

Along these lines, exocytosis of TRPC6 channels to the plasma membrane was also analysed. Most interestingly, both mechanisms, receptor activation and store depletion by thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} (SERCA) pumps, induced translocation of TRPC6 channels to the plasma membrane, indicating a store-dependent contribution to TRPC6 channel density at the plasma membrane (Cayouette et al. 2004).

Mechanosensitivity of TRPC6 is also a matter of debate (see below). Several reports, however, agreed that TRPC6 is not per se mechanosensitive at physiological mechanical stress levels (reviewed in Sharif-Naeini et al. 2008).

2.2 Regulation by Ca^{2+}/Calmodulin and Phosphoinositides

Extracellular Ca^{2+} ($[Ca^{2+}]_o$) has complex effects on TRPC6 activity. A $[Ca^{2+}]_o$ of 2 mM inhibited vasopressin-induced TRPC6 activity in the rat A7r5 smooth muscle cell line. However, reduction of $[Ca^{2+}]_o$ to 50–200 μM facilitated TRPC6 cation currents, whereas complete removal of Ca^{2+} led to a decrease in currents (Jung et al. 2002). This observation of TRPC6 activation and further enhancement by the extracellular calcium concentration involves phosphorylation by calmodulin-dependent kinase II (see below and (Shi et al. 2013)), an effect that was not noted for the closely related TRPC7 protein (Shi et al. 2004).

Most interestingly, binding of phosphoinositides, especially phosphatidylinositol 3,4,5-trisphosphate (PIP_3), was demonstrated to be at the calmodulin-binding site (Kwon et al. 2007) originally identified as calmodulin/IP_3 receptor-binding site in TRPC3 (Zhang et al. 2001) (calmodulin/IP3 receptor/PI binding (CIRPIB) site in Fig. 1c). Mutations in this binding site decreasing the affinity of PIP_3 enhanced calmodulin binding and reduced the TRPC6-dependent current. Vice versa, mutations resulting in increased PIP_3 binding led to a reduced affinity of calmodulin and enhanced TRPC6 currents, while a triple amino acid substitution (R853Q/K860Q/R861Q in human TRPC6) resulted in reduced binding of both interaction partners (Kwon et al. 2007). Very recently, binding of S100A, a dimeric protein with

two EF-hand domains and highly expressed in cardiomyocytes, to the same site was also reported (Bily et al. 2013 and see http://trpchannel.org/proteins/show?id=TRPC6 for an update of TRPC6 interaction partners).

To conclude, TRPC6 is subject to a complex regulation by intracellular and extracellular Ca^{2+}, calmodulin and phosphoinositides.

2.3 Regulation by Phosphorylation

TRPC6 ion channels are also regulated by protein serine and tyrosine phosphorylation, and numerous phosphorylation sites were identified in recent years (see Fig. 1c).

The protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) has no acute effect on basal TRPC6 activity, but inhibited carbachol-induced TRPC6 activation by more than 90 % (Estacion et al. 2004). Pharmacological inhibition of PKC by calphostin C resulted in a significantly retarded inactivation time course of TRPC currents (Shi et al. 2004), and TRPC1/5 activation in vascular myocytes results in Ca^{2+}-mediated PKC inhibition of angiotensin II (Ang II)-induced TRPC6 activity (Shi et al. 2010). PKC phosphorylation at a defined TRPC6 phosphorylation site (serine 768) not only is responsible for TRPC6 inhibition by PKC but also enables binding of FK506-binding protein 12 kDa (FKBP12) and calcineurin/calmodulin resulting in the release of the activated G protein-coupled-receptor from a multiprotein complex centred around TRPC6 channels (Kim and Saffen 2005). Recently, however, PKC-mediated phosphorylation and inhibition of TRPC6 at serine 768 have been questioned and an alternative PKCδ phosphorylation site at serine 448 has been advocated. Thus, a TRPC6 S448A mutation (substitution of serine 448 by alanine) showed no PKC-dependent channel inhibition or channel activation by a PKC-specific inhibitor when compared to wild-type TRPC6 (Bousquet et al. 2010). The same group identified a phosphorylation at serine 814 phosphorylated under basal conditions and not modifying TRPC6 activity (Bousquet et al. 2011).

TRPC6 activity is also diminished by threonine phosphorylation. In a heterologous expression system and in A7r5 vascular myocytes, protein kinase G (PKG) activated by the NO-donor SNAP is able to inhibit murine TRPC6 activity by phosphorylation of threonine 69 (T70 in human TRPC6) (Takahashi et al. 2008). The proposed signalling pathway represents an interesting alternative mechanism for endothelial vasorelaxation. Thus, TRPC6 phosphorylation might be an important pharmacological strategy to combat cardiac hypertrophy. Atrial natriuretic peptide (ANP) exerts its anti-hypertrophic effects through guanylyl cyclase (GC)-A and PKG-mediated TRPC6 phosphorylation at threonine 69 (Kinoshita et al. 2010) which can be also elicited by cGMP accumulation through application of the phosphodiesterase 5 (PDE5) inhibitor sildenafil (Nishida et al. 2010; Koitabashi et al. 2010). The latter report identified an additional TRPC6 phosphorylation site at serine 322 responsible for decreased Ca^{2+} influx through TRPC6 and subsequent inhibition of the calcineurin/NFAT-dependent cardiac hypertrophy

(Koitabashi et al. 2010). Moreover, sildenafil regulates TRPC6 as well as TRPC1 and TRPC3 expression in cultured neonatal rat cardiomyocytes by a yet unidentified mechanism (Kiso et al. 2013).

Cyclic AMP (cAMP) also seems to play an important role in the regulation of TRPC6 activity because cilostazol, a potent PDE3 inhibitor, attenuated Ang II-induced vasoconstriction of thoracic aorta by threonine 69 phosphorylation of TRPC6 (Nishioka et al. 2011). In contrast to these results, ERK1/2 phosphorylation of serine 281 in murine TRPC6 (S282 in human TRPC6) involving a cAMP-PI3K-PKB-MEK-ERK1/2 signalling pathway increased TRPC6 activity in rat mesangial cells (Shen et al. 2011).

Recently, two Ca^{2+}calmodulin-dependent kinase II (CaMK II) phosphorylation sites were detected in TRPC6 by constructing channel chimaeras of the CaMK II sensitive TRPC6 and the insensitive TRPC7 channel. Phosphorylation of threonine 487 (T488 in human TRPC6) and a distal part of the CIRPIB domain are crucial for positive feedback regulation of Ca^{2+} influx through TRPC6 (Shi et al. 2013).

While there is clear evidence for tyrosine phosphorylation of the TRPC3 channel by the Src family kinase fyn (Kawasaki et al. 2006), phosphorylation of the corresponding tyrosine residue (Y230) in TRPC6 is still a matter of debate (Hisatsune et al. 2004; Kawasaki et al. 2006). A recent publication suggests that fyn-induced phosphorylation of tyrosine 284 (Y288 in human TRPC6) increases surface expression of TRPC6 in HEK293T cells and in cultured podocytes. Most interestingly, nephrin, an essential protein in the podocyte slit diaphragm, interacts with the phosphorylated domain of TRPC6, while PLCγ needs another additional domain including tyrosine 31 of TRPC6 phosphorylated by src. While the nephrin–TRPC6 interaction reduces channel surface expression, the PLCγ interaction promotes TRPC6 translocation to the plasma membrane (Kanda et al. 2011). Moreover, gain-of-function mutations of TRPC6 in patients with focal segmental glomerulosclerosis (FSGS) showed reduced interaction with nephrin, but increased PLCγ interaction resulting in increased surface expression and TRPC6 activity at the cell membrane (Kanda et al. 2011).

2.4 Pharmacology of TRPC6

In recent years some novel specific TRPC6 blockers and activators were identified, while others turned out to be too unspecific to be used for therapeutic purposes.

Like all other TRPs (except the insensitive TRPM2), TRPC3 and 6 are inhibited by ions of rare earth minerals like La^{3+} and Gd^{3+}. TRPC6 is blocked by La^{3+} ions with an IC_{50} of 4–6 μM (Inoue et al. 2001; Jung et al. 2002) similar to TRPC3 (4 μM; (Halaszovich et al. 2000)). Electrophysiological whole-cell recordings on TRPC6-expressing HEK 293 cells reveal an IC_{50} value of 1.9 μM (Inoue et al. 2001) for a Gd^{3+} block similar to results obtained with the heterologously expressed TRPC3 channel (2.3 μM; Dietrich et al. 2003). In clear contrast to TRPC3 and TRPC6, TRPC4 and 5 show a concentration-dependent stimulation in the presence of these trivalent ions (Jung et al. 2003).

Clotrimazole, an azole compound clinically used as antifungal drug, was first described as specific TRPM2 blocker (Hill et al. 2004) but also blocks TRPC6, TRPM3 and TRPV4 (Harteneck et al. 2011), while TRPV1 and TRPA1 are activated (Meseguer et al. 2008).

2-APB (2-aminoethoxydiphenyl borate) was used as a tool to study store-operated Ca^{2+} entry (SOCE) mechanisms (Bootman et al. 2002). In this context, it has been repeatedly shown that TRPC channels including TRPC6 (Tesfai et al. 2001) are blocked by 2-APB. However, after identification of Orai channels as molecular targets of SOCE, a complex regulation of these proteins by 2-APB was demonstrated (Zhang et al. 2008).

GsMTx-4 is a peptide isolated from the Chilean rose tarantula *Grammostola spatulata* and has been known to inhibit mechanosensitive cation channels (Suchyna et al. 2000). Along these lines, mechanosensitivity of TRPC6 was proposed, and the resulting channel activity was blocked by GsMTx-4 (Spassova et al. 2004). Two other reports, however, demonstrated that TRPC6 is not per se mechanosensitive at physiological mechanical stress levels (Mederos y Schnitzler et al. 2008; Gottlieb et al. 2008).

SKF-96365 1-[ß-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride was originally described as an inhibitor of the receptor- and store-operated elevation of intracellular Ca^{2+} through voltage-independent Ca^{2+} channels (Clementi and Meldolesi 1996). Especially the function of DAG-sensitive TRPC channel was analysed by applying SKF-96365 to different cell types (Shlykov et al. 2003; Boulay et al. 1997). A recent report demonstrated that TRPC6 ($IC_{50} = 2$ μM) was more efficiently blocked than TRPM3 ($IC_{50} = 12$ μM), TRPV4 ($IC_{50} = 25$ μM) and TRPM2 ($IC_{50} = 75$ μM) by this compound in a heterologous expression system (Harteneck et al. 2011).

A higher selectivity for TRPC3 and TRPC6 was recently discovered for the synthetic gestagen norgestimate with IC_{50} values of 3.0 and 5.2 μM, respectively (Miehe et al. 2012). Other TRPC channels like TRPC5 needed significantly higher compound concentrations (>10 μM).

The highest specificity for a TRPC6-specific blocker with an IC_{50} value of 3.2 μM and an at least 2.5-fold higher selectivity for TRPC6 than for the closely related TRPC3 was however demonstrated recently (Urban et al. 2012). Most interestingly, compound 8009-5364 was also effective in blocking TRPC6-induced acute hypoxic vasoconstriction of lung arteries in vivo and is therefore a promising drug candidate to treat pulmonary hypertension in the future (Urban et al. 2012).

As opposed to channel blockers, TRPC6 activators may be useful to stimulate acute hypoxic pulmonary vasoconstriction in non-ventilated regions of the lungs to avoid arterial hypoxaemia (see below). Accordingly, the membrane-permeable DAG analogue OAG was able to promote hypoxic vasoconstriction of isolated lungs from wild-type mice, but not from *Trpc6*-deficient lungs (Fuchs et al. 2011).

The arachidonic acid metabolite, 20-HETE, was also identified as activator of TRPC6 (Basora et al. 2003), but its specificity and physiological relevance remain elusive.

A non-specific cation channel blocker, flufenamate (FFA; 100 μM), turned out to be an activator of heterologously expressed TRPC6 channels in HEK293 cells, while closely related TRPC3 and TRPC7 channel activities were inhibited. Moreover, FFA was able to stimulate the α_1-adrenoceptor induced non-selective cation influx in smooth muscle cells from portal vein myocytes (Inoue et al. 2001) and the vasopressin-induced cation currents of the rat smooth muscle cell line A7r5 (Jung et al. 2002), thus identifying native TRPC6 currents. Similar results were obtained with niflumic acid (100 μM; Jung et al. 2002). FFA also allowed to identify native TRPC6 currents in primary lung endothelial cells (Weissmann et al. 2012).

Hyperforin is a natural compound of the antidepressant drug St. John's wort. Most interestingly, 10 μM of the compound activates TRPC6, but not TRPC3 in a heterologous expression system (Leuner et al. 2007). These data open up the intriguing possibility that TRPC6 activation might increase synaptic serotonin and norepinephrine concentration after hyperforin stimulation. In this regard, hyperforin also induced neurite outgrowth in PC12 cells in a TRPC6-specific manner (Leuner et al. 2007). Although this compound also induces unspecific cellular effects (Tu et al. 2010) at similar concentrations, hyperforin-induced vascular leakage was clearly reduced in *Trpc6*$^{-/-}$ mice (Chen et al. 2013). A further comparative analysis of hyperforin and its derivatives (Leuner et al. 2010) on *Trpc6*$^{-/-}$ and wild-type cells is important to obtain additional evidence arguing in favour of TRPC6 specificity.

In the future, some of these drugs might be helpful to identify native TRPC6 currents in tissues and primary cells and to develop new therapeutic strategies for patients afflicted with pulmonary hypertension, arterial hypoxaemia after partial lung blockade, depression as well as proteinuria induced by TRPC6 gain-of-function mutations (see below).

3 Biological Relevance and Emerging/Established Biological Roles for TRPC6

The exact physiological role of TRPC6 is still unknown. However, there is growing evidence that TRPC6 is an intrinsic constituent of receptor-operated cation entry involved in numerous physiological processes.

3.1 TRPC6 in the Heart and the Cardiopulmonary Vasculature

It is now clear that TRPC6 plays an important role in the heart and the cardiopulmonary vasculature and experimental evidence for its function is listed in this paragraph of the book chapter.

The control of **heart** function is one of the most important physiological processes in the body, and there are numerous manuscripts describing TRPC6 expression and function in the heart. Cardiac output is mediated by altered size and functionality of the heart resulting from inotropic and chronotropic changes.

There is mounting evidence to support a role of Ca^{2+} influx in slowly progressive remodelling processes of the heart like cardiac hypertrophy induced by pressure overload such as chronic hypertension and aortic stenosis. Initially, cardiac hypertrophy is a physiologically helpful adaptive response of the heart to many forms of cardiac stress, including cardiac arrhythmias and mutations in cardiac contractile protein genes. But after an initially compensatory mechanism that increases cardiac performance, sustained hypertrophy is maladaptive and frequently leads to ventricular dilatation and heart failure. Intracellular Ca^{2+} acts to induce the hypertrophic response, and previous reports have underscored the relevance of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin in mediating cardiac hypertrophy and progressive heart failure. Calcineurin dephosphorylates transcription factors of the **n**uclear **f**actor of the **a**ctivated T-cell (**NFAT**) family, thus allowing translocation into the nucleus and resultant activation of hypertrophic response genes (reviewed in Molkentin and Dorn 2001; Goonasekera and Molkentin 2012).

Several studies implicated TRPC channels, especially TRPC1, 3 and 6, in cardiac hypertrophy (reviewed in Dietrich et al. 2007; Dietrich and Gudermann 2011). Knock-down of TRPC6 reduced hypertrophic signalling induced by the α_1-adrenoceptor agonist phenylephrine and ET-1, while transgenic mice overexpressing TRPC6 showed increased NFAT-dependent expression of the β-myosin heavy chain, a sensitive marker for cardiomyopathy (Kuwahara et al. 2006). Interestingly, both TRPC3 and TRPC6 mediate Ang II-induced Ca^{2+} influx in rat neonatal cardiomyocytes, and either knock-down of TRPC3 or TRPC6 channels completely suppresses Ang II-induced hypertrophy (Onohara et al. 2006). Thus, TRPC3 and TRPC6 may form heteromeric channel complexes in cardiomyocytes as postulated for smooth muscle cells (Dietrich et al. 2005) described below. Amino acid Thr69 in TRPC6 (Takahashi et al. 2008) is the target for negative regulation by the NO-cGMP-PKG pathway (see above). It was also demonstrated that phosphorylation of TRPC6 might be essential for the anti-hypertrophic effects of phosphodiesterase 5 (PDE5) inhibition (Nishida et al. 2010), because specific inhibitors of the cGMP-dependent PDE5 like sildenafil prevented TRPC6-mediated Ca^{2+} influx in cardiomyocytes (Nishida et al. 2010). Interestingly, cardiac atrial natriuretic peptide ANP is also able to counteract cardiac hypertrophy via cGMP production by guanylyl cyclase A (GC-A). The inhibition of the hypertrophic growth response induced by angiotensin II, and not by β-adrenergic receptor stimulation, was selectively thwarted in mice deficient in TRPC3 and TRPC6 (Klaiber et al. 2010). In chronic cardiac hypertrophy, however, the entire TRPC3/6 complex in myocytes is activated by physical interaction with GC-A after receptor desensitisation and blunted cGMP production by markedly increased ANP levels (Klaiber et al. 2011). Therefore, ANP is able to inhibit cardiac hypertrophy by cGMP-mediated TRPC3/6 phosphorylation, but supports cardiac hypertrophy by direct interaction of GC-A and the TRPC3/6 channel complex inducing Ca^{2+} influx through voltage-gated Ca^{2+} channels (Klaiber et al. 2011). Very recently, the cardioprotective effect of Klotho, a membrane protein with multiple anti-ageing effects, was identified. Soluble Klotho from the systemic

vasculature inhibits TRPC6 currents in cardiomyocytes by blocking phosphoinositide-3-kinase-dependent exocytosis of TRPC6 channels (Xie et al. 2012).

The systemic and pulmonary vasculature consists of two major cell types: endothelial and smooth muscle cells. The **endothelium** is a thin layer of cells lining the interior surface of all blood vessels and forms an interface between circulating blood in the lumen and the vessel wall. Vascular inflammation induces changes in endothelial cell shape and consequently increases in endothelial permeability brought about by gaps between endothelial cells subsequent to Ca^{2+} influx.

An involvement of Ca^{2+} entry through TRPC6 compromising the barrier function in pulmonary arteries has been proposed (Singh et al. 2007). For this reason, we set out to analyse endothelial function in lung ischaemia–reperfusion-induced oedema (LIRE) in wild-type and *Trpc6*$^{-/-}$ mice. Most interestingly, LIRE was completely absent in *Trpc6*$^{-/-}$ mice, and *Trpc6*$^{-/-}$ lung endothelial cells displayed reduced hypoxia-induced shape change. An involvement of TRPC6 function in immune cells could be excluded by bone marrow transplantation experiments in *Trpc6*$^{-/-}$ and wild-type mice. An in-depth analysis of signalling pathways leading to TRPC6 activation revealed that *Nox2*$^{y/-}$ mice were also protected from LIRE, while phosphorylation of PLCγ as well as DAG kinase inhibition increased TRPC6 activity. Our observations led to a novel mechanistic model for LIRE comprising endothelial Nox2-derived production of superoxide, activation of PLCγ, inhibition of diacylglycerol (DAG) kinase and DAG-mediated activation of TRPC6 (Weissmann et al. 2012). Along these lines, disruption of the lung vascular endothelium during acute lung injury (ALI) induced by binding of endotoxins like lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR4) also depends on TRPC6, as *Trpc6*$^{-/-}$ mice are protected from sepsis-induced lung injury (Tauseef et al. 2012). In addition to LPS, platelet-activating factor (PAF) is also known to increase endothelial cell permeability. Accordingly, recruitment of TRPC6 channels to caveolae after stimulation of acid sphingomyelinase is essential for PAF-induced formation of lung oedema (Samapati et al. 2012). Most interestingly, histamine-provoked vascular leakage as assessed by intravital microscopy studies of the mouse cremaster circulation was absent in *Trpc6*$^{-/-}$ mice, and ANP- or sildenafil-induced cGKI-dependent phosphorylation of threonine 69 of TRPC6 inhibited this effect in wild-type mice similar as in myocytes (Chen et al. (2013) and see above).

Vascular endothelial growth factor (VEGF) increases vascular permeability by stimulating endothelial Ca^{2+} entry. In human microvascular endothelial cells, the VEGF-induced cation current has characteristics similar to those of VEGF-mediated TRPC currents in cells heterologously expressing VEGFR2 and TRPC3 or TRPC6 (Cheng et al. 2006). VEGF is important for endothelial cell migration, sprouting and proliferation as well as for other functions necessary for VEGF-induced angiogenesis. Thus, a role for TRPC6 in VEGF-induced angiogenesis promoting tumour progression was proposed (Ge et al. 2009). Moreover, it was reported that the phosphatase and tensin homologue (PTEN) receptor interacts with TRPC6 enabling its surface expression in endothelial cells, thus promoting TRPC6 function in endothelial permeability and angiogenesis (Kini et al. 2010).

In human umbilical vein endothelial cells (HUVEC), 11,12-epoxygenase-derived epoxyeicosatrienoic acids (EETs), some of the predominant mechanically induced metabolites resulting from arachidonic acid (AA) metabolism via phospholipase A_2 (PLA_2) and cyclooxygenase (COX) are found to facilitate the translocation of the TRPC6 protein to caveolin-1-rich cell membrane areas. This event leads to enhanced Ca^{2+} influx into endothelial cells in response to bradykinin (Fleming et al. 2007).

A TRPC5/6 activation cascade has been shown to take part in the regulation of endothelial cell migration. Transfection of bovine aortic endothelial cells with siRNA against either TRPC5 or TRPC6 reduced lysoPC-induced increases in $[Ca^{2+}]_i$ and cell migration (Chaudhuri et al. 2008). Similar results were obtained in *Trpc6*-deficient endothelial cells (Chaudhuri et al. 2008). However, this report is the only one demonstrating the existence of TRPC5/6 heteromeric channels, while other studies excluded this sort of heteromultimerisation (see above and Hofmann et al. 2002; Goel et al. 2002; Strubing et al. 2003). Very recently, it was reported that atheroprone shear stress induced TRPC6 and TRPV1 mRNA expression in endothelial cells, but a putative role of TRPC6 in atherosclerosis remains elusive (Thilo et al. 2012).

Smooth muscle cells provide not only structural integrity for the vessel but also precise regulation of vascular tone and blood pressure. By comparative biophysical characterisation and gene suppression using antisense oligonucleotides, TRPC6 has been shown to be the molecular correlate of the α_1-adrenoceptor-activated non-selective cation channel in vascular smooth muscle cells (Inoue et al. 2001) and the vasopressin-activated cation channel in the aortic smooth muscle cell line A7r5 (Jung et al. 2002). Most recently, it was demonstrated that the PI3-kinase (PI3K)/PTEN pathway regulates vasopressin-induced translocation of TRPC6 to the plasma membrane and vasopressin-induced Ca^{2+} entry in this cell line (Monet et al. 2012).

In addition, TRPC6 has been proposed to play a critical role in the intravascular pressure-induced depolarisation and constriction of small arteries and arterioles (Welsh et al. 2002) known as the Bayliss effect. Myogenic constriction of resistance arteries results from Ca^{2+} influx through voltage-gated Ca^{2+} channels subsequent to membrane depolarisation. However, *Trpc6*-deficient mice did not display a decreased Bayliss effect excluding an exclusive role of TRPC6 for this effect (Dietrich et al. 2005). Moreover, aortic rings of *Trpc6*$^{-/-}$ mice showed higher smooth muscle contractility and an elevated systemic blood pressure that was further increased by inhibition of nitric oxide (NO) synthase (Dietrich et al. 2005). These effects could be explained by in vivo replacement of TRPC6 by TRPC3-type channels which are closely related, but constitutively active (Dietrich et al. 2003), resulting in enhanced basal and agonist-induced cation entry into smooth muscle cells leading to increased smooth muscle contractility (Dietrich et al. 2005). Because the expression pattern of TRPC3 and TRPC6 overlaps in most tissues containing smooth muscle cells (reviewed in Beech et al. 2004), a heteromeric TRPC3/6 channel complex may be the native molecular correlate of the non-selective cation influx into smooth muscle cells.

Expression studies revealed that PDGF-mediated proliferation of pulmonary artery smooth muscle cells (PASMC) is associated with c-jun/STAT3-induced up-regulation of TRPC6 expression (Yu et al. 2003). In this context, it is intriguing to note that excessive PASMC proliferation, a major cause of the elevated pulmonary vascular resistance in patients with idiopathic pulmonary arterial hypertension (IPAH), also correlates with overexpression of TRPC6 and TRPC3 proteins in these tissues. In line with these data, down-regulation of TRPC6 by TRPC6-specific small interfering RNAs resulted in attenuated proliferation of PASMC from IPAH patients (Yu et al. 2004). A more detailed analysis of genomic DNAs revealed a single-nucleotide polymorphism (SNP) in the promoter of the TRPC6 gene in IPAH patients which enhanced nuclear factor kappa B-mediated promoter activity and stimulated TRPC6 expression (Yu et al. 2009). TRPC6 channels are also up-regulated in pulmonary arteries of rats kept under chronic hypoxic conditions to induce pulmonary hypertension. As expected, OAG-induced cation entry was significantly increased in hypoxia-treated PASMC as compared to control cells (Lin et al. 2004).

In contrast to the systemic vasculature, the pulmonary circulation responds to hypoxia by constricting pulmonary arteries and diverting blood flow to the well-ventilated areas of the lungs to ensure maximal oxygenation of the venous blood. This hypoxia-mediated vasoconstriction phenomenon is known as acute hypoxic pulmonary vasoconstriction (HPV) and was first described by von Euler and Liljestrand in 1946. Sustained pulmonary vasoconstriction (chronic HPV) is often accompanied by vascular remodelling, i.e. the muscularisation of smaller arteries and arterioles due to SMC proliferation and migration. TRPC1 and 6 up-regulation was shown to require the expression of hypoxia-inducible factor 1 (HIF-1) (Wang et al. 2005, 2006). As for the systemic vasculature, small arterial vessels rather than large vessels are responsible for the regulation of vascular tone. For these reasons, we analysed pulmonary arterial pressure (PAP) in isolated lungs during acute (<20 min) and prolonged (60–160 min) hypoxia. Acute HPV was completely absent in *Trpc6*$^{-/-}$ mice, while the vasoconstriction after prolonged hypoxia was not significantly different in *Trpc6*$^{-/-}$ mice compared to wild-type mice (Weissmann et al. 2006). These data show for the first time that the acute hypoxic vasoconstrictor response and the prolonged phase are regulated by different molecular mechanisms. Moreover, the lack of acute HPV in *Trpc6*$^{-/-}$ mice has profound physiological relevance because partial occlusion of alveolar ventilation provoked severe hypoxaemia in *Trpc6*$^{-/-}$, but not in wild-type mice (Weissmann et al. 2006). We also analysed Ca^{2+} influx in small precapillary pulmonary arterial smooth muscle cells (PASMC) from *Trpc6*-deficient and wild-type mice after priming the cells with endothelin-1. Hypoxic incubation of wild-type PASMC resulted in an increase in $[Ca^{2+}]_i$, which was completely absent in PASMC from *Trpc6*$^{-/-}$ mice (Weissmann et al. 2006). Similar to the situation in endothelium (see above), epoxyeicosatrienoic acids (EETs) are also active in smooth muscle cells. 11,12 EETs are able to induce vasoconstriction and to increase acute HPV in wild-type but not in *Trpc6*$^{-/-}$ lungs (Keseru et al. 2008). Moreover, translocation of TRPC6

to the plasma membrane was increased in pulmonary arterial smooth muscle cells after application of 11,12 EET (Keseru et al. 2008).

Most interestingly, Ca^{2+} influx was completely dependent on extracellular Ca^{2+} (Weissmann et al. 2006), excluding a proposed contribution of STIM1 and Orai, as well as store-operated TRPC channels (Yuan et al. 2009). It is notable that entry of Ca^{2+} ions in response to hypoxia is mainly carried by voltage-gated Ca^{2+} channels, because nicardipine, a potent blocker of these channels, almost completely inhibited acute HPV in isolated lungs and Ca^{2+} influx in wild-type PASMC (Weissmann et al. 2006). These data support a model in which Na^{+} influx through TRPC6 channels leads to membrane depolarisation and activation of voltage-gated Ca^{2+} channels (Estacion et al. 2006; Gudermann et al. 2004; Soboloff et al. 2005). Most interestingly, TRPC6 seems to be activated by hypoxia-induced DAG accumulation, which can be mimicked by inhibition of DAG degradation to phosphatidic acid through DAG kinases (Weissmann et al. 2006), suggesting a similar mechanism for TRPC6 activation as identified in endothelial cells (see above; Weissmann et al. 2012). Further studies involving siRNA-mediated down-regulation of these proteins in precapillary pulmonary arterial smooth muscle cells will identify the exact molecular mechanism of TRPC6 activation during acute HPV.

3.2 TRPC6 in the Kidney

In the kidney, TRPC6 in heteromeric complexes with TRPC3 is detected in the glomerulus and along the collecting duct (Hsu et al. 2007) and co-localises with aquaporin 2 (Goel et al. 2006). Moreover, native TRPC3/6 heteromers have also been identified in Madin-Darby canine epithelial (MDCK) cells (Bandyopadhyay et al. 2005). In polarised cultures of M1 and IMCD-3 collecting duct cells, however, TRPC3 localises exclusively to the apical domain, whereas TRPC6 is found on basolateral and apical membranes (Goel et al. 2007).

An important function of TRPC6 in the kidney was discovered by "reverse genetics". Several studies identified "gain-of-function" mutations in TRPC6 (see Fig. 1c and Mottl et al. 2013) for a recent summary of all mutations) in patients with focal segmental glomerular sclerosis (FSGS) and massive proteinuria (reviewed in Dietrich et al. 2010). Along these lines, TRPC-deficient mice are protected from Ang II-induced albuminuria (Eckel et al. 2011), while in vivo delivery of cDNA encoding TRPC6 to mice induces proteinuria (Moller et al. 2007). Therefore overactive TRPC6 together with other proteins, e.g. nephrin and podocin (reviewed in Kriz 2005), is responsible for podocyte dysfunction and finally kidney failure in FSGS. Ultrafiltration of plasma to dispose of metabolic end products, excess electrolytes and water is one of the key functions of the kidney glomerulus which is destructed in FSGS patients. Glomeruli comprise mesangial cells and podocytes contributing to a slit diaphragm additionally formed by the fenestrated endothelium and a basal membrane. Until recently podocytes were assumed not to be replaceable as these cells are not able to proliferate, and podocyte dysfunction was believed to

be the primary cause of FSGS. For this reason TRPC6 function was extensively analysed in immortalised podocyte cell lines, because freshly isolated podocytes do not proliferate in vivo. Channel mutants characterised by increased Ca^{2+} influx (P112Q; Winn et al. 2005) or larger current amplitudes (R895C and E897K; Reiser et al. 2005) in a heterologous expression system were expressed in this podocyte cell line and resulted in basal NFAT-mediated transcription (Schlondorff et al. 2009) similar to the scenario as described for the heart (see above). Moreover, TRPC6 phosphorylation of erk1/2 (Chiluiza et al. 2013) and association with PLCγ and nephrin is altered by the mutations and may increase TRPC6 levels and activity at the plasma membrane (Kanda et al. 2011). These changes result in a disruption of the actin cytoskeleton in cultured podocytes (Moller et al. 2007) responsible for the defective filtration process. Most interestingly, TRPC6-induced Ca^{2+} influx induced calcineurin activation and synaptopodin dephosphorylation which led to decreased protection of RhoA from proteasomal degradation (Faul et al. 2008). These results contrast with findings by other researchers suggesting an antagonistic action of TRPC6 as a RhoA activator and TRPC5 as a Rac1 activator inhibiting and promoting cell migration in cultured podocytes, respectively (Tian et al. 2010). TRPC6 might also work in close interaction with podocin which detects mechanical forces exerted by the glomerular filtration process, resulting in TRPC6 activation. Accordingly, heterologously expressed podocin regulates TRPC6 activity in a cholesterol-dependent manner (Huber et al. 2007). The second TRPC6 interacting protein, nephrin, is an essential component of the slit diaphragm. Nephrin deficiency leads to overexpression and mislocalisation of TRPC6 in podocytes supporting the concept of a signalling complex with nephrin, podocin and probably AT_1 receptors (reviewed in Gudermann 2005).

However, limiting TRPC6 function in kidney only to podocytes may not present the whole truth. Parietal epithelial cells (PEC) were discovered in the wall of the glomerulus and could be differentiated to podocytes by incubation with all-trans retinoic acid (ATRA) in vitro (Zhang et al. 2012). In light of this discovery, a comparative analysis of TRPC6 function in PECs and freshly isolated podocytes as well as in other cell types of the glomerulus like endothelial and mesangial cells is highly desirable.

Other forms of glomerulosclerosis are induced by type 2 diabetes and may result in diabetic nephropathy (DN) the most frequent form of end-stage renal disease in the USA (Brownlee 2001). Apart from podocytes and renal endothelial cells, mesangial cells are held responsible for DN. Rats with streptozotocin-induced diabetes display reduced TRPC6 expression in mesangial cells (Graham et al. 2007). Moreover, chronic application of high glucose to cultured mesangial cells induced ROS production and PKC activation followed by decreased TRPC6 expression levels (Graham et al. 2011) by binding of NF-κB transcription factors to the TRPC6 promoter (Wang et al. 2013). Therefore, decreased TRPC6 function may be responsible for mesangial hypo-contractility and increased glomerular filtration rates that will initiate proteinuria like in FSGS patients.

3.3 TRPC6 in Immune and Blood Cells

Evidence for a functional TRPC3/6 heteromeric channel which can be blocked by specific small hairpin (sh) RNAs was published recently in human **T cells** (Carrillo et al. 2012). Most interestingly, ovalbumin-challenged *Trpc6*$^{-/-}$ mice exhibited reduced allergic responses as evidenced by a decrease in airway eosinophilia and blood IgE levels as well as levels of T-helper type 2 cytokines (IL-5, IL-13) in the bronchoalveolar lavage (Sel et al. 2008). However, the precise function of TRPC6 in these cells is still elusive. TRPC6 was also identified in other immune cells of the lungs like alveolar macrophages, and TRPC6 mRNA expression was significantly increased in macrophages obtained from COPD patients compared to healthy controls, while TRPC3 and TRPC7 levels remained unchanged (Finney-Hayward et al. 2010).

Migration of *Trpc6*$^{-/-}$ **neutrophils** in response to macrophage inflammatory protein-2 (MIP2 also known as CXCL2) was reduced compared to WT neutrophils (Damann et al. 2009). In the same report an involvement of TRPC6 in cytoskeletal rearrangements during neutrophil migration was demonstrated, suggesting an important role of TRPC6 in migrating lung neutrophils (Damann et al. 2009). Moreover, TRPC6 channels are essential for CXR2-mediated intermediary chemotaxis, but not for *N*-formyl-methionine-leucine-phenylalanine (fMLP) receptor-mediated end-target chemotaxis of neutrophils, indicating that not all receptors in neutrophils are in signalling complexes with TRPC6 (Lindemann et al. 2013).

Erythrocytes have a life span of some 100–120 days in the blood, and eryptosis is a coordinated, programmed cell death removing erythrocytes without rupture of the cell membrane and release of intracellular material (Lang et al. 2012). This process is different to apoptosis in other cells, because erythrocytes lack nuclei and mitochondria. The trigger of eryptosis is influx of Ca^{2+} and although the exact molecular make-up of the channels involved is still elusive, TRPC6 is considered to be an essential part of it. Cell shrinkage and phospholipid scrambling, two hallmarks of eryptosis, were significantly lower in Cl^--depleted erythrocytes from *Trpc6*$^{-/-}$ mice than from wild-type mice (Foller et al. 2008). Moreover, Ca^{2+} entry in these cells was inhibited after pre-incubation with specific TRPC6 antibodies, but not with antibodies directed against TRPC3 or TRPM2 (Foller et al. 2008). A postulated activation of the channel by prostaglandins and reactive oxygen species is also in line with the characteristics of TRPC6 (see above).

In human **platelets**, thrombin-activated cation influx through TRPC6 seems to be independent of store depletion (Hassock et al. 2002), although interaction of this channel with IP3 receptors, SERCA pumps, STIM proteins and Orai channels was reported (Jardin et al. 2009), (Redondo et al. 2008). It is well documented that phosphoinositide 3-kinase (PI3K) activation resulting in the production of PIP_3 triggers platelet aggregation by inducing an influx of Ca^{2+} (Lu et al. 1998). In accord with this concept, TRPC6 was recently identified as the putative molecular correlate of a PIP_3-sensitive calcium entry system in platelets, Jurkat T cells and RBL-2H3 mast cells (Tseng et al. 2004). However, platelet function in *Trpc6*$^{-/-}$ mice in vivo and in vitro was completely unaltered, although DAG-induced

Ca^{2+} influx was missing (Ramanathan et al. 2012). Most interestingly, *Stim1*$^{-/-}$ (Varga-Szabo et al. 2008) and *Orai1*$^{-/-}$ (Braun et al. 2009) platelets exhibit severe defects in thrombosis. Therefore, TRPC6 is dispensable for hemostasis and thrombosis (Ramanathan et al. 2012) and seems to work independently of Orai1 and STIM1. Very recently however, coincidence activation of platelets by thrombin and glycoprotein VI was shown to be dependent on a heteromeric TRPC3/6 channel complex which drives the Na^+/Ca^{2+} exchanger (NCX) to the reverse mode by Na^+ influx in murine platelets (Harper et al. 2013).

3.4 TRPC6 in Tumour Development

In addition to a function of TRPC6 in VEGF- and PTEN-induced angiogenesis supporting tumour progression (see above), there are numerous reports published in the last years describing TRPC6 function in different tumour entities. OAG-induced TRPC6 currents were detected in a breast carcinoma cell line MCF-7 as well as in primary cultured human breast carcinoma epithelial cells (hBCE), while TRPC6 expression was selectively elevated in breast cancer carcinoma specimen in comparison to normal tissues (Guilbert et al. 2008). In human breast ductal adenocarcinoma, however, TRPC6 did neither correlate with proliferative parameters as shown for TRPC1, TRPM7 and TRPM8, nor was it overexpressed in invasive cancer cells like TRPV6 (Dhennin-Duthille et al. 2011). In tumour samples from the liver of a cancer patient, TRPC6 was expressed at a higher level than in normal liver tissues (El Boustany et al. 2008).

A possible mechanism for TRPC6-induced carcinogenesis was proposed for gastric (Cai et al. 2009) and oesophageal tumours (Shi et al. 2009) as well as prostate cancer (Wang et al. 2010) and renal cell carcinoma (Song et al. 2013). Blockage of TRPC6 by transfection of a dominant negative form of the channel (DNTRPC6) or TRPC6-specific siRNAs inhibited cdc42 activation and led to cell growth arrest at the G2 phase as well as suppressed tumour formation after injection of the respective tumour cells [e.g. oesophageal squamous cell carcinoma (OSCC) (Shi et al. 2009)] into nude mice. Another interesting signal transduction pathway involving TRPC6 was also discovered in prostate epithelial cells. Agonist-mediated stimulation of the α_1-adrenergic receptor promotes proliferation of primary human prostate cancer epithelial cells by inducing Ca^{2+} entry mostly relying on TRPC6 and subsequent activation of NFAT (Thebault et al. 2006).

A recent report further supports an important role of TRPC6 in oesophageal cancer, as TRPC6 overexpression correlated with a poor prognosis (Zhang et al. 2013).

In glioblastoma multiforme (GBM), a highly malignant primary brain tumour, hypoxia has been demonstrated to drive cancer cells in a more aggressive and malignant state. The molecular mechanism involves Notch1-induced TRPC6 expression entailing activation of the calcineurin-NFAT pathway (Chigurupati et al. 2010). Thus, TRPC6 was elevated in GBM specimen compared to normal tissues (Chigurupati et al. 2010).

Recently, elevated TRPC6 expression was identified in five cell lines derived from head and neck squamous cell carcinoma (HNSCC) tissues and in HNSCC tumour samples (Bernaldo de Quiros et al. 2013). Most interestingly, siRNA-induced knock-down in the cell lines resulted in inhibition of HNSCC cell invasion, while cell proliferation was not altered (Bernaldo de Quiros et al. 2013).

In the future, specific TRPC6 inhibitors may be attractive candidates to complement classical chemotherapeutic strategies in the fight against tumour progression.

3.5 TRPC6 and Neuronal Function

TRPC6 expression in the brain is lower than that of other TRPCs. Thus TRPC3, but not TRPC6, is essential for metabotropic glutamate receptor signalling in mouse cerebellar Purkinje cells (Hartmann et al. 2008). However, overexpression of TRPC6 induces excitatory synapse formation, and TRPC6 transgenic mice show better performance in water maze tests than wild-type mice, indicating a better spatial learning and memory in these mice (Zhou et al. 2008). Along the same line, *Trpc6*$^{-/-}$ mice demonstrated reduced exploration behaviour in the square open field and the elevated star maze, while anxiety behaviour was not distinguishable from wild-type mice (Beis et al. 2011).

The highly specific expression of TRPC6 in the dentate gyrus (Bonaventure et al. 2002) may be due to its highly specific function in the brain, but has still to be investigated. TRPC6 and TRPC3 play an important role in brain-derived neurotrophic factor (BDNF)-mediated survival of granule cells in the cerebellum, because down-regulation of these channels induced apoptosis and blocked BDNF protective effects (Jia et al. 2007). Moreover, in cultured rat cerebellar granule cells, elevation of $[Ca^{2+}]_i$ and growth cone steering induced by BDNF are abolished when TRPC3 and 6 are inhibited by down-regulation or dominant negative mutants of TRPC3 and 6 (Li et al. 2005). In the hippocampus, expression of TRPC4, 5 and 6 is already detectable during embryonic development and TRPC6 is suggested to promote (Tai et al. 2008), while TRPC4 and 5 seem to inhibit dendritic growth (Tai et al. 2009). Along these lines, TRPC6 is able to activate a transcriptional pathway involving calmodulin kinase IV (CAMKIV) and cAMP response element-binding protein (CREB) to increase spine number in hippocampal neurons (Tai et al. 2008).

Sufficient oxygen supply is crucial for neuronal function and focal cerebral ischaemia results in severe brain injury. High levels of glutamate lead to overactivation of *N*-methyl-D-aspartate receptors (NMDARs) which induce Ca^{2+} overload and activation of the protease calpain. Most interestingly, cleavage of an amino-terminal calpain recognition site (see Fig. 1c) in TRPC6 leads to channel inactivation, while inhibition of TRPC6 degradation suppresses ischaemic brain damage in rats (Du et al. 2010). Along these lines, the antidepressant drug hyperforin which activates TRPC6 channels (see above and Leuner et al. 2007) was also able to suppress brain damage induced by transient middle cerebral artery occlusion (MCAO) in rats via inhibition of channel degradation (Lin et al. 2013b). Moreover, the same research group also recently reported that resveratrol, a

polyphenol found in grapes, exerts its neuroprotective effects via inhibition of calpain proteolysis of TRPC6 and protects rat from MCAO (Lin et al. 2013a).

A novel class of microvillous secondary chemosensory cells in the mammalian olfactory system were identified that express TRPC6 colocalised with PLCβ_2 and components of the cytoskeleton of microvilli (Elsaesser et al. 2005). But they represent only 5 % of all olfactory cells and their function remains unclear.

3.6 Other Possible Roles of TRPC6 and Outlook

TRPC6 was also selectively localised to the cell body of rods, while voltage- gated calcium channels were expressed in the synaptic terminal and in the ellipsoid and subellipsoid regions of the retina (Krizaj 2005). However it remains unclear how TRPC6 function might substitute voltage-gated calcium channels in the cell body of rods.

In human keratinocytes, TRPC6 is sufficient to induce differentiation. Thus, TRPC6 activation by hyperforin triggers, while TRPC6-specific siRNAs inhibit keratinocyte differentiation (Muller et al. 2008). Moreover, triterpenes which also promote keratinocyte differentiation are able to increase TRPC6 expression in human keratinocytes (Woelfle et al. 2010).

Fibroblast transdifferentiation into myofibroblasts is an essential step in wound healing and tissue remodelling. In a genome-wide screen, TRPC6 was identified as sufficient for myofibroblast transformation. Thus, *Trpc6*-deficient mice showed impaired dermal and cardiac wound healing, because their fibroblasts were not able to initiate transforming growth factor β (TGFβ)- and Ang II-induced transdifferentiation (Davis et al. 2012).

Parasites like *Plasmodium falciparum* and *Toxoplasma gondii* use cellular signal transduction cascade for egress from the host cell. Very recently it was demonstrated that Ca^{2+} influx through TRPC6 activates host calpain to proteolyse the host cytoskeleton, allowing the release of parasites (Millholland et al. 2013).

To conclude, the exact physiological role of TRPC6 channels in numerous tissues is still not completely clear. Therefore, future investigations of human pathophysiology involving TRPC6 should be highly rewarding in years to come. Hopefully, more TRPC6-specific blockers and activators will be identified in the near future and might be beneficial for therapeutic intervention in diseases coupled to pathological functions of TRPC6 channels.

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Transient Receptor Potential Canonical 7: A Diacylglycerol-Activated Non-selective Cation Channel

Xuexin Zhang and Mohamed Trebak

Contents

Abstract

Transient receptor potential canonical 7 (TRPC7) channel is the seventh member of the mammalian TRPC channel family. TRPC7 mRNA, protein, and channel activity have been detected in many tissues and organs from the mouse, rat, and human. TRPC7 has high sequence homology with TRPC3 and TRPC6, and all three channels are activated by membrane receptors that couple to isoforms of phospholipase C (PLC) and mediate non-selective cation currents. TRPC7, along with TRPC3 and TRPC6, can be activated by direct exogenous application of diacylglycerol (DAG) analogues and by pharmacological maneuvers that increase endogenous DAG in cells. TRPC7 shows distinct properties of activation, such as constitutive activity and susceptibility to negative regulation by extracellular Ca^{2+} and by protein kinase C. TRPC7 can form heteromultimers with TRPC3 and TRPC6. Although TRPC7 remains one of the least studied TRPC channel, its role in various cell types and physiological and pathophysiological conditions is beginning to emerge.

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TRPC7 • Non-selective cation channel • Diacylglycerol • Ca^{2+} signaling • Phospholipase C

1 Gene, Splicing, Etc.

TRPC7 gene was first cloned by Okada et al. (1999) from mouse brain. The murine TRPC7 gene has 12 exons and is mapped to the chromosomal region 13 B2 (Numaga et al. 2007). Eight transcripts from mouse TRPC7 gene have been reported so far. Subsequently, Riccio et al. (2002a) cloned human TRPC7 from brain. The human TRPC7 gene mapped to the chromosomal region 5q31.1 and has also 12 exons. Thus far, 10 transcripts of human TRPC7 have been identified. Both human and mouse TRPC7 genes consist of an open reading frame of 2,589 bp yielding a protein of 862 amino acids (Okada et al. 1999; Riccio et al. 2002a). Human TRPC7 shares the common structural features of the larger TRP channel superfamily with a predicted six transmembrane segments and a pore region. Human TRPC7 has 98 % overall identity with mouse TRPC7 and 81 % and 75 % overall identity with human TRPC3 and TRPC6, respectively (Numaga et al. 2007). Rat TRPC7 gene consists of 12 exons and is mapped to the chromosomal region 17p14. In rabbit, TRPC7 gene is mapped to the third chromosome. No splice variants were reported for either rat or rabbit; detailed information on various TRPC7 splice variants in different species is shown in Table 1.

2 Expression

TRPC7 mRNA and protein have been detected in many tissues and organs from the human, mouse, rabbit, and rat.

In the human, Riccio et al. (2002b) reported that human TRPC7 mRNA was broadly expressed in the central nervous system (CNS) as well as some peripheral tissues (pituitary gland, kidney, intestine, prostate, and cartilage). Myometrium from pregnant women and human myometrial cell lines expressed TRPC7 mRNA (Dalrymple et al. 2002; Yang et al. 2002). TRPC7 mRNA was also detected in human coronary artery endothelial cells (Yip et al. 2004), in human undifferentiated gingival keratinocytes (Cai et al. 2005), in differentiated IMR-32 neuroblastoma cells (Nasman et al. 2006), in HaCaT cells (Beck et al. 2006), and in lung tissue (Finney-Hayward et al. 2010). Human TRPC7 protein was detected in HEK293 cells by Zagranichnaya et al. in 2005. Studies failed to detect TRPC7 mRNA in human MG-63, SaOS, and U2 OS osteoblasts (Abed et al. 2009) and in human non-small cell lung cancer (Zhang et al. 2010).

In the mouse, abundant TRPC7 mRNA expression was detected in mouse heart, lung, and eye and moderate expression in the brain, spleen, and testis (Okada

Table 1 TRPC7 gene in different species

Species	Chromosomal location	Splicing	mRNA (bp)	aa	Exon
Human	5q31.1	hTRPC7-001	2,987	862	12
		hTRPC7-002	2,603	380	11
		hTRPC7-003	2,239	746	9
		hTRPC7-004	2,022	261	8
		hTRPC7-005	2,404	801	10
		hTRPC7-006	2,422	807	10
		hTRPC7-007	817	?	4
		hTRPC7-008	414	?	2
		hTRPC7-201	2,752	801	11
		hTRPC7-202	2,587	746	10
Mouse	13 B2	mTRPC7-001	3,483	862	12
		mTRPC7-002	3,519	861	12
		mTRPC7-003	3,502	451	13
		mTRPC7-004	2,424	807	11
		mTRPC7-005	2,024	261	9
		mTRPC7-006	2,406	801	11
		mTRPC7-008	2,241	746	10
		mTRPC7-009	2,605	380	12
Rabbit	3	ND	4,077	862	12
Rat	17p14	ND	3,055	862	12

? No protein product

et al. 1999). TRPC3/6/7 mRNA was detected in retinas from mice (Warren et al. 2006; Hartwick et al. 2007; Sekaran et al. 2007). Boisseau et al. reported that TRPC7 mRNA was less abundant in embryonic brain and cortex of E13 C57BL6/J mice (Boisseau et al. 2009). TRPC7 mRNA was also detected in murine MC3T3 osteoblasts (Abed et al. 2009), while skeletal muscle (Jang et al. 2012) and inner ear organs from embryonic (E) and early postnatal (P) Swiss Webster mice (Asai et al. 2010) showed no TRPC7 mRNA expression. Western blots revealed TRPC7 protein expression in rhythmically active ventral respiratory medullary brain slice preparation (Ben-Mabrouk and Tryba 2010).

In the rat, TRPC7 mRNA was present in neurons throughout ganglia (Buniel et al. 2003). Rat A7r5 vascular smooth muscle cells expressed transcripts encoding TRPC7 (Maruyama et al. 2006). In rat hippocampal H19–7 cells, the levels of mRNA and protein for TRPC7 are high in proliferating cells and decline dramatically upon differentiation (Wu et al. 2004; Numaga et al. 2007). In Dahl salt-sensitive rats, TRPC7 mRNA expression was increased in the failing myocardium (Satoh et al. 2007). TRPC7 mRNA was also expressed in cultured rat microglia (Ohana et al. 2009).

TRPC7 protein was observed in rat striatal cholinergic interneurons (Berg et al. 2007) and had a strikingly high level of expression in the neuropil in the rat globus pallidus (GP) (Chung et al. 2007).

TRPC7 mRNA was not detected in freshly isolated rat renal resistance vessels, glomeruli, and aorta (Facemire et al. 2004), in rat distal pulmonary arterial smooth muscle (Wang et al. 2004), and in rat dorsal root ganglia (DRG) neurons (Wu et al. 2008) (Table 2).

Table 2 Expression of TRPC7

Species	mRNA	Protein
Human	Lung tissue[P] (Finney-Hayward et al. 2010) Differentiated IMR-32 neuroblastoma cells[P] (Nasman et al. 2006) HaCaT cells[P] (Beck et al. 2006) Undifferentiated gingival keratinocytes[P] (Cai et al. 2005) HEK293 cells[P] (Zagranichnaya et al. 2005) Coronary artery endothelial cells[P, ISH] (Yip et al. 2004) Pregnant women myometrium and myometrial cell lines[P] (Dalrymple et al. 2002; Yang et al. 2002) Central nervous system(CNS) and pituitary gland, kidney, intestine, prostate, and cartilage[P] (Riccio et al. 2002b)	HEK293 cells[W] (Zagranichnaya et al. 2005)
Mouse	Brain, testis, lung, liver, heart, kidney, and DRG[P] (Jang et al. 2012) MnPO glutamatergic neurons[P] (Tabarean 2012) Melanopsin-expressing ganglion cells[P] (Perez-Leighton et al. 2011) MC3T3 osteoblasts[P] (Abed et al. 2009) Embryonic brain and cortex of E13 C57BL6/J mice[P] (Boisseau et al. 2009) Retinas[P] (Warren et al. 2006; Hartwick et al. 2007; Sekaran et al. 2007) Smooth muscle cells[P] (Walker et al. 2001) Heart, lung, eye, brain, spleen, and testis[N] (Okada et al. 1999)	Cerebellum and ventral respiratory group island[W] (Ben-Mabrouk and Tryba 2010)
Rabbit	Not determined	Portal vein and mesenteric artery[IP] (Ju et al. 2010) Portal vein myocytes[W] (Saleh et al. 2008) Coronary artery myocytes[I] (Peppiatt-Wildman et al. 2007)
Rat	Mammary tissues[P] (Anantamongkol et al. 2010) Hypocretin/orexin neurons[P] (Cvetkovic-Lopes et al. 2010) Cultured rat microglia[P] (Ohana et al. 2009) Failing myocardium of Dahl salt-sensitive rats[P] (Satoh et al. 2007) Striatal cholinergic interneurons[ISH] (Berg et al. 2007) Hippocampal H19-7 cells[P] (Wu et al. 2004; Numaga et al. 2007) A7r5 vascular smooth muscle cells[P] (Maruyama et al. 2006) Myometrium[P] (Babich et al. 2004)	GH4C1 pituitary cells[W] (Lavender et al. 2008) Neuropil in globus pallidus (GP)[I] (Chung et al. 2007) Striatal cholinergic interneurons[I] (Berg et al. 2007) Hippocampal H19-7 cells[W] (Wu et al. 2004; Numaga et al. 2007) A7r5 vascular smooth muscle cells[W] (Maruyama et al. 2006) Ganglion neurons[I] (Buniel et al. 2003) Brain[W] (Goel et al. 2002)

[I]Protein detected by immunostaining
[ISH]mRNA detected by in situ hybridization
[N]mRNA detected by northern blotting
[P]mRNA detected by RT-PCR
[W]Protein detected by western blotting
[IP]Immunoprecipitation

In the rabbit, immunocytochemical studies demonstrated preferential TRPC7 expression in the plasmalemma of the freshly dispersed rabbit coronary artery myocytes (Peppiatt-Wildman et al. 2007). Saleh et al. (2008) and Ju et al. (2010) reported that TRPC7 protein was expressed in portal vein myocytes. To date rabbit TRPC7 mRNA detection was not reported.

3 The Channel Protein Including Structural Aspects

TRPC channels share sequence similarity and transmembrane topology with voltage-gated K^+ and Na^+ channels, and by analogy to these channels, functional TRPC is predicted to form tetramers. TRPC7 protein contains four N-terminal ankyrin repeats, six transmembrane-spanning domains, a putative pore region located between transmembrane domains 5 and 6, and a highly conserved TRP box consisting of the EWKFAR motif and a proline-rich region (Dietrich et al. 2005) (Fig. 1).

4 Interacting Partners and Regulation

TRPC7 along with other TRPC channels has been proposed to constitute the pore-forming units of store-operated calcium entry (SOCE) channels (Worley et al. 2007; Cheng et al. 2013). However, the more recent discovery of STIM1 and Orai1 as the bona fide molecular correlates of the SOCE pathway has challenged this earlier view. STIM1 is the Ca^{2+} sensor residing in the endoplasmic reticulum (ER), while Orai1 is the SOCE channel at the plasma membrane mediating the highly Ca^{2+}-selective Ca^{2+} release-activated Ca^{2+} (CRAC) current (Potier and Trebak 2008). Nevertheless, STIM1 was shown to bind to TRPC1, TRPC2, TRPC4, and TRPC5 but not to TRPC3, TRPC6, and TRPC7, suggesting a more general role for STIM1 in regulating various ion channels at the plasma membrane (Worley et al. 2007). DeHaven et al. showed that TRPC7-mediated currents are receptor activated and were not dependent on either STIM1 or Orai1 expression (DeHaven et al. 2009), questioning the requirement for STIM1 in TRPC channel function. Regardless of whether STIM1 is involved in TRPC channel regulation, it is widely accepted that TRPC7 is activated by downstream stimulation of PLC-coupled receptors in a manner independent of store depletion, presumably through production of diacylglycerol (DAG); DAG analogues such as 1-oleolyl-2-acetyl-sn-glycerol (OAG) applied in bath solutions can activate TRPC7 currents in a PKC-independent manner (Okada et al. 1999). TRPC3 and TRPC6 channels are also activated by a similar mechanism involving DAG produced downstream PLC activity (Hofmann et al. 1999; Trebak et al. 2003a, b; Putney et al. 2004; Vazquez et al. 2004). It is unclear how DAG activates TRPC7 and its closest homologues, TRPC3 and TRPC6, and whether DAG acts directly on these channels or through some intermediary proteins. In the cell-attached configuration, TRPC7 channels expressed in HEK293 cells could be activated by OAG, but

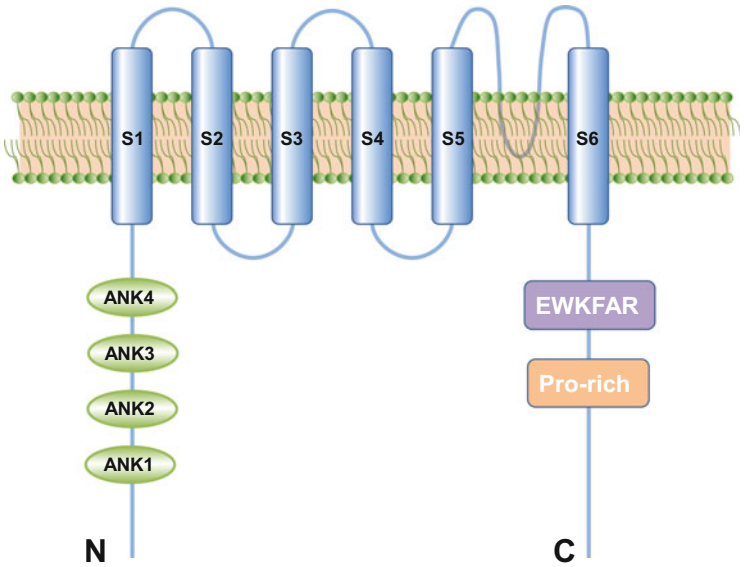

Fig. 1 Domain structure of TRPC7. TRPC7 contains six transmembrane domains (S1–S6) with the pore region between S5 and S6. N-terminal obtains 4 ankyrin-like repeats (ANK1–4). C-terminal obtains a highly conserved TRP box containing the EWKFAR motif and a proline-rich region

OAG failed to activate TRPC7 channels in excised patches suggesting that DAG action on TRPC7 channels is not a direct one and requires cytosolic proteins or factors lost in excised patches (Lemonnier et al. 2008).

The inositol 1,4,5-trisphosphate (IP_3) receptor is one of the major proteins that was shown to interact with TRPC7 to positively regulate its activity (Vazquez et al. 2006; Zhang et al. 2008; Ju et al. 2010). Polyphosphoinositides (PIPs) were shown to interact with and regulate a number of TRP channels, including TRPC channels (Lemonnier et al. 2008; Trebak et al. 2009). PIPs are positive modulators of TRPC7 channels whereby inhibition of PIPs synthesis inhibits the ability of OAG to activate TRPC7 currents (Lemonnier et al. 2008). In excised inside-out patches, TRPC7 channels can be activated by application of phosphatidylinositol-4,5 bisphosphate (PIP_2) or ATP, but not by inositol 1,4,5-trisphosphate (IP_3) (Lemonnier et al. 2008). Using ectopic expression of a voltage-sensing PIP phosphatase gene from zebra fish, Imai et al. showed that dephosphorylation of PIPs robustly inhibited TRPC7 currents induced by either the muscarinic agonist carbachol (CCh), OAG, or the DAG lipase inhibitor RHC80267 (which causes endogenous DAG accumulation) (Imai et al. 2012). These authors showed that that depletion of PIP2 using the voltage-sensing PIP phosphatase inhibits TRPC3/C6/C7 channel activity. These data suggest that while PIP_2 degradation is necessary for TRPC3/6/7 channel activation by producing DAG, PIP2 degradation is also necessary for the closing of TRPC3/6/7 channels, in agreement with previous studies documenting the complex nature of PIPs in the regulation of TRPC channels

(Lemonnier et al. 2008; Trebak et al. 2009). A study by Ju et al. used inhibitory antibodies against TRPC in inside-out patch clamp recordings and co-immunoprecipitations to conclude that in portal vein myocytes, noradrenaline-activated native cationic currents are mediated by TRPC6/TRPC7 heteromultimers (Ju et al. 2010). They also showed that PIP_2 inhibited OAG-induced native TRPC6/TRPC7 activity, and this inhibition by PIP_2 could be normalized by IP_3 acting independently of IP_3 receptors (Ju et al. 2010). This study is in stark contrast to the data described above indicating a positive regulation of TRPC7 channels by PIP_2. This discrepancy might be due to the heteromeric nature of the TRPC6/TRPC7 channels identified in portal vein myocytes by Ju et al. (2010).

TRPC7 channels are negatively regulated by Ca^{2+} through a direct extracellular action and via Ca^{2+}/calmodulin (CaM)- and protein kinase C (PKC)-dependent mechanisms (Shi et al. 2004; Lemonnier et al. 2006). The negative regulation of TRPC7 channels by PKC also extends to its closest homologues, TRPC3 and TRPC6, and PKC phosphorylates a C-terminal site, Ser712 on TRPC3 (Trebak et al. 2005) and Ser714 on TRPC6 (Kim and Saffen 2005). TRPC7 inhibition by Ca^{2+} is due to Ca^{2+} entry through TRPC7 channels themselves and this negative feedback by Ca^{2+} is mediated by calmodulin and attenuated under physiological conditions by the presence of closely associated SERCA pumps, which buffers Ca^{2+} to the endoplasmic reticulum (Lemonnier et al. 2006). Shi et al. reported whole-cell and single-channel data suggesting voltage-dependent inhibitory actions of extracellular Ca^{2+} on TRPC7 currents likely mediated through Ca^{2+} interaction with an extracellular site capable of sensing the membrane potential. The same authors also reported a concentration-dependent inhibitory effect of intracellular Ca^{2+} on TRPC7 currents mediated by calmodulin (Shi et al. 2004).

Lussier et al. used yeast two-hybrid screen, GST pull-down, and co-immunoprecipitation assays to show that a member of the dynamin superfamily, MxA, interacts with TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7; the interaction of MxA with TRPC6 involved the second ankyrin-like repeat domain of TRPC6, and co-expression of MxA and TRPC6 enhanced agonist-activated and OAG-activated calcium entry, suggesting a positive regulatory role of MxA on TRPC channel activity. MxA mutants defective in GTP binding marginally potentiated OAG-induced TRPC6 channel activity, suggesting that MxA positive regulation of TRPC channels is dependent on GTP binding (Lussier et al. 2005). A subsequent study by the same group used a similar approach to reveal that RNF24, a new membrane RING-H2 finger protein, interacted with the ankyrin-like repeat domain of TRPC6 and interacted with all TRPCs (Lussier et al. 2008). The significance of this RNF24/TRPCs interaction is not clear as RNF24 did not affect receptor activation of TRPC channels. The authors proposed that RNF24 interacts with TRPCs channels in the Golgi apparatus, thus likely impacting TRPC intracellular trafficking (Lussier et al. 2008).

Yuasa et al. investigated the potential regulation of TRPC7 channels by cyclic GMP (cGMP) and cGMP-dependent protein kinase (cGK) using ectopic expression in HEK293 and COS-7 cells. They found that TRPC7 has three putative cGK phosphorylation sites, and using in vitro and in vivo kinase assays, they revealed

that the cytosolic cGK-Iα isoform specifically phosphorylates mouse TRPC7 on threonine 15 without any effect on mouse TRPC3. This cGK-Iα-mediated phosphorylation of TRPC7 caused a significant reduction in carbachol-activated calcium signaling and phosphorylation of the transcription factor, CREB (Yuasa et al. 2011). In the same report, Yuasa et al. used co-immunoprecipitation assays and showed interaction between cGK-Iα and TRPC7 N-terminal ankyrin repeat region (Yuasa et al. 2011).

Sinkins et al. reported differential association between TRPC channel isoforms and immunophilins. Using immunoprecipitations assays in cells co-expressing different TRPC and immunophilin isoforms, they showed that TRPC3, TRPC6, and TRPC7 interact with FKBP12, whereas TRPC1, TRPC4, and TRPC5 interact with FKBP52. Similar results were found with membrane lysates from the rat cerebral cortex and in both cases the association of immunophilins with TRPC channels could be displaced by FK506 (Sinkins et al. 2004). Finally, in HEK293 cells stably expressing TRPC6 channels, FK506 disrupted the interaction between TRPC6 and immunophilins and inhibited TRPC6 activation by the muscarinic agonist carbachol, suggesting a role of immunophilins in the receptor activation of all TRPC channels (Sinkins et al. 2004).

5 A Biophysical Description of the Channel Function, Permeation, and Gating

When ectopically expressed in HEK293 cells, TRPC7 forms a non-selective cation channel with reversal potentials around ~0 mV and permeability ratios P_{Cs}:P_{Na}:P_{Ca}:P_{Ba} of 1:1:1.9:3.5 for the spontaneous current and 1:1.1:5.9:5.0 for the ATP-activated current (Okada et al. 1999). Lievremont et al. (2005b) reported that in TRPC7-deficient avian B-lymphocytes DT40 cells, Ba^{2+} entry induced in response to either OAG or muscarinic receptor stimulation was significantly inhibited, suggesting that TRPC7 channel permeates Ba^{2+} ions. When expressed in HEK293 cells, the typical current–voltage (I/V) relationship of mouse or human TRPC7 currents is almost linear with a slight flattening around the reversal potentials (Fig. 2, left panel). Mouse TRPC7 single-channel conductance is 24.8 pS and 24.3 pS (Fig. 2, right panel) under bath solutions containing 1 mM Ca^{2+} and 10 mM Ca^{2+}, respectively (Shi et al. 2004).

As discussed above, TRPC7 is a receptor-activated non-selective cation channel activated through PLC-mediated metabolism of PIP_2 and production of DAG (Okada et al. 1999; Trebak et al. 2003b). Okada et al. (1999) showed that heterologously expressed mouse TRPC7 behaves as a receptor-activated cation channel not activated by store depletion. Earlier reports on human TRPC7 described its activation by the SERCA pump inhibitor thapsigargin, suggesting that at least under certain expression conditions, TRPC7 could behave as a store-operated channel (Riccio et al. 2002a; Lievremont et al. 2004). However, subsequent studies on native TRPC7 in DT40 cells and on TRPC7 ectopically expressed in HEK293 cells have questioned the role of human TRPC7 in store-operated Ca^{2+} entry and demonstrated that human TRPC7

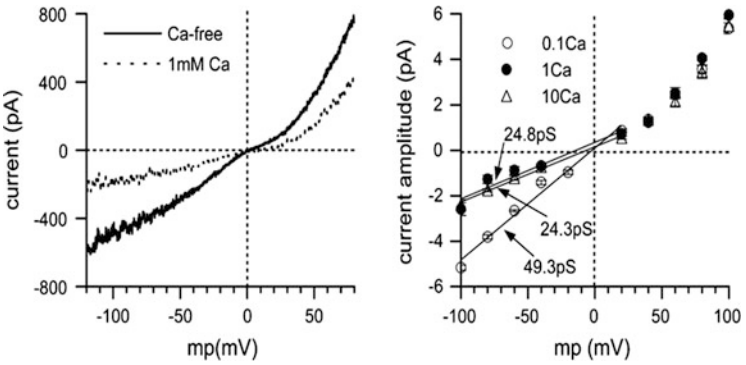

Fig. 2 Current–voltage relationship and single-channel conductance of carbachol-stimulated TRPC7 channel currents in HEK293 cells. Nystatin-perforated whole-cell recordings (*left panel*) were performed in HEK293 cells expressing TRPC7. The pipette solution contained (in mM) 140 CsCl, 2 $MgCl_2$, 10 Hepes, and 10 glucose (pH = 7.2, adjusted with Tris base); Ca^{2+}-free external solution contained (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 EGTA, 10 Hepes, and 10 glucose (pH = 7.4, adjusted with Tris base); for Ca^{2+}-containing external solution, 1 mM $CaCl_2$ was added to Ca^{2+}-free solution with the omission of EGTA. For cell-attached (C/A) configuration single-channel recording (*right panel*), cells were bathed in high potassium solution with the following composition to null the transmembrane potential (in mM): 140 KCl, 2 EGTA, 2 $MgCl_2$, and 10 Hepes (pH = 7.2, adjusted with Tris base); the pipette solution contained (in mM) 140 NaCl, 5 TEA-Cl, 1.2 $MgCl_2$, 0.1 $CaCl_2$, 10 Hepes, 10 glucose, and 0.1 carbachol (CCh) (pH = 7.4, adjusted with Tris base). Taken from Shi et al. (2004)

forms a store-independent receptor-activated channel (Lievremont et al. 2005b; DeHaven et al. 2009). Currently, there are no specific pharmacological modulators of TRPC7. Okada et al. showed 100 μM lanthanum (La^{3+}) and 25 μM SKF96365 are more efficient at inhibiting ATP-activated Ca^{2+} entry in HEK293 cells expressing mouse TRPC7 than 100 μM gadolinium (Gd^{3+}) (Okada et al. 1999). Riccio et al. (2002a, b) used 250 μM Gd^{3+}, 250 μM La^{3+}, and 25 μM SKF96365 to completely block the Ca^{2+} entry in cells expressing human TRPC7. Amiloride at 300 μM was also used to inhibit constitutively activated TRPC7 currents (Okada et al. 1999), and the drug 2-aminoethoxydiphenyl borate (2-APB) originally described and IP_3 receptor blocker partially inhibited TRPC3, TRPC6, and TRPC7 channels at concentrations of 10-100 μM by a mechanism not involving IP_3 receptors (Lievremont et al. 2005a) (Table 3).

6 Physiological Functions in Native Cells, Organs, and Organ Systems

Using ectopic expression, Hofmann et al. (2002) showed that TRPC channel isoforms can heteromultimerize with preferential associations between members of the TRPC3/6/7 subfamily, and several investigations suggested widespread TRPC channel heteromultimerization in native tissues (for review Eder and

Table 3 Activation and inhibition of TRPC7 channels

	Activation	Inhibition
Human TRPC7	CCh, TG (Riccio et al. 2002b) TG, MeCh (Lievremont et al. 2004) TG, MeCh (Lievremont et al. 2005a) CCh, ATP (Beck et al. 2006) ET-1 (Horinouchi et al. 2011) ATP,OAG (Nishioka et al. 2011) OAG, PIP_2 (Lemonnier et al. 2008)	La^{3+}, Gd^{3+}, SKF96365 (Riccio et al. 2002b) 2-APB, SKF96365 (Lievremont et al. 2005a; Zagranichnaya et al. 2005) TG/Ca^{2+} (Lemonnier et al. 2006) Cilostazol (Nishioka et al. 2011)
Mouse TRPC7	DOG, OAG, ATP (Okada et al. 1999) Constitutive active (Okada et al. 1999) CCh, GTPγS, OAG (Shi et al. 2004) CCh (Yuasa et al. 2011) CCh, OAG, RHC80267 (Imai et al. 2012)	La^{3+}, SKF96365, amiloride (Okada et al. 1999) DrVSP (Imai et al. 2012)
Rabbit TRPC7	OAG, IP_3 (Ju et al. 2010)	PIP_2 (Ju et al. 2010)
Rat TRPC7	AVP, Ang II (Maruyama et al. 2006) CCh (Satoh et al. 2007)	SKF96365 (Satoh et al. 2007)

CCh, carbachol; TG, thapsigargin; MeCh, methacholine; ET-1, endothelin-1; ATP, adenosine-5′-triphosphate; SKF96365, 1-[beta-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride; 2-APB, 2-aminoethoxydiphenyl borate; DOG, 1,2-dioctanoylsn-glycerol; OAG, 1-oleoyl-2-acetyl-sn-glycerol; AVP, arginine vasopressin; Ang II, angiotensin II; DrVSP, *Danio rerio* voltage-sensing phosphatase; RHC80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; DiC8-PIP_2, a water-soluble PIP_2 analogue

Groschner 2008). Wu et al. (2010) reported that the activity of the TRPC3/6/7 subfamily was inhibited in the heart of cardiac-specific transgenic mice that express dominant-negative TRPC4, suggesting that coordinated TRPC heteromultimers might occur between different TRPC subfamilies under native conditions. Although this has not been described specifically for TRPC7 channels, studies have reported associations between TRPC1 and a member of the vallinoid family of TRP channels, TRPV4, in vascular endothelial cells (Ma et al. 2010). Most data currently available on TRPC7 and its involvement in various native conditions are correlative. TRPC7 mRNA is upregulated in human myometrium obtained from pregnant women at term active labor when compared with samples from non-pregnant patients (Dalrymple et al. 2004). Anantamongkol et al. showed rat mammary tissues from pregnant and lactating rats upregulated the expression of TRPC1, TRPC5, and TRPC7 while downregulating that of TRPC3 and TRPC4 in the early lactating period (Anantamongkol et al. 2010). A study by Ben-Mabrouk et al. suggested that substance P activates TRPC3 and TRPC7

channels to enhance respiratory rhythm regularity. These authors suggested that in mouse brainstem slices containing the pre-Bötzinger complex, TRPC3/7 underlies the rhythmic inward nonspecific cation current necessary for pacemaker activity (Ben-Mabrouk and Tryba 2010).

7 Lessons from Knockouts

Xue et al. (2011) reported the existence of an intrinsic component of the eye pupillary light reflex in nocturnal and crepuscular non-primates mammals that was dependent on the pigment melanopsin and PLCβ4 isoform. TRPC6$^{-/-}$ and TRPC7$^{-/-}$ double knockout mice showed abrogated light response in the M1 subtype of melanopsin-expressing, intrinsically photosensitive retinal ganglion cells (M1-ipRGCs) but had a normal intrinsic pupillary light reflex. These authors concluded that in M1-ipRGCs, the depolarizing light response is presumably generated by cation entry through heteromeric TRPC6/TRPC7 channels activated downstream of PLC, while there is no role for either TRPC3/6/7 or TRPC1/4/5 in light-induced contraction of the sphincter muscle. Shortly after, a study by Perez-Leighton et al. (2011) reported that TRPC3$^{-/-}$ and TRPC7$^{-/-}$ single knockouts showed no effect on the light response of intrinsically photosensitive retinal ganglion cells, while the TRPC6$^{-/-}$ knockout mice displayed smaller light responses compared to wild-type mice. The reasons for the discrepancy between the results obtained by these two independent groups are unknown. Xue et al. (2011) suggested in a post-scriptum that the differences could be attributed to their use of the perforated-patch clamp recording technique as opposed to the classical whole-cell configuration adopted by Perez-Leighton et al. (2011).

8 Role in Hereditary and Acquired Diseases

Miyagi et al. (2009) showed data suggesting heteromeric associations between a novel TRPP2 mutant and either TRPC3 or TRPC7. These heteromultimeric channels are involved in enhanced receptor-activated Ca^{2+} entry potentially leading to dysregulated cell growth in autosomal dominant polycystic kidney disease. Miyagi et al. identified a TRPP2 frameshift mutant with a 17-amino acid addition after glutamic acid residue 697 from a family with symptoms of autosomal dominant polycystic kidney disease. In HEK293 expression system, this TRPP2 mutant showed predominant plasma membrane localization and increased muscarinic receptor-activated Ca^{2+} influx in cells co-expressing either TRPC3 or TRPC7. Biochemical studies showed a direct association between the TRPP2 mutant, but not wild-type TRPP2, and either TRPC3 or TRPC7. This TRPP2 mutant induced a depolarizing shift of reversal potentials and an enhancement of single-channel conductance of muscarinic receptor-activated currents. These data suggest that heteromultimers of TRPP2 mutant and either TRPC3 or TRPC7 can enhance

receptor-activated Ca^{2+} entry responsible for disruption of cell growth in autosomal dominant polycystic kidney disease (Miyagi et al. 2009). Alvarez et al. described a potentially pathological role of TRPC3/67 channels in ventricular cardiomyocytes. These authors proposed that TRPC3/7 heteromultimers mediate Ca^{2+} entry upon activation of the purinergic receptor $P2Y_2$ by ATP/UTP. The sustained receptor-activated TRPC3/7 currents could cause depolarization, thus inducing cell automaticity and arrhythmias in response to the ATP/UTP release that occurs during early infarcts (Alvarez et al. 2008). In the failing myocardium of Dahl salt-sensitive rats, Satoh et al. reported increased TRPC7 expression, suggesting that TRPC7 might mediate pathological Ca^{2+} signaling and causing myocardial apoptosis (Satoh et al. 2007). Wu et al. suggested a role of TRPC in cardiac hypertrophy as inhibition of TRPC channel expression using a dominant-negative strategy in transgenic mice or in cultured neonatal cardiomyocytes inhibited NFAT transcriptional activity and pathological cardiac hypertrophy (Wu et al. 2010). In light of the established role of SERCA2 gene mutations Darier's disease [for review see Hovnanian (2004)], the study by Lemonnier et al. (2006), demonstrating that the maintained activation of TRPC7 channels depends on closely associated SERCA pumps, might indicate an involvement of TRPC7 channels in the clinical manifestations of Darier's disease.

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Part II

The TRPV Subfamily

TRPV1

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Contents

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Abstract

TRPV1 is a well-characterised channel expressed by a subset of peripheral sensory neurons involved in pain sensation and also at a number of other neuronal and non-neuronal sites in the mammalian body. Functionally, TRPV1 acts as a sensor for noxious heat (greater than ~42 °C). It can also be activated by some endogenous lipid-derived molecules, acidic solutions (pH < 6.5) and some pungent chemicals and food ingredients such as capsaicin, as well as by toxins such as resiniferatoxin and vanillotoxins. Structurally, TRPV1 subunits have six transmembrane (TM) domains with intracellular N- (containing 6 ankyrin-like repeats) and C-termini and a pore region between TM5 and TM6 containing sites that are important for channel activation and ion selectivity. The N- and C-termini have residues and regions that are sites for phosphorylation/dephosphorylation and PI(4,5)P2 binding, which regulate TRPV1 sensitivity and membrane insertion. The channel has several interacting proteins, some of which (e.g. AKAP79/150) are important for TRPV1 phosphorylation. Four TRPV1 subunits form a non-selective, outwardly rectifying ion channel permeable to monovalent and divalent cations with a single-channel conductance of 50–100 pS. TRPV1 channel kinetics reveal multiple open and closed states, and several models for channel activation by voltage, ligand binding and temperature have been proposed. Studies with TRPV1 agonists and antagonists and *Trpv1*$^{-/-}$ mice have suggested a role for TRPV1 in pain, thermoregulation and osmoregulation, as well as in cough and overactive bladder. TRPV1 antagonists have advanced to clinical trials where findings of drug-induced hyperthermia and loss of heat sensitivity have raised questions about the viability of this therapeutic approach.

Keywords

TRPV1 • Vanilloid receptor • Nociceptor • Capsaicin • Heat • Pain

TRPV1 is the most intensively studied mammalian TRP channel with a wealth of studies investigating its biophysical properties, cellular function and regulation, as well as its role in health and disease. TRPV1 has long attracted a great interest as a nociceptive transduction molecule, since it is a polymodal receptor for heat, low pH and some pungent chemicals that evoke a sensation of burning pain. Early studies were aided by the highly selective TRPV1 agonists capsaicin (the pungent ingredient in hot chilli peppers) and resiniferatoxin (RTX, from *Euphorbia resinifera*), which were used to selectively stimulate heat-sensitive unmyelinated nociceptive neurons (Holzer 1991). Capsaicin was eventually used to clone TRPV1 using an elegant expression cloning strategy (Caterina et al. 1997). The initial discovery of TRPV1 expression in a subset of sensory neurons that are involved in the detection and transmission of painful stimuli prompted numerous studies on the role of TRPV1 in sensory function. For this reason many studies have investigated the

properties of TRPV1 in sensory nerves as well as in heterologous expression systems. The development of specific TRPV1 antagonists as potential therapeutic agents has provided tools that together with genetically modified mice have greatly facilitated investigations into TRPV1 function. This chapter summarises the key properties and functions of TRPV1.

1 Gene

The human *Trpv1* gene is located on chromosome 17p13 and organised over 17 exons (Birnbaumer et al. 2003) and encodes an 839 amino acid protein. In addition to the full-length protein, expression of two N-terminal *Trpv1* splice variants has been reported, *Trpv1b* and *VR.5'sv* (Xue et al. 2001). *Trpv1b* mRNA does not contain exon 7, which encodes 60 amino acid residues (Xue et al. 2001), and the translated protein does not respond to heat, low pH or capsaicin but does associate with TRPV1 and acts as a dose-dependent inhibitor or negative regulator of TRPV1 (Vos et al. 2006). The second splice variant, *VR.5'sv*, lacks exons 1–4 as well as exon 7, and like TRPV1b, the protein has been shown not to respond to TRPV1 agonists but to interact with TRPV1 and inhibit channel activation (Eilers et al. 2007). Truncated N-terminal TRPV1 splice variants have been suggested to contribute to central osmosensation in the hypothalamus and to transduction of salt taste in taste buds (Lyall et al. 2004; Sharif Naeini et al. 2006; Sudbury et al. 2010).

Several non-synonymous TRPV1 polymorphisms have been reported and two of these, P91S and I315M, are associated with an increased surface expression of the channel in HEK293 cells (Xu et al. 2007). However, the physiological significance of these polymorphisms is unknown. The frequent polymorphism I585V, which was reported not to alter channel function significantly (Hayes et al. 2000), has been shown to reduce the risk of active childhood asthma (Cantero-Recasens et al. 2010). Intriguingly, I585V has been found to be associated with a reduced sensitivity to cold pain in healthy volunteers (Kim et al. 2004) and in neuropathic pain patients (Binder et al. 2011).

A mechano-sensitive homologue of the TRPV1 channel, initially named 'stretch-inactivated channel' (SIC), was cloned from the rat kidney and found to be activated by cell shrinkage and insensitive to capsaicin (Suzuki et al. 1999). Later analysis identified SIC as a chimeric construct composed of a truncated TRPV1 sequence and a C-terminal domain sequence belonging to the TRPV4 gene, thought to have arisen from an anomalous cDNA construct (Xue et al. 2001).

Expression of TRPV1 is regulated by the neurotrophic growth factors nerve growth factor (NGF), brain-derived neurotrophic factor and members of the glial cell-derived neurotrophic factor (GDNF) family in sensory neurons expressing the cognate receptors (Bevan and Winter 1995; Winter et al. 1988; Bron et al. 2003). This longer term regulation of TRPV1 expression is in addition to acute effects of neurotrophins on TRPV1 function outlined below.

2 Expression

2.1 Neuronal Expression

TRPV1 is expressed in about half of all somatic and visceral sensory neurons, with expression restricted to neurons of small to medium size in the dorsal root, trigeminal and vagal ganglia (Caterina et al. 1997; Helliwell et al. 1998). These neurons form unmyelinated or thinly myelinated nerve fibres, classified as C or Aδ fibres, that project to most organs and tissues. TRPV1 is also present in the central projections of sensory neurons in the dorsal horn of the spinal cord, trigeminal nucleus caudalis and the nucleus of the solitary tract (Tominaga et al. 1998; Szallasi and Blumberg 1999). TRPV1 is expressed in two distinct populations of DRG neurons. One class of TRPV1-expressing neurons is nerve growth factor (NGF)-sensitive and CGRP- and substance P-positive (peptidergic), and the second class contains glial cell-derived neurotrophic factor (GDNF)-sensitive IB4-binding neurons (Tominaga et al. 1998).

NGF is required as a trophic signal to maintain the expression of TRPV1, and in cultured neurons, TRPV1 expression is lost in a matter of days if the neurons are deprived of NGF (Bevan and Winter 1995; Winter et al. 1988). A similar regulation of TRPV1 expression by brain-derived neurotrophic factor is seen in vagal sensory neurons which express the cognate neurotrophin receptor (Bron et al. 2003). Elevated levels of NGF and the GDNF family member, artemin, occur in conditions such as inflammation and upregulate TRPV1 expression in vivo, which is thought to contribute to thermal hypersensitivity (Ji et al. 2002; Malin et al. 2006). In addition to enhancing TRPV1 expression, both NGF and artemin sensitise TRPV1 acutely (Malin et al. 2006; Shu and Mendell 1999), at least in part by increasing the membrane insertion of TRPV1 channels (Zhang et al. 2005).

It has long been recognised that TRPV1 is also present in the central nervous system (CNS), notably the preoptic area of the hypothalamus, where it is essential for thermoregulatory responses to avoid hyperthermia (Jancso-Gabor et al. 1970b; Szolcsanyi et al. 1971). Furthermore, pharmacological studies have demonstrated capsaicin-evoked physiological or electrophysiological responses that are consistent with TRPV1 expression in a number of brain nuclei or regions (Steenland et al. 2006). In situ hybridisation has further indicated the presence of TRPV1 in several brain nuclei (Mezey et al. 2000), and $^{[3]}$H-RTX autoradiography in *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice demonstrated a widespread expression of TRPV1 in the central nervous system (Roberts et al. 2004). A role for TRPV1 producing postsynaptic long-term depression (LTD) in response to production of the endogenous agonist anandamide has also been demonstrated in the dentate gyrus and the nucleus accumbens (Chavez et al. 2010; Grueter et al. 2010). Finally, a *Trpv1* splice variant expressed in the supraoptic nucleus (SON) and organum vasculosum lamina terminalis (OVLT) has been proposed to act as transduction channel responding to fluctuations in cerebrospinal fluid osmolarity (Ciura and Bourque 2006; Sharif Naeini et al. 2006). With reporter gene expression driven from the *Trpv1* locus, TRPV1 expression was confirmed in a restricted number of neurons in discrete CNS

areas including the entorhinal cortex, the periaqueductal grey, the supramammillary nucleus, hypothalamus and hippocampus (Cavanaugh et al. 2011). However, in contrast to the reports implicating TRPV1 in central osmosensation and LTD, Cavanaugh et al. (2011) found no evidence of TRPV1 expression in SON, OVLT, dentate gyrus or the nucleus accumbens.

2.2 Non-neuronal Expression

Activation of TRPV1 in perivascular sensory neurons produces vasorelaxation of resistance arteries by releasing the neuropeptide CGRP (Kawasaki et al. 1988; Zygmunt et al. 1999). However, in addition to these sensory neuron-mediated effects, TRPV1 expression has also been reported in arteriolar smooth muscle in some tissues (Kark et al. 2008; Cavanaugh et al. 2011). TRPV1 expressed in arteriolar smooth muscle may control blood flow in skeletal muscle and in certain thermoregulatory tissues, such as skin, trachea and cremaster muscle (Cavanaugh et al. 2011; Kark et al. 2008), but the physiological function of heat-induced arteriolar constriction in thermoregulatory tissues has yet to be explored in detail. It has been proposed that TRPV1 or a TRPV1 splice variant expressed in fungiform taste receptor cells contributes to salt taste transduction (Lyall et al. 2004), but later studies (Treesukosol et al. 2007; Breza and Contreras 2012) demonstrated that chorda tympani responses evoked by NaCl were unaffected by TRPV1 antagonists and were identical in $Trpv1^{+/+}$ and $Trpv1^{-/-}$ mice. Thus, the expression and possible physiological function of TRPV1 in taste receptor cells remain unclear. TRPV1 expression has been reported in bladder urothelium, where the channel was proposed to regulate bladder contractions by mediating urothelial ATP release in response to stretch (Birder et al. 2002). However, other studies have not confirmed expression in urothelial cells but found TRPV1 restricted to sensory nerve fibres innervating the bladder (Everaerts et al. 2009; Yamada et al. 2009). Several studies have reported expression of functional TRPV1 in human keratinocytes (Inoue et al. 2002; Southall et al. 2003), but this does not appear to be the case in the mouse (Chung et al. 2004).

3 Structure and Function

The topology of TRPV1 and other TRP channels is homologous to that of voltage-gated potassium channels (Kv) (Yu and Catterall 2004). TRPV1 is thus assembled as a homotetramer with each subunit containing six transmembrane (TM) segments and intracellularly located N- and C-termini.

The N-terminus of TRPV1, like other TRPV channels, contains six ankyrin repeats (Lishko et al. 2007). The structure of the ankyrin repeat domain has been determined with high resolution using x-ray crystallography (Lishko et al. 2007), and the structure of the complete channel has been resolved to 19 Å with single-particle electron cryo-microscopy (Moiseenkova-Bell et al. 2008). The compact

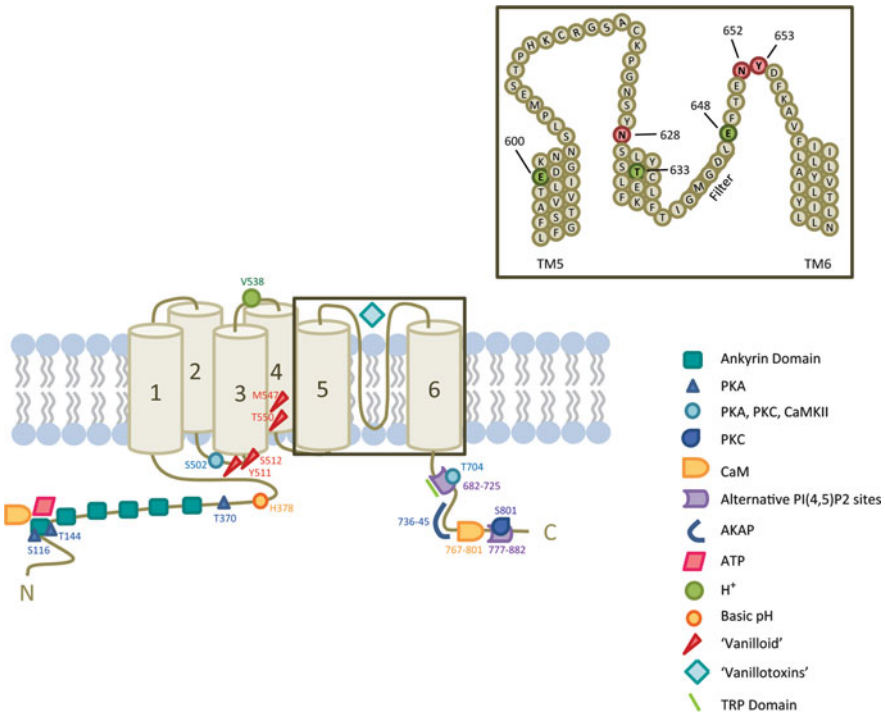

Fig. 1 Schematic diagram illustrating some key structural features of TRPV1 and some of the amino acid residues that are important for the control and regulation of channel activity and ion permeation. *Inset* shows expanded schematic view of the pore loop region. Residues important for heat and pH sensitivity are *colour coded red* and *green*, respectively

structure obtained with electron cryo-microscopy is largely consistent with the channel architecture determined by FRET measurements (De-la-Rosa et al. 2013). A pore-forming loop containing an outer pore 'turret', a pore helix and a selectivity filter is located between TM segments 5 and 6 (Venkatachalam and Montell 2007; Latorre et al. 2009). Cysteine accessibility studies have revealed that residues in TM6 contribute to the formation of an activation gate (Salazar et al. 2009). A number of amino acid residues that influence agonist responses have been identified in the pore loop, and phosphorylation sites for a number of different kinases have been identified in both termini and in the intracellular loops between TM segments. The overall structure of TRPV1 and the location of many of the residues discussed below are outlined in the diagrams in Fig. 1. Very recently, the structure of a functional, TRPV1 channel construct was determined at a resolution of 3.4 Å using electron cryo-microscopy (Liao et al. 2013). The same construct was also used to examine conformational rearrangements associated with agonist-induced activation of TRPV1 (Cao et al. 2013b). These investigations validated

many previous structural findings and provided several novel conclusions. For example, the 4 most N-terminal ankyrin repeats were shown to be arranged as previously described by Lishko et al. (2007), but the last 2 ankyrin repeats appear to be involved in channel assembly. Elements of both the N-terminal linker domain that connects the ankyrin repeat domain with TM1 and the C-terminal interact with the last two ankyrin repeats from an adjacent TRPV1 subunit (Liao et al. 2013). A short motif often referred to as the TRP domain is found in the membrane proximal part of the C-terminal in many TRP channels. Liao et al. (2013) demonstrated that the TRP domain forms an α-helix that resides parallel with the plasma membrane and which interacts with both the TM4–5 linker and the N-terminal pre-TM1 linker.

3.1 Ionic Permeability

TRPV1 is a non-selective cation channel which displays nearly equal permeabilities to Na^+, K^+, Cs^+, Li^+ and Rb^+ (Caterina et al. 1997; Oh et al. 1996; Samways and Egan 2011). The permeability to the physiologically relevant divalent cations is higher than that of Na with $P_{Ca}/P_{Na} \sim 5$ and $P_{Mg}/P_{Na} \sim 10$ (Ahern et al. 2005; Caterina et al. 1997; Mohapatra and Nau 2003) when activated by capsaicin. A similar P_{Ca}/P_{Na} ratio of 3–4 was reported for TRPV1 channels when activated by heat (Tominaga et al. 1998) although slightly lower estimates were given for heat-activated channels in sensory neurons [$P_{Ca}/P_{Na} \sim 2$, (Nagy and Rang 1999); ~1.3 (Cesare and McNaughton 1996)]. Direct measurements of the fraction of current carried by Ca^{2+} ions (Pf) using a combination of electrophysiology and fluorometric Ca^{2+} measurements yielded Pf values of 4.3 % for capsaicin-evoked TRPV1 responses in sensory neurons (Zeilhofer et al. 1997) and 3.5 % for heterologously expressed TRPV1 in response to pH 5.5 (Egan and Khakh 2004). A lower Pf value (1.65 %) was noted for sensory neuron responses to a pH 5.1 solution, but this may result from activation of non-TRPV1 channels in these neurons (Zeilhofer et al. 1997).

As well as sensitising or activating TRPV1 (discussed below), protons are highly permeant with estimated P_{H+}/P_{Cs} ratios of over 1,000 at pH 5.5. This proton permeability is likely to contribute to intracellular acidification noted upon TRPV1 activation (Hellwig et al. 2004). TRPV1 is also permeable to larger polyvalent cations including guanidinium (Winter et al. 1990) and NMDG, albeit with a relatively low relative permeability—$P_{NMDG}/P_{Cs} = 0.05–0.14$ (Hellwig et al. 2004; Nagy and Rang 1999; Cesare and McNaughton 1996). Other permeant bulkier cations include TEA (Hellwig et al. 2004) and polyamines (putrescine, spermidine and spermine), which display relative permeabilities P_x/P_{Na} of 3–16 (Ahern et al. 2006), as well as aminoglycoside antibiotics (Myrdal and Steyger 2005). In some cases the permeability of larger cations may be associated with pore dilation as discussed below.

The permeability of TRPV1 to some larger cations has been explored for agonist-induced intracellular delivery of the local anaesthetic QX314 (263 Da). Although membrane impermeant and therefore inactive as a local anaesthetic,

QX314 is able to permeate activated TRPV1 channels without the need for pore dilation (Puopolo et al. 2013). Co-administration of QX314 together with a TRPV1 activator can therefore deliver QX314 intracellularly to specifically block neuronal activity in TRPV1-expressing nerve fibres (Binshtok et al. 2007).

3.2 Structural Basis for Ion Selectivity

Amino acid residues in the pore-forming loop between TM5 and TM6 have been implicated in the control of ion selectivity. The sequence motif of the putative selectivity filter is relatively well conserved among TRPV1–4 and shares significant similarities to that of the bacterial potassium ion channel, KcsA (Owsianik et al. 2006); TRPV1–T^{641}IGMG^{645}; KcsA–TVGYG. Other residues in this region appear to regulate cation permeability as well as channel activation. Neutralisation of the adjacent aspartate residue, D646N, was reported to reduce the relative Mg^{2+} permeability as well as block by the charged TRPV1 inhibitor ruthenium red (Garcia-Martinez et al. 2000). Furthermore, neutralisation of a neighbouring glutamic acid (E648A) reduced TRPV1 activation by protons and other extracellular cations (Ahern et al. 2005, 2006). A different mutation at this site (E648Q) was reported to increase Ca^{2+} permeability (Welch et al. 2000), although another study reported little effect of this mutation (Garcia-Martinez et al. 2000).

Mutation of E636 (E636Q), which is thought to be located in the pore helix region, greatly increased calcium permeability (Welch et al. 2000). In addition, a more dramatic mutation of this residue (E636K) completely inactivated the channel although normal channel function could be restored by introduction of the reciprocal mutation to a nearby amino acid (K639E) consistent with the presence of a salt bridge between these two residues within the pore helix (Garcia-Martinez et al. 2000).

The transmembrane S6 domain has also been reported to play a key role in permeation. A Y671K mutation in S6 causes a tenfold reduction in Ca permeability (P_{Ca}/P_{Na} 9.0 to 0.8), leading to a loss of calcium-mediated desensitisation (Mohapatra and Nau 2003), and use of a Y671C mutant further suggested that this residue is near the gate that restricts permeation of smaller cations (Salazar et al. 2009). The residue I679 was shown to form the narrowest, most constricted point along the ion permeation path in the structure determined by electron cryomicroscopy (Liao et al. 2013). Comparisons of the position of pore-lining residues in the closed and open channel states confirmed that I679 not only forms a sufficiently tight hydrophobic seal to prevent permeation of hydrated ions in the closed state but also demonstrated that rearrangements in the activated state disrupt the seal and widen the permeation path, consistent with I679 forming an activation gate (Cao et al. 2013b).

3.3 Pore Dilation

Activation of native or recombinant rat TRPV1 results in time- and agonist concentration-dependent increases in relative permeability to large cations and changes in Ca^{2+} permeability without any change in sensitivity to the agonist used to activate the channel (Chung et al. 2008). For example, the permeability to the bulky cation NMDG (195.2 Da) increases within tens of seconds with prolonged channel activation. This permeability increase can also be monitored optically by the influx of the fluorescent, divalent dye YO-PRO1 (376 Da), which is generally membrane impermeant but permeates TRPV1 with prolonged channel activation (Banke et al. 2010; Chung et al. 2008). At physiologically relevant external Ca^{2+} concentrations, prolonged TRPV1 activation leads to an increase in P_{Ca}/P_{Na}. A study of the accessibility of different residues to chemical modification and the associated effects on ion permeabilities concluded that the permeability changes were attributable to alterations in the selectivity filter (Chung et al. 2008).

The structural basis for the change in ion selectivity has been ascribed to pore dilation, a phenomenon that has been observed in purinergic receptor/channels (Virginio et al. 1999) as well as some other TRP channels (Banke et al. 2010; Chung et al. 2005). Studies of relative permeabilities at various times after TRPV1 activation and other investigations of ion channel block using cations of different sizes suggest that the initial pore diameter is 10.1 Å but increases to 12.3 Å during dilation (Chung et al. 2008; Jara-Oseguera et al. 2008), although these studies did not exclude the possibility that the change is due to an increased conformational flexibility within the pore selectivity filter that increases the flux of larger cations. Intriguingly, the ability to elicit pore dilation appears to depend on the ligand or mode of activation (piperine = capsaicin = RTX= > NADA > heat > camphor) although this does not correlate with any obvious difference in the amplitude or speed of response (Chung et al. 2008). The mechanism(s) responsible for this differential agonist effect is unknown.

3.4 Heat

TRPV1 is a major heat sensor in a subset of peripheral sensory neurons that innervate tissues throughout the body. Typically, thermal activation of these neurons shows a temperature threshold of about 42 °C although this can be lower in conditions of inflammation. Expression of TRPV1 is sufficient to confer heat sensitivity to cells, and, importantly, the threshold activation temperature for heterologously expressed TRPV1 is the same as in sensory neurons (Caterina et al. 1997). Purified TRPV1 in proteoliposomes retains its characteristic heat sensitivity which indicates that thermal activation is an intrinsic channel property (Cao et al. 2013a).

TRPV1 displays a high temperature sensitivity with Q_{10} values of about 25 measured for both whole cell and single-channel currents (Caterina et al. 1997; Liu et al. 2003; Vlachova et al. 2003). This Q_{10} value is much higher

than noted for most other types of ion channels where Q_{10} values are typically ~2–3 (Hille 2001). The region or regions of the molecule that are responsible for heat activation have not been firmly established. Studies using either amino acid mutations, truncation of the C-terminal or deletion of sections of either the C- or N-terminal have provided evidence that all of these regions can influence the temperature responses of TRPV1.

C-terminal Studies with chimeras between TRPV1 and TRPM8 indicated that the C-terminal can determine whether the channel is heat or cold sensitive. Chimeric channels with part of the C-terminal of TRPV1 replaced with a cassette of TRPM8 are activated by cooling and not heating, and conversely, TRPM8 channels bearing a TRPV1 C-terminal cassette (686–752) are activated by heating. However, the temperature sensitivity of the TRPV1 C-terminal/TRPM8 chimera is weakened (Q_{10} ~ 10) compared to TRPV1 and the threshold temperature is lowered towards 20 °C (Brauchi et al. 2006). Some weak heat sensitivity was conferred on TRPM8 in a chimeric channel incorporating the amino acids 727–752 of TRPV1 but not in a chimera with only the 741–752 TRPV1 sequence (Brauchi et al. 2007).

Consistent with a role for the C-terminal, progressive truncation of the C-terminal reduced sensitivity to capsaicin and low pH and lowered the temperature threshold for activation with an associated reduction in Q_{10}. Removal of the terminal 78 amino acids resulted in a channel with a low Q_{10} similar to non-thermosensitive channels (Vlachova et al. 2003). In agreement with this finding, TRPV1 lacking the terminal 88 amino acids (751–838) could not be activated by heat in normal external calcium solutions. However, in this study the truncated channel could be activated by heat, as well as capsaicin and low pH solutions, when calcium was omitted from the recording solutions suggesting that the effect of C-terminal truncation on heat sensitivity was indirect, perhaps due to TRPV1 desensitisation (Liu et al. 2004). In other studies, mutation of several amino acids (notably I696A, W697A and R701A) in the C-terminal region TRP box resulted in loss or reduction in heat, capsaicin and voltage activation (Valente et al. 2008).

Interestingly, a truncated C-terminal variant of TRPV1 lacking the terminal 62 amino acids is found in TG, but not DRG, neurons that innervate the facial pit organs in vampire bats (Gracheva et al. 2011). This TRPV1 variant results from cell-specific alternative splicing of the TRPV1 transcript and has a reduced thermal threshold (~30 °C) and a reduced Q_{10} (~5), similar to the features of experimentally truncated TRPV1. The lowered thermal threshold is thought to facilitate detection of blood vessels in the prey.

N-terminal There is also evidence that residues in the N-terminus influence thermal activation. Chimeras between TRPV1 and TRPV2 showed that the introduction of the foreign TM1–6 region of TRPV2 into TRPV1 did not modify either the kinetics of channel opening or the enthalpy of activation in response to heat, although the activation temperature threshold was increased. Transfer of the

N-terminal region of TRPV1 that connects the ankyrin repeats to TM1 (358–434) to TRPV2 conferred the kinetic characteristics of TRPV1 on the chimeric channel. Furthermore, insertion of this region into temperature-insensitive channels such as human TRPV2 channel resulted in heat-sensitive channels, although with a high activation temperature (Yao et al. 2011).

Pore Region Other studies have implicated the pore region in thermal activation. A random mutagenesis screen for gain-of-function mutants identified the pore helix region (~627–641) as a domain with a strong influence on channel gating (Myers et al. 2008). In particular, the mutation F640L enhanced channel sensitivity to heat and capsaicin but abrogated the potentiating effect of protons, whereas T641S and T650S produced constitutively active channels (Myers et al. 2008). A separate unbiased mutagenesis screen identified three mutations (N638K, N652T and Y653T) in the pore region that specifically reduced heat sensitivity but resulted in normal sensitivity to capsaicin, protons and membrane depolarisation (Grandl et al. 2010). Replacement of a 14 amino acid segment of the pore turret with an artificial sequence eliminated the heat response while having little effect on the response to capsaicin (Yang et al. 2010). FRET studies of fluorophore-tagged cysteines in the pore turret sequence also indicated that heat but not capsaicin or depolarisation induced conformational changes leading to interactions across channel subunits (Yang et al. 2010). In addition, cysteine labelling studies have shown that higher temperatures evoke some structural changes in the pore region (Kim et al. 2013).

The available information thus suggests that several TRPV1 domains are essential for the heat sensitivity of TRPV1. Specifically, domains in the C-terminal and several residues in the pore are of profound importance for responses to heat but have little influence over responses evoked by low pH or capsaicin.

3.5 Vanilloid Agonists

To examine the structural determinants for activation, several laboratories have used chimeric constructs and exploited species differences in the sensitivity of TRPV1 to agonists and the insensitivity of the related ion channel TRPV2. The avian TRPV1 orthologue is virtually insensitive to capsaicin but displays heat and pH sensitivity very similar to that of mammalian TRPV1. Chimeric constructs produced by swapping domains between rat and chicken TRPV1 identified residues in TM2 and TM3 as important for capsaicin and RTX binding (Jordt and Julius 2002). Rabbit TRPV1 is insensitive to capsaicin and displays a markedly reduced affinity for RTX. Remarkably, chimeric rat/rabbit constructs led to the identification of a single amino acid residue substitution, I550T, which was sufficient to gain capsaicin sensitivity comparable to that of the rat orthologue (Gavva et al. 2004). An additional residue was required to gain full affinity for RTX, L547M

(Gavva et al. 2004). An inverse substitution, T550I, was sufficient to substantially reduce the affinity of human as well as rat TRPV1 channels for capsaicin. In a separate study, chimeric channels produced between TRPV1 and the vanilloid-insensitive TRPV2 channel led to the identification of TM3 and residues in the N- and C-termini as necessary for conferring sensitivity to capsaicin and for resiniferatoxin binding. Deleting either R114 or E761 was found to be sufficient to prevent RTX binding to TRPV1 completely (Jung et al. 2002).

The first selective TRPV1 antagonist identified, capsazepine (Bevan et al. 1992), inhibits pH evoked responses in the human and guinea pig TRPV1 channels much more potently than those mediated by rat TRPV1 (McIntyre et al. 2001). Chimeric constructs produced by domain swapping between human and rat channels identified I514M, V518L and M547L as the residues responsible for the species differences. L547M also strongly enhanced the agonist activity of the RTX derivative phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) on the human channel, without affecting the agonist activities of capsaicin, RTX or olvanil (Phillips et al. 2004).

Thus, evidence obtained using several different chimeric strategies has identified TM3 and TM4 as essential domains for binding of the classical TRPV1 agonists capsaicin and RTX, as well as the antagonist capsazepine. Distinct but overlapping binding sites for capsaicin and RTX were identified by Cao et al. (2013b). The structure of TRPV1 with RTX or capsaicin bound suggests that these agonists bind to the channel in a pocket that involves the TM3–4 residues mentioned above, but that may also influence the TM4–5 linker and TM6 from an adjacent subunit (Cao et al. 2013b).

In addition to capsaicin, a number of other plant products act as TRPV1 agonists. These include piperine (from black pepper), zingerone, gingerol and shogaol (from ginger), and various components found in some essential oils (rose, thyme, palmarosa) such as citronellol and geraniol [for a review, see Nilius and Appendino (2013)]. The binding site(s) for these ligands has not been elucidated but is likely to overlap or coincide with the sites responsible for capsaicin activation.

3.6 Vanillotoxins

In addition to plant-derived TRPV1 agonists, several animal-derived toxins have been shown to act on TRPV1. Some of these probably act indirectly by stimulating pathways that can sensitise TRPV1 function (Bohlen and Julius 2012), but the toxins from tarantula spiders act directly. These cysteine-rich (inhibitor cysteine knot, ICK) toxins contain internal disulfide bonds that stabilise the structure of the toxin. Three ICK 'vanillotoxins' from *Psalmopoeus cambridgei* (VaTx1-3) activate TRPV1 with micromolar affinity (VaTx3 > VaTx2 > VaTx1, 0.5–10 μM) by acting at an external site (Siemens et al. 2006). A toxin from another tarantula species (*Ornithoctonus huwena*, DkTx) containing two separately folded ICK domains binds with even higher affinity due to its bivalent structure. Analysis of various chimeric channels and point mutations demonstrated that DkTx binds within the

outer pore region and activation is abrogated by a quadruple mutation in this region (I599A, F649A, A657P, F659A). Single-channel analysis revealed that DkTx-evoked channel openings were long-lasting suggesting that the toxin traps the channel in the open state (Bohlen et al. 2010). This indicates that the pore domain undergoes significant conformational change during gating, which is consistent with other studies showing the importance of the pore region for channel gating (Grandl et al. 2010; Myers et al. 2008). Structural analysis of TRPV1 channels with DkTx bound demonstrates that this toxin binds to the interface between subunits, with each toxin molecule interacting both with the pore helix from one subunit and the outer pore loop from a neighbouring subunit (Cao et al. 2013b).

3.7 Protons

Extracellular acidification directly activates TRPV1 channels in DRG neurons and heterologously transfected cells at pH values below about 6.2 (Bevan and Yeats 1991; Tominaga et al. 1998; Davis et al. 2000; Caterina et al. 2000). In addition, TRPV1 sensitivity to heat and capsaicin is modulated by variations in $[H^+]$ through the range pH 6–9 (McLatchie and Bevan 2001; Jordt et al. 2000). Mild acidification sensitises TRPV1 to stimulation with capsaicin with a pKa of about 7.5 (McLatchie and Bevan 2001) and to stimulation with heat with a midpoint of about pH 7 (Jordt et al. 2000). The potentiating effect of pH on capsaicin is associated both with an increased affinity for capsaicin and by enhanced channel gating (Ryu et al. 2003). In the rat, acid potentiation of capsaicin-evoked TRPV1 responses appears to primarily be explained by a reduction in the slope factor of the concentration–response relationship coupled with an increase in apparent agonist affinity (McLatchie and Bevan 2001) or a parallel leftward shift in the agonist concentration–response relationship (Ryu et al. 2007), without a significant increase in the maximal capsaicin-induced current amplitude. In contrast, capsaicin is not a full agonist at the human channel, and acidification of hTRPV1 significantly increases the maximal amplitude of capsaicin-evoked currents (Wang et al. 2010). In addition to the agonist and potentiating activity of low pH, protons also reduce the unitary conductance of TRPV1 in sensory neurons and of TRPV1 expressed in cell lines (Baumann and Martenson 2000; Ryu et al. 2003). This inhibition is independent of the mechanisms that mediate proton activation and potentiation of TRPV1 (Liu et al. 2009). The effects of extracellular pH on TRPV1 channel function are thus complex and involve distinct mechanisms, with different concentration dependencies.

Mutagenesis studies have identified several residues that are specifically required for the proton modulation, activation or block of TRPV1 (Jordt et al. 2000; Welch et al. 2000; Wang et al. 2010; Liu et al. 2009). The potentiating effect of protons on other modes of activation can be recapitulated by neutralising the negative charge of the glutamate residue E600 (Jordt et al. 2000). A second glutamate residue (E648) located between the selectivity filter and the TM6 was proposed to mediate the agonist effect of protons, without affecting pH modulation

(Jordt et al. 2000). Studies of chimeric channels constructed by systematically exchanging extracellular domains between TRPV1 and TRPV2 identified amino acid residues in the linker between S3 and S4 (V538) as well as in the pore helix (T633) as necessary for proton-induced activation of TRPV1 (Ryu et al. 2007). Since neither of these two residues can be protonated, they likely influence the coupling of proton binding to channel gating. Finally, protonation of E636 and D646, probably influenced by K639, has been proposed to mediate the $[H^+]$-dependent inhibition of the unitary conductance (Liu et al. 2009).

3.8 Voltage Dependence

TRPV1 can be activated by membrane depolarisation in the absence of any other stimulus (Vlachova et al. 2003; Voets et al. 2004), and this can be demonstrated at both the whole cell and single-channel level. The conductance-voltage relationship can be described by a Boltzmann relationship (Eq. 1) with weak voltage sensitivity ($z \sim 0.5$–07) (Matta and Ahern 2007; Voets et al. 2004; for review cf. Nilius et al. 2005):

$$P_0 = {P_0}^{\max}/(1 + \exp\ [-zF(V - V_{0.5})/RT]), \tag{1}$$

where V is the membrane potential, z the gating charge, F the Faraday constant, R the universal gas constant, T the temperature and $V_{0.5}$ the membrane potential at which half of the channels are activated.

Without additional stimulation, channel opening occurs only at very positive potentials that are never experienced by the cells. However, a rise in temperature or addition of a TRPV1 agonist is associated with a shift in voltage-dependent activation to more negative potentials, such that a voltage change in the physiological range has an effect on channel function.

Several models have been proposed to explain the importance of voltage sensitivity for TRPV1 activation by temperature or agonists. These models of channel gating have been described and discussed by Voets and colleagues [see Voets (2012, 2014) and others (e.g. Latorre et al. 2007; Matta and Ahern 2007)] and will not be considered in great detail here.

The main models differ in the degree to which the voltage sensor and temperature (or agonist) sensor are coupled. Voets et al. (2004) proposed a simple linear two-state model for TRPV1 activation in which movement of the voltage sensor is required for channel activation and an increase in temperature shifts the voltage sensitivity to more negative potentials. This model makes no assumption about the location of the voltage sensor which could be located in one or multiple regions in the channel. According to this two-state model, the relationship between temperature and the $V_{0.5}$ for channel activation is given by Eq. (2):

$$V_{0.5} = (\Delta H - T\Delta S)/zF, \quad (2)$$

where ΔH and ΔS are the changes in enthalpy and entropy associated with the transitions between the closed and open states (Nilius et al. 2005).

The enthalpy for TRPV1 activation is large (Yao et al. 2010) but is balanced by a large entropy such that the free energy difference ($\Delta G = \Delta H - T\Delta S$) is small allowing reversibility of the reaction. The large ΔH and the low value of z are responsible for the marked effects of temperature on voltage sensitivity.

This two-state model for TRP channel activation can be modified to incorporate the effects of chemical ligands by the addition of a ligand binding step that acts to shift the voltage sensitivity of the voltage sensor to more negative potentials for agonists and more positive potentials for antagonists (see Voets et al. 2007).

Other linear models for heat activation have been proposed that accommodate the multiple closed and open channel states revealed by single-channel analysis (Grandl et al. 2010; Yao et al. 2011). One hypothesis is that the high temperature sensitivity of TRPV1 results from sequential transitions between multiple open states each with a relatively low temperature sensitivity where the overall Q_{10} comprises all individual components (Grandl et al. 2010). However, another analysis concluded that the temperature dependences of the gating steps before the first main open state determine the overall Q_{10} (Voets 2012). Here, the temperature sensor may be structurally distinct from the gating mechanism in the channel pore, but the voltage sensor and gating mechanisms are strongly coupled such that the channel is open when the voltage sensors are active and closed when the sensors are inactive. A Monod–Wyman–Changeux allosteric model with one or more temperature sensors has been used to describe TRP channel activity under such conditions (Voets 2012). This gives rise to several closed and active states of the channel with temperature modifying $V_{0.5}$ as described for the linear model.

In another more modular, allosteric model of TRPV1 activation, the voltage, temperature and ligand sensors and the channel gate are separate but allosterically coupled (Brauchi et al. 2004; Matta and Ahern 2007). The equilibrium between the inactive and active state of the temperature sensor follows the form of Eq. (2), while the equilibrium between the inactive and active states of the voltage sensor is described by the Boltzmann equation:

$K_V = K_0 \exp(zFV/RT)$, where K_0 is the equilibrium at 0 mV.

The experimental data favour the more complex models with various degrees of allosteric interactions to describe TRPV1 activity. For example, analyses of single-channel currents have revealed multiple open and closed states incompatible with a simple two-state model. One study also concluded that voltage is only a partial activator of TRPV1 and that the efficacy of voltage-activated channel opening can be augmented by an increase in temperature or chemical activation of TRPV1 (Matta and Ahern 2007). Similarly in single-channel studies, heat potentiated maximally effective capsaicin (10 μM) responses (Yang et al. 2010). Such an increase in efficacy is not predicted by the simple linear model. Furthermore, at very negative membrane potentials, capsaicin and RTX were reported to evoke voltage-independent gating consistent with an allosteric model where the ligand

binding is not tightly coupled to the voltage sensor (Matta and Ahern 2007) although data in another report did not confirm voltage-independent gating by capsaicin (Wang et al. 2010). Reports that the thermal sensitivities of TRPV1 (heat) and TRPM8 (cold) could be exchanged by swapping the C-termini to the other channel without losing the sensitivity to their normal agonists (Brauchi et al. 2006) and that modifications to the pore turret lose temperature sensitivity but retain near normal sensitivity to capsaicin (Yang et al. 2010) also argue in favour of independent temperature and chemical sensing domains in TRPV1.

4 Single-Channel Properties

4.1 Conductance

The single-channel conductance of capsaicin-activated TRPV1 in either DRG neurons or in an expression system shows outward rectification with slope conductances of ~90–100 pS at positive potentials (e.g. +60 mV) but a lower conductance (~50 pS) at negative potentials (Caterina et al. 1997; Oh et al. 1996; Premkumar et al. 2002). The presence of occasional openings to a lower, sub-conductance level has been reported in some studies (e.g. Hui et al. 2003; Nagy and Rang 1999; Premkumar et al. 2002), but the origin and properties of these openings have not been investigated.

Heat-activated TRPV1 channels show a similar voltage-sensitive behaviour to capsaicin-activated channels although the single-channel current amplitudes at all potentials are slightly higher for heat-activated than for capsaicin-activated TRPV1 (Nagy and Rang 1999). The Q_{10} for the increase in current amplitude is similar to that for other ion channels and can be explained by the effect of temperature on ion permeation (Liu et al. 2003). The rectification of single-channel current amplitudes combined with an increase in open channel probability at positive potentials gives rise to the outward rectification of TRPV1 current observed in whole cell recordings (Nilius et al. 2005).

The TRPV1 single-channel current amplitude is not only reduced by low extracellular pH (discussed above in Sect. 3, *Protons*) but also modulated by the extracellular concentration of the divalent cations Ca^{2+} and Mg^{2+} (Samways and Egan 2011). The inhibition of the single-channel conductance by the divalent cations is concentration-dependent with an IC_{50} value of ~2 mM for Ca^{2+} and somewhat higher for Mg^{2+} (8 mM).

4.2 Single-Channel Kinetics

Ligand-Gated TRPV1 Several studies have examined the single-channel kinetics of TRPV1 in sensory neurons and in heterologous expression systems. All studies agree that TRPV1 has multiple open and closed states when activated. Hui et al. (2003) and Studer and McNaughton (2010) described three components to

the capsaicin-evoked open dwell time distribution with time constants ($\tau \sim 0.3$–0.5, 1.5–2 and 10–30 ms) that were independent of capsaicin concentration. Both studies also reported that the distribution of closed times could be fitted by either four or five components with the durations of the three shortest components ($\tau \sim$ 0.15–0.3, 1–1.5 and 7–30 ms) independent of agonist concentration. In contrast the time constants of longer duration components ($\tau \sim 800$ ms or 800 and 100 ms) decreased with increasing agonist concentration ($\tau = 850$–180 ms) associated with a reduced probability of occupancy in this state. Although the data describing channel kinetics cannot be unambiguously described by a simple model of receptor activation, they demonstrate that channel opening can occur from partially liganded states (Hui et al. 2003; Studer and McNaughton 2010).

Potentiation of capsaicin-evoked TRPV1 activity by PKC-mediated phosphorylation (see **Regulation of TRPV1 by phosphorylation** below) with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), was shown to resemble the effect of raising the agonist concentration. Channel opening was promoted by an increase in the occupancy of the longest-lived open state while there was no effect on the time constant of any open state. Similarly, PMA treatment did not change the time constants or relative occupancies of the three shortest closed states but reduced the relative occupancy and time constant of the longest closed state. Conversely, treatment with the broad-spectrum kinase inhibitor staurosporine reduced baseline single-channel activity by reducing the proportion of long open times and increasing the time constant and occupancy of the longest closed state, resembling the effect of reducing the concentration of agonist (Studer and McNaughton 2010).

TRPV1 channels in DRG neurons show a similar complexity of channel openings and closures with 2–4 components in the distribution of channel open times ($\tau \sim 0.1$, 0.5–1, ~2–5 and 15 ms) with a similar number of components for the closed time distribution (McLatchie and Bevan 2001; Oh et al. 1996; Premkumar et al. 2002).

Temperature Activation Multiple channel open states have also been found for heterologously expressed TRPV1 at 10–30 °C in the absence of any chemical agonist with mean open times of ~1 ms and ~10 ms. Interestingly, mutations in the pore region that selectively reduced heat sensitivity shifted the gating equilibrium and eliminated the longer openings at all temperatures (Grandl et al. 2010).

At higher temperatures corresponding to the typical activation range for TRPV1, the open time distribution is composed of between two and five components, with time constants ranging from sub-millisecond to ~10 ms (Liu et al. 2003). As found for capsaicin activation, heat activation leads to bursts of short openings separated by longer gaps. With increased temperature, the gaps are shortened and the bursts are prolonged. From the temperature dependence of the probability of channel opening, Liu et al. (2003) calculated enthalpy changes of 150 kcal/mol indicative of large conformational changes associated with channel opening. The large

enthalpy is balanced by a large entropy ~470 kcal/mol such that the free energy difference between the open and closed conformations is relatively small.

The high temperature dependence of channel opening was associated with a reduction in the long gaps (hundreds of msec) between bursts of channel openings (Q_{10} ~ 7) and an increase in the burst durations (Liu et al. 2003). Within bursts, the distributions of closed and open dwell times for temperature-activated channels showed 3 or more open (τ ~ 0.25, ~1 and ~5 ms) and closed (τ ~ 0.2, ~1 and ~10 ms) components. An increase in temperature from 42 °C to 50 °C had no significant effect on the open channel time constants but reduced the proportion of brief openings and promoted the longer (>5 ms) openings. Conversely, the proportions of longer closures (>5 ms) within bursts decreased and short closures predominated as the temperature was raised. The consistency of the time constants at different temperatures indicates that thermal activation mostly affects the equilibrium between the different states.

5 Regulation of TRPV1 by Phosphorylation and Phosphoinositides

5.1 Phosphorylation

The activity of TRPV1 can be regulated by phosphorylation of several key residues which leads to increased sensitivity to both chemical and thermal stimuli. This is an important feature since a number of inflammatory mediators activate protein kinase A (PKA) and/or protein kinase C (PKC) through GPCR-related mechanisms or engage other phosphorylation pathways (Vellani et al. 2010; Sugiura et al. 2002; Bhave et al. 2002; Mohapatra and Nau 2003; Dai et al. 2004; Amadesi et al. 2006). Conversely, dephosphorylation is thought to underlie calcium-mediated desensitisation of TRPV1. Some of the actions of these enzymes are promoted by interactions with the scaffolding protein AKAP79/150.

TRPV1 contains many putative sites for kinase-mediated phosphorylation, and the available evidence has highlighted the importance of phosphorylation at several key residues. For TRPV1, S502 located in the intracellular loop between transmembrane domains 2 and 3 and the C-terminal T704 are phosphorylated by both PKA and PKC, and S800 in the C-terminal tail is a substrate for PKC-mediated phosphorylation (Bhave et al. 2002; Numazaki et al. 2002; Mandadi et al. 2006; Bhave et al. 2003). These sites play major roles in the functional modification of TRPV1, and mutants lacking these residues generally cannot be sensitised by treatments that activate PKA and PKC pathways (Studer and McNaughton 2010). PKA also phosphorylates other residues including S116, T144 and T370 in the N-terminal region as well as T704 in the C-terminal, and these appear to be involved in regulating TRPV1 activity (Bhave et al. 2002, 2003; Numazaki et al. 2002; Patwardhan et al. 2006). Other site-directed mutagenesis studies have also indicated that phosphorylation of other residues in the N- and C-terminal regions may contribute to TRPV1 regulation (Jung et al. 2004; Mohapatra and Nau 2003).

The finding that the broad-spectrum kinase inhibitor staurosporine reduces TRPV1 single-channel activity similarly in wild-type TRPV1 and S501A/S800A double mutants is consistent with regulation at other sites (Studer and McNaughton 2010).

The effects of PKA- and PKC-mediated phosphorylation appear to differ. PKC sensitisation is associated with a shift in voltage-dependent activation to more negative potentials thereby increasing the probability of channel opening at normal membrane potentials or in response to other TRPV1 stimuli (Ahern and Premkumar 2002; Matta and Ahern 2007; Vellani et al. 2001). In contrast the effects of PKA-mediated phosphorylation appear to be more related to reversal of desensitisation—see below.

Src kinase activation has also been implicated in TRPV1 sensitisation (Jin et al. 2004; Zhang et al. 2005), and phosphorylation of Y199 in the N-terminus of TRPV1 promotes channel trafficking to the plasma membrane (Zhang et al. 2005). The increase in surface expression by regulated exocytosis is responsible for the acute sensitising effects of NGF on TRPV1 in sensory neurons (Stein et al. 2006; Zhang et al. 2005). Several studies have concluded that NGF stimulation leads to activation of PI3 kinase, but there is no consensus about the subsequent steps. One proposal is that Src kinase is activated by PI3 kinase activity (Zhang et al. 2005), while others have argued either that PIP3 binding to TRPV1 promotes trafficking (Stein et al. 2006) or that there is crosstalk between PI3 kinase and PKC pathways such that PI3 kinase stimulates PKC-mediated phosphorylation of TRPV1, leading to TRPV1 sensitisation (Zhu and Oxford 2007).

There is also evidence for a role of calcium-calmodulin kinase II (CaMKII) and MAP kinases in TRPV1 regulation. Inclusion of CaMKII/calmodulin in the pipette in whole cell recordings promoted recovery from desensitisation (Jung et al. 2004). Although TRPV1 activation leads to desensitisation, some stimulation protocols can potentiate TRPV1 activity, and this potentiation can be inhibited by CaMKII and MEK inhibitors but not inhibitors of PKC or PKA signalling (Firner et al. 2006; Zhang et al. 2011). In addition, MAPK inhibitors have been reported to reduce NGF sensitisation of TRPV1 (Bonnington and McNaughton 2003; Zhu and Oxford 2007; Zhuang et al. 2004).

5.2 Desensitisation

A striking feature of TRPV1 is its calcium-mediated inactivation or desensitisation, which leads to a loss of sensitivity to capsaicin and other chemical agonists and a reduction in heat sensitivity (Vyklicky et al. 1999). Desensitisation of TRPV1 by capsaicin is associated with a decrease in the apparent affinity for this agonist as responses can be recovered by raising the capsaicin concentration (Novakova-Tousova et al. 2007). Also, desensitisation is less marked at positive than at negative membrane potentials (Piper et al. 1999), and this is not simply due to reduced calcium influx at positive potentials leading to reduced desensitisation but appears to reflect a change in the voltage dependence of desensitised TRPV1.

Several mechanisms have been proposed to explain desensitisation. One mechanism is dephosphorylation of TRPV1 driven by the calcium influx through open TRPV1 channels which activates the Ca^{2+}-calmodulin-dependent phosphatase, calcineurin (PP3) (Docherty et al. 1996; Koplas et al. 1997; Mohapatra and Nau 2005). This mechanism is consistent with the findings that phosphorylation at several consensus sites for PKC and PKA is able to reduce Ca^{2+}-mediated desensitisation of TRPV1 (Bhave et al. 2002; Mandadi et al. 2004; Mandadi et al. 2006; Mohapatra and Nau 2003; Numazaki et al. 2002). The second is Ca^{2+}-dependent stimulation of PLC leading to hydrolysis of PI(4,5)P2 (see below). A third proposed mechanism of inactivation involves binding of Ca^{2+}-calmodulin to TRPV1 (Numazaki et al. 2003; Rosenbaum et al. 2004; Lishko et al. 2007; Grycova et al. 2008; Lau et al. 2012).

5.3 Phosphoinositides

Phosphoinositides have important roles in regulating the activity of various integral membrane proteins, and some ion channels, such as KCNQ channels and TRPM8, require PI(4,5)P2 to retain activity (Delmas and Brown 2005; Zakharian et al. 2010). For TRPV1 the available data for the role of PI(4,5)P2 are conflicting, although all reports show that treatments that alter PI(4,5)P2 levels modify TRPV1 function.

The location of the PI(4,5)P2 binding site responsible for TRPV1 modulation is disputed. Jeske and colleagues proposed that PI(4,5)P2 binds to an accessory protein, AKAP79/150 (see Sect. 6), which acts as a scaffolding protein facilitating PKA- and PKC-mediated phosphorylation of TRPV1. Sensitisation of TRPV1 by treatments that activate PLC was lost when AKAP79/150 was genetically ablated (Jeske et al. 2011). In their scheme PI(4,5)P2 anchors AKAP79/150 to the plasma membrane, and hydrolysis liberates AKAP79/150 resulting in increased TRPV1–AKAP-79/150 interactions. Another proposed site of action is a 135 amino acid membrane protein, PIRT, that binds PI(4,5)P2 and was reported to co-precipitate with the C-terminal region of TRPV1 (Kim et al. 2008). This conclusion was based on the observations that DRG neurons from $Pirt^{-/-}$ mice showed smaller responses to capsaicin and heat, less bradykinin-evoked potentiation of capsaicin sensitivity and did not show the potentiating effect of PI(4,5)P2 on capsaicin-evoked currents. In addition co-expression of PIRT with TRPV1 was reported to increase the amplitude of the responses to heat and capsaicin without affecting TRPV1 expression. A subsequent study challenged the involvement of PIRT, reporting that deletion of PIRT had no effect on either the ability of phosphoinositide chelation to inhibit TRPV1 or the ability of PI(4,5)P2 to restore TRPV1 activity. Furthermore, in these latter studies, co-expression of PIRT with TRPV1 did not affect capsaicin sensitivity, and FRET analyses failed to show an interaction between TRPV1 and PIRT but indicated a direct interaction between PI(4,5)P2 and TRPV1 (Ufret-Vincenty et al. 2011).

Direct binding of PI(4,5)P2 to TRPV1 has been proposed to underlie the various effects of PI(4,5)P2. One proposed PI(4,5)P2 binding site in the distal C-terminal of TRPV1 (amino acids 777–882) was based on the loss of TRPV1 potentiation evoked by PLC activation when these residues were deleted (Prescott and Julius 2003). However, a later study found that deletion of these residues had no effect on the potentiating effect of PI(4,5)P2 and proposed that a more proximal polybasic/hydrophobic domain close to the plasma membrane at the bottom of TM6 acted as the PI(4,5)P2 binding site. Deletion of these latter sites eliminated TRPV1 activity precluding a functional analysis, but FRET studies revealed a direct interaction between fluorescent PI(4,5)P2 and the labelled proximal C-terminal region (amino acids 682–725) (Ufret-Vincenty et al. 2011). Studies showing modulatory effects of PI(4,5)P2 on purified TRPV1 also support a direct action of PI(4,5)P2 on TRPV1 (Cao et al. 2013a).

Functionally, PI(4,5)P2 has been proposed to be an activator (Klein et al. 2008; Yao and Qin 2009; Ufret-Vincenty et al. 2011), an inhibitor (Prescott and Julius 2003) or to cause bidirectional modulation of TRPV1 (Lukacs et al. 2007; Stein et al. 2006). Cellular studies using full-length TRPV1 and a TRPV1 mutant lacking a putative PI(4,5)P2 binding region in the distal region of the C-terminal concluded that PI(4,5)P2 binding exerts an inhibitory effect on TRPV1 (Chuang et al. 2001; Prescott and Julius 2003). In this scenario, activation of $G_{q/\alpha 11}$-linked GPCRs by agonists such as bradykinin leads to hydrolysis of PI(4,5)P2 by PLC which relieves the inhibition of TRPV1 and therefore promotes channel opening. Other groups have presented data in accord with this mechanism for low levels of TRPV1 stimulation in cells by capsaicin, low pH or moderate heat. Here, various treatments to deplete PI(4,5)P2 levels potentiated TRPV1 responses, whereas procedures to increase PI(4,5)P2 inhibited the responses (Jeske et al. 2011; Lukacs et al. 2007). The conclusion that PI(4,5)P2 exerts an inhibitory effect on TRPV1 is also supported by recent studies on purified TRPV1 in proteoliposomes lacking endogenous phosphoinositide lipids (Cao et al. 2013a). In this preparation, TRPV1 was activated by either capsaicin or a heat stimulus, consistent with the view that TRPV1 function does not require the presence of PI(4,5)P2 or other phosphoinositides. Furthermore, addition of PI, PI(4)P or PI(4,5)P2 (but not PI(3,4,5)P3) to the proteoliposomes decreased capsaicin sensitivity and raised thermal thresholds to higher temperatures indicating an inhibitory effect of several phosphoinositide species.

Other studies using excised membrane patches demonstrated that chelation of phosphoinositides inhibited capsaicin-evoked currents whereas application of PI(4,5)P2, PI(4)P or PI(3,4,5)P3 to the cytoplasmic side of the membrane restored channel activity, consistent with a stimulatory role for these molecules (Klein et al. 2008; Lukacs et al. 2007; Stein et al. 2006; Ufret-Vincenty et al. 2011). One possible explanation for the different results and conclusions is that the effects on TRPV1 function depend on the concentrations of the various phosphoinositides and that other negatively charged phospholipids can also regulate TRPV1 activity (Rohacs 2013; Lukacs et al. 2013a, b). Possible PI(4,5)P2 interactions with other proteins, such as AKAP79/150, may also contribute to variable outcomes in different experimental situations.

6 Interacting Proteins

TRPV1, like many other ion channels, is organised in a multimeric complex with other proteins that are important in regulating the location and function of the channels. These include AKAP79/150, various protein kinases, calmodulin, motor proteins such as KIF13B, and vesicular SNARE proteins. It has also been proposed that TRPV1 subunits can form heteromeric channels with TRPA1 (Staruschenko et al. 2010), although the effect of such a combination on the properties of channels activated by TRPV1 agonists is unknown.

AKAP79/150 A kinase-anchoring protein (AKAP79 in human and AKAP150 in mouse) acts as a scaffolding protein that binds PKA, PKC and calcineurin (PP3) and positions these enzymes close to TRPV1. It therefore has an important role in the regulation of TRPV1 phosphorylation, and functionally the interaction between TRPV1 and AKAP79/150 is required for TRPV1 sensitisation or recovery from desensitisation. AKAP79/150 is expressed in 70–90 % of TRPV1-positive DRG neuron (Schnizler et al. 2008; Zhang et al. 2008; Brandao et al. 2012), and co-precipitation studies have demonstrated a direct interaction between AKAP79/150 and TRPV1 (Schnizler et al. 2008; Zhang et al. 2008).

The regions responsible for the interaction between TRPV1 and AKAP79 have been identified. Key residues in the 736–745 C-terminal of TRPV1 are required for binding to AKAP79/150 [D738, R740, C742 and V745] (Fischer et al. 2013). Complementary experiments identified a unique site for TRPV1 binding within amino acids 326–336 on AKAP79 (Btesh et al. 2013).

The important functions of AKAP79/150 have been demonstrated in several ways. For PKA-mediated regulation, a cell-permeable peptide inhibitor (St-HT31) that disrupts the coupling of PKA to AKAP79/150 inhibited forskolin-induced sensitisation of TRPV1 in DRG neurons and TRPV1-HEK293 cells (Rathee et al. 2002; Schnizler et al. 2008). Similarly, deletion of the PKA binding domain in AKAP or knockdown of AKAP by RNA interference also reduced the ability of PKA stimulation (with forskolin, 8Br-cAMP or PGE2) to inhibit TRPV1 desensitisation or to sensitise TRPV1 (Schnizler et al. 2008; Jeske et al. 2008; Zhang et al. 2008).

AKAP79/150 is also responsible for many PKC-mediated effects on TRPV1. Expression of a mutated AKAP lacking the PKC binding site (AKAPΔPKC) resulted in a reduction in capsaicin sensitivity (Zhang et al. 2008) and reduced the ability of PDBu to sensitise TRPV1 (Jeske et al. 2009). Similarly, PDBu-induced sensitisation was reduced in trigeminal ganglion neurons from mice lacking AKAP150 (Jeske et al. 2009), and AKAP150 siRNA treatment inhibited BK and PGE2-evoked sensitisation of TRPV1 in DRG neurons (Zhang et al. 2008) and PDBu-induced phosphorylation of TRPV1 in TG neurons (Jeske et al. 2009).

Sensitisation can also be inhibited by disrupting the TRPV1/AKAP79 interaction. Intracellular application of small peptides containing the interacting sequence of TRPV1 inhibited PKC- (PMA and bradykinin) and PKA-mediated (PGE2)

sensitisation of TRPV1 in DRG neurons and TRPV1-HEK293 cells (Zhang et al. 2008; Fischer et al. 2013). Similarly, an 11aa TRPV1 binding peptide from the AKAP sequence (326–336) blocked PMA- and forskolin-mediated sensitisation in DRG neurons as well as the sensitising effects of PMA in TRPV1-HEK293 cells (Btesh et al. 2013).

TRPV1 phosphorylation is also regulated by the phosphatase calcineurin (PP3; Docherty et al. 1996), and this may be mediated by PP3 binding to AKAP79/150. In HEK293 cells, over-expression of AKAP79 strongly enhanced desensitisation of capsaicin-evoked currents, while siRNA downregulation of AKAP79 and deletion of the PP3 binding site on AKAP79/150 reduced the degree of TRPV1 desensitisation (Zhang et al. 2008). In contrast another study found similar degrees of TRPV1 desensitisation in CHO cells transfected with TRPV1, TRPV1 plus AKAP150 or TRPV1 plus AKAPΔPP3 and equivalent desensitisation of capsaicin-evoked currents in TG neurons from wild-type or AKAP150 knockout mice (Por et al. 2010). Furthermore, it is unclear whether PP3 co-precipitates with TRPV1 or AKAP150 (Efendiev et al. 2013; Por et al. 2010). Therefore, although the evidence for PP3 involvement in TRPV1 desensitisation is strong, this may be independent of the interaction with AKAP79/150.

Calmodulin (CaM) Two CaM-binding sites have been identified in the cytoplasmic domains of TRPV1. For the N-terminus, Ca^{2+}-CaM binds to isolated peptides from the ankyrin repeat domain (ARD) of TRPV1 (Rosenbaum et al. 2004) and competes with ATP for ARD binding (Lishko et al. 2007). In vitro binding assays also identified a 35 amino acid sequence in the C-terminus that binds CaM, and deletion of this region inhibits TRPV1 desensitisation (Numazaki et al. 2003). A more recent study concluded that although the C-terminal region contains a high-affinity binding site for Ca^{2+}-CaM, this region has a relatively minor role in TRPV1 desensitisation. In contrast mutational studies showed that the lower affinity N-terminus binding of Ca^{2+}-CaM is important for desensitisation although whether this involves a direct CaM-TRPV1 interaction is unclear (Lau et al. 2012).

PI3 Kinase Co-precipitation studies have provided evidence that the 85 kDa subunit of PI3 kinase binds to the N-terminal of TRPV1 (Stein et al. 2006) and this interaction may play a role in the increased trafficking of TRPV1 to the cell surface in response to NGF stimulation.

Cdk5 Cyclin-dependent kinase 5 (Cdk5) is a neuron-specific, proline-directed serine/threonine kinase that can regulate heat and capsaicin sensitivity in sensory neurons associated with phosphorylation of TRPV1 at T407. Inhibition of Cdk5 results in a significant decrease in capsaicin-evoked calcium influx (Pareek et al. 2007).

GABARAP GABA receptor-associated protein is expressed in some but not all TRPV1-positive DRG neurons, and pull-down experiments indicate that it can interact with TRPV1 through the N-terminal of the channel. Expression of GABARAP with TRPV1 led to an increase in surface expression associated with increased clustering of TRPV1 in the plasma membrane (Lainez et al. 2010).

7 Trafficking

The delivery of TRPV1 to the cell surface is a regulated process that can be modified in conditions such as inflammation where it contributes to the increased sensory neuron sensitivity evoked by some, but not all, inflammatory mediators. Some mediators (NGF, ATP and IGF-I) promote the recruitment of TRPV1 to the cell surface, whereas others (bradykinin, artemin and IL-1β) act by altering the function of TRPV1 without affecting the surface density (Camprubi-Robles et al. 2009). Inflammatory mediators that do stimulate membrane insertion of TRPV1 activate PKC-, PKA- and Src-linked signalling pathways, and the exocytosis of vesicles is known to be regulated by signalling molecules such as PKC (Morgan et al. 2005). NGF stimulates surface expression of TRPV1 through an Src-mediated pathway that promotes phosphorylation of Y199 in the N-terminus and insertion into the surface membrane (Zhang et al. 2005), although how this stimulates trafficking is unclear.

Some of the mechanisms of TRPV1 trafficking are known but much detail remains to be discovered. Trafficking requires several TRPV1 interacting proteins, including AKAP79/150, KIF13B and some vesicular SNARE proteins. TRPV1 interacts with two vesicular proteins, snapin and synaptotagmin IX (SytIX), which modulate SNARE-mediated neuronal exocytosis. Over-expression of SNARE proteins abrogates SNARE-dependent exocytosis, and co-expression of snapin or SytIX with TRPV1 reduced the PKC-mediated increase in surface expression and capsaicin responses in a similar manner to botulinum toxin (Morenilla-Palao et al. 2004).

AKAP79/150 plays an important role in TRPV1 trafficking as well as in the control of channel properties. Over-expression of AKAP79/150 increased TRPV1 trafficking to the cell surface while knockdown reduced surface levels. Deletion of either the PKA or PKC binding sites on AKAP 79/150 largely removes the effects of over-expression (Zhang et al. 2008).

Cdk5 has also been implicated in TRPV1 trafficking by phosphorylating a kinesin ‘motor protein’ KIF13B which can bind to TRPV1 (Xing et al. 2012) and promote intracellular transport of cargos including transmembrane receptors (Hirokawa et al. 2009). Over-expression of cdk5 or its activator p35 increased surface TRPV1 while a dominant negative cdk5 or a cdk5 inhibitor reduced surface levels (Xing et al. 2012).

8 Physiological Functions

8.1 Heat Transduction

A major role of TRPV1 is to act as a thermosensor in a subset of primary afferent sensory neurons responsible for transducing physical, chemical and thermal nociception. TRPV1 is activated by temperatures in the noxious heat range (typically >42 °C), and activation of TRPV1-expressing sensory neurons with either high temperatures or a chemical agonist, such as capsaicin, gives rise to the sensation of 'burning' heat (Szolcsanyi 1977). Studies with *Trpv1*$^{-/-}$ mice revealed that the sensation of painfully hot temperatures is not solely reliant upon the presence of TRPV1 (Caterina et al. 2000; Davis et al. 2000; Woodbury et al. 2004). However, clinical studies with TRPV1 antagonists have revealed that in humans, antagonism of TRPV1 leads to a reduction in heat sensitivity such that normally noxious, and potentially tissue damaging, temperatures are not always sensed as painful (Eid 2011; Rowbotham et al. 2011).

TRPV1 has a more prominent physiological role as a thermosensor in conditions of tissue inflammation when temperatures lower than 42 °C can elicit a painful sensation (thermal hyperalgesia). Studies on *Trpv1*$^{-/-}$ mice have demonstrated an important role for TRPV1 in inflammatory thermal hyperalgesia. In contrast to wild-type animals, *Trpv1*$^{-/-}$ mice do not exhibit an increased sensitivity to noxious heat after tissue injury or inflammation (Bolcskei et al. 2005; Caterina et al. 2000; Davis et al. 2000). This matches well with the in vitro findings that inflammatory mediators sensitise TRPV1 and lower the temperature threshold for activation (see Sect. 5) and reports that in vivo a wide range of TRPV1 antagonists inhibit thermal hyperalgesia after inflammation (Immke and Gavva 2006).

8.2 Thermoregulation

There is now good evidence that TRPV1 is involved in the maintenance of normal body temperature. Hypothermia can be induced by administration of capsaicin either systemically or by microinjection into the preoptic area of the hypothalamus (Jancso-Gabor et al. 1970a, b). Intriguingly, although animals exposed to desensitising doses of capsaicin subsequently have an impaired ability to regulate their body temperature at high ambient temperatures (Jancso-Gabor et al. 1970b), *Trpv1*$^{-/-}$ mice have normal core body temperatures (Toth et al. 2011). In view of the latter finding, it was surprising that in clinical trials TRPV1 antagonists evoked an increase in body temperature of several degrees (Gavva et al. 2008; Krarup et al. 2013). It is now clear that administration of TRPV1 antagonists in multiple species elicits a hyperthermic response (Gavva et al. 2007).

Both central and peripheral mechanisms have been identified as important for TRPV1-mediated thermoregulation. Neurons residing in the median preoptic nucleus (MnPO) have been postulated as the important effectors of capsaicin-mediated hypothermia (Jancso-Gabor et al. 1970b; Szolcsanyi et al. 1971).

TRPV1 receptors expressed on these neurons are thought not to be active under normal conditions (Romanovsky et al. 2009). TRPV1 antagonists that induce hyperthermia are thought to act predominantly in the periphery via tonically activated TRPV1 channels expressed on sensory neurons innervating the viscera (Romanovsky et al. 2009). This hypothesis is supported by the finding that TRPV1 antagonists are not more effective at causing hyperthermia when administered intracerebroventricularly or intrathecally than when given intravenously (Steiner et al. 2007). Tonic activation of TRPV1 channels in visceral sensory neurons is not thought to be heat-mediated as the hyperthermic response evoked by TRPV1 antagonism was unaffected by either core body temperature or tail skin temperature (Steiner et al. 2007). The mechanism responsible for the strong hyperthermic effects of some, but not all, TRPV1 antagonists is not entirely clear. However, a link has been proposed between the ability of antagonists to inhibit proton activation of TRPV1 and their hyperthermia-inducing potential, such that antagonists that do not block activation of TRPV1 by acidic solutions (<pH 5.5) are devoid of the hyperthermia liability (Garami et al. 2010; Kort and Kym 2012; Reilly et al. 2012).

8.3 Osmosensation

TRPV1 has also been proposed to play a role in osmosensory transduction in the CNS. An N-terminal splice variant of TRPV1 is expressed on magnocellular neurosecretory cells (MNCs) in the supraoptic nucleus (SON). Increases in membrane conductance associated with a hyperosmotic-induced decrease in cell volume were reduced by the promiscuous TRPV antagonist ruthenium red (RR) and absent in *Trpv1*$^{-/-}$ mice. *Trpv1*$^{-/-}$ mice also had a higher basal serum osmolality compared to wild-type mice and a reduced vasopressin response to increased osmolality (Sharif Naeini et al. 2006). A further study examining the intrinsic osmosensitivity of the organum vasculosum lamina terminalis (OVLT) demonstrated that OVLT neurons responded to elevated osmolality by increased membrane conductance and amplified action potential discharge. This process was ruthenium red sensitive and absent in *Trpv1*$^{-/-}$ mice. In addition *Trpv1*$^{-/-}$ mice consumed significantly less water than wild-type mice in response to a systemic hyperosmotic challenge, suggesting that TRPV1 plays a role in the generation of thirst (Ciura and Bourque 2006).

8.4 Endogenous Agonists

Anandamide was identified as the first endogenous TRPV1 agonist (Zygmunt et al. 1999). In addition to anandamide, several lipoxygenase-generated lipid products, including 12- and 15-(*S*)-hydroperoxyeicosatetraenoic acids (HPETE), 5- and 15-(*S*)-hydroxyeicosatetraenoic acids (HETE) and leukotriene B_4, have been identified as having agonist activity at the TRPV1 channel (Hwang et al. 2000). Another endogenous lipid, *N*-arachidonoyl-dopamine (NADA), is found within the

brain and is a potent TRPV1 agonist. Intradermal NADA evokes a TRPV1-dependent thermal hyperalgesia and activation of native TRPV1 receptors expressed on DRG neurons by NADA induces neurotransmitter release. In addition NADA amplifies paired-pulse depression in hippocampal neurons, suggesting a role for both NADA and TRPV1 in synaptic plasticity mechanisms (Huang et al. 2002).

Other studies have also identified oxidation products of linoleic acid, hydroxyoctadecadienoic acids (HODEs), as endogenous agonists of TRPV1 (Patwardhan et al. 2010). Very recently, the DAG lipase product 2-arachidonylglycerol has been identified as an endogenous agonist which formation can be induced by stimulation of PLC-coupled receptors (Zygmunt et al. 2013).

In addition to the several lipid 'endovanilloids', the TRPV1 channel can also be directly activated and sensitised by protons, and this may be relevant in inflammatory condition where the local extracellular pH can be decreased.

8.5 TRPV1 Antagonists and Agonists In Vivo

Studies with genetically modified mice and TRPV1 agonists and antagonists have also provided clues about the physiological functions of TRPV1.

Pain TRPV1 antagonists reverse hyperalgesia associated with both inflammation and neuropathy in rodents (Honore et al. 2005; Kitagawa et al. 2013a; Lehto et al. 2008; Walker et al. 2003). Additionally, inhibition of TRPV1 can attenuate pain behaviours in a rat model of bone cancer (Ghilardi et al. 2005). Intriguingly, the effects are not restricted to thermal (heat) stimuli, and mechanical hypersensitivities can also be reversed by TRPV1 antagonists (Immke and Gavva 2006). Comparison of the effects of peripheral versus intrathecal (spinal) administration of TRPV1 antagonists has helped to elucidate the important sites of action for TRPV1-mediated analgesia. TRPV1 in the CNS plays a predominant role in the suppression of pain in conditions such as osteoarthritis, where the involvement of central sensitisation of neuronal pathways is likely to be important. In contrast, both peripherally and intrathecally administered TRPV1 antagonists reverse inflammatory hyperalgesia, suggesting contribution of channels at both sites (Cui et al. 2006).

TRPV1 agonists have also been investigated as potential therapeutic agents. This is based on the observation that exposure to TRPV1 agonists functionally desensitises TRPV1-expressing sensory neurons. The effect is probably due to excessive Ca^{2+} influx triggering a variety of intracellular biochemical events, leading to local cell damage (Caterina et al. 1997). As these neurons are responsible for the transmission of painful stimuli, functional desensitisation can lead to analgesia (Bevan and Szolcsanyi 1990). Capsaicin-containing ointments are used clinically as topical analgesics. Different TRPV1 agonists display different rates of channel activation and therefore different abilities to produce rapid depolarisation

and action potential firing (Ursu et al. 2010). Based on these observations, efforts to produce agonists that cause desensitisation with a minimum of discomfort and pain were initiated. This approach led to the identification of TRPV1 agonists that were devoid of pungency and effective oral analgesics in rodent models in vivo (Urban et al. 2000).

Cough In addition to their analgesic effects, TRPV1 antagonists have also demonstrated efficacy in the modulation of other physiological mechanisms. Sensory nerves expressing TRPV1 innervate the airways, and inhalation of capsaicin elicits cough (Laude et al. 1993). TRPV1 antagonists have shown efficacy in suppressing cough and airways hyper-responsiveness in guinea pigs induced by capsaicin and citric acid (Bhattacharya et al. 2007; Lalloo et al. 1995) and, more relevantly, by ovalbumin (Delescluse et al. 2012). Cough suppression has been an end point in at least one clinical trial for a TRPV1 antagonist (Eid 2011).

Overactive Bladder/Colonic Hypersensitivity TRPV1 antagonists have also highlighted a role for TRPV1 expressed by sensory nerves innervating the urinary bladder. Here, TRPV1 antagonists inhibit capsaicin-induced increases in pelvic nerve discharge and intravesical pressure in rats and are able to suppress bladder overactivity induced by resiniferatoxin and acetic acid (Charrua et al. 2009; Kitagawa et al. 2013b). In addition, TRPV1 antagonists have been demonstrated to be effective in reducing colonic hypersensitivity in rodents (Kiyatkin et al. 2013; Wiskur et al. 2010).

TRPV1 agonists have also been used to modify the activity of sensory neurons innervating the bladder, and intravesical resiniferatoxin has been reported to show some efficacy in improving bladder function in patients with overactive bladder (Kissin and Szallasi 2011).

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TRPV2

Itaru Kojima and Masahiro Nagasawa

Contents

Abstract

Transient receptor potential vanilloid type 2, TRPV2, is a calcium-permeable cation channel belonging to the TRPV channel family. This channel is activated by heat (>52 °C), various ligands, and mechanical stresses. In most of the cells, a large portion of TRPV2 is located in the endoplasmic reticulum under unstimulated conditions. Upon stimulation of the cells with phosphatidylinositol 3-kinase-activating ligands, TRPV2 is translocated to the plasma membrane and functions as a cation channel. Mechanical stress may also induce translocation of TRPV2 to the plasma membrane. The expression of TRPV2 is high in some types of cells including neurons, neuroendocrine cells, immune cells involved in innate

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immunity, and certain types of cancer cells. TRPV2 may modulate various cellular functions in these cells.

Keywords
Calcium • Calcium-permeable channel • Translocation • Trafficking • Mechanosensitive channel • Stretch-activated channel

1 Gene/Expression

Transient receptor potential vanilloid type 2 (TRPV2) is a calcium-permeable cation channel belonging to the TRPV family and was identified independently by two groups in 1999 using different approaches. Caterina et al. (1999) identified TRPV2 as vanilloid receptor-like protein-1 (VRL-1), a molecule structurally related to vanilloid receptor TRPV1 (Caterina et al. 1997). They cloned rat and human cDNA for VRL-1 which encode 761 and 764 amino acids, respectively, both of which are 49 % identical and 66 % similar to rat TRPV1.

We have been studying the calcium-permeable cation channel activated by growth factors such as insulin-like growth factor-I (IGF-I) (Kojima et al. 1988, 1993). We identified mouse TRPV2 as a growth factor-regulated channel (GRC). Mouse TRPV2 encodes 756 amino acids, which are 44 % identical to rat TRPV1. The gene encoding TRPV2 is on chromosome 11 and is located ~10 Mb apart from TRPV1 (Park et al. 2011a).

In general, TRPV2 is expressed ubiquitously. It is also true that the expression level of TRPV2 differs depending upon the type of tissues and cells. Its expression is high in certain types of cells in certain organs and tissues. By Northern blotting, the expression of TRPV2 is high in the brain, lung, and spleen (Kanzaki et al. 1999).

In the central nervous system, unlike TRPV1 (Cavanaugh et al. 2011), TRPV2 is expressed in various regions of the brain. In the forebrain, TRPV2 is expressed abundantly in neurons, and in fact, the expression of TRPV2 is the highest among various members of the TRPV family (Cahoy et al. 2008). The expression of TRPV2 in the brain has been extensively studied recently (Nedungadi et al. 2012a). The results show that the expression of TRPV2 is particularly high in supraoptic nucleus, paraventricular nucleus, arcuate nucleus, nucleus of the solitary tract, hypoglossal nucleus, and nucleus ambiguous. These results indicate that TRPV2 is abundantly expressed in the brain regions involved in the osmoregulation and autonomic regulation, including appetite and cardiovascular regulation. TRPV2 is also expressed in Purkinje cells of the cerebellum (Kowase et al. 2003). In the eye, TRPV2 is expressed in retinal pigment epithelial cells and mediates the action of IGF-I (Cordeiro et al. 2010). TRPV2 is also expressed in the spinal cord (Caterina et al. 1999; Lewinter et al. 2004; Park et al. 2011a), trigeminal ganglia (Ichikawa and Sugimoto 2001; Park et al. 2011a), and dorsal root ganglia (DRG) (Caterina et al. 1999; Park et al. 2011a, b). The expression of TRPV2 is significant in myelinated A- and C-fiber sensory neurons (Ma 2001; Lewinter et al. 2004; Koike et al. 2004). A large portion of the TRPV2-expressing neurons are thought to be

peptidergic. Thus, approximately one third of the TRPV2-positive rat DRG neurons express CGRP (Qin et al. 2008), and rat TRPV2-positive neurons express substance P (Yamamoto and Taniguchi 2005). TRPV2 is also expressed in intestinal intrinsic sensory neurons and inhibitory motor neurons and regulates motility of the intestine (Mihara et al. 2010).

TRPV2 is also expressed abundantly in various types of endocrine cells. In the pituitary, growth hormone-producing somatotrophs express a relatively large amount of TRPV2. Chromogranin-positive neuroendocrine cells in the stomach, duodenum, and intestine abundantly express TRPV2 (Kowase et al. 2003). In the pancreas, insulin-producing β-cells express a relatively large amount of TRPV2 (Kanzaki et al. 1999; Hisanaga et al. 2009). During development, pancreatic ductal cells are also strongly positive for TRPV2 (Kowase et al. 2003).

TRPV2 is abundantly expressed in the cells of the immune system. mRNA for TRPV2 is strongly expressed in the spleen (Caterina et al. 1999; Kanzaki et al. 1999). Among various types of blood cells involved in the immune system, macrophages are densely positive for TRPV2 (Nagasawa et al. 2007; Link et al. 2010). TRPV2 is also expressed in resident macrophages. Thus, alveolar macrophages in the lung are strongly positive for TRPV2 (Kowase et al. 2003). Likewise, Kupffer cells in the liver express TRPV2 abundantly (Link et al. 2010). In addition, osteoclasts in the bone, which are derived from progenitor of monocytes, express TRPV2 (Kajiya et al. 2010), and TRPV2 may be involved in osteoclastogenesis. Besides macrophages, TRPV2 is expressed significantly in mast cells (Stokes et al. 2004; Zhang et al. 2012), in neutrophils (Heiner et al. 2003), and in both T and B lymphocytes (Saunders et al. 2007; Santoni et al. 2013). TRPV2 is expressed not only in peripheral blood cells but also in hematopoietic stem cells, from which all lineages of blood cells are derived. Thus, $CD34^+/CD45^+/CD133^+/CD73^+$ hematopoietic stem cells express TRPV2 (Park et al. 2011b).

Immunoreactivity of TRPV2 is detected in various types of epithelial cells. Epithelial cells in submandibular and parotid glands are strongly positive for TRPV2 (Kowase et al. 2003). Also, epithelial cells of the mammary gland, tracheal gland, and pancreatic duct express TRPV2 abundantly (Kowase et al. 2003). TRPV2 immunoreactivity is observed in oral and laryngeal mucosa (Yamamoto and Taniguchi 2005; Hamamoto et al. 2008; Shimohira et al. 2009) as well as in dental pulp (Gibbs et al. 2011). TRPV2 and TRPV4 are expressed in bladder epithelial cells which are exposed to mechanical stimuli (Yu et al. 2011).

The expression of TRPV2 is also significant in the cardiovascular system, and TRPV2 may play an important role in the regulation of circulation and blood pressure. TRPV2 is expressed in arterial smooth muscle cells (Muraki et al. 2003; Park et al. 2003) and venous smooth muscle cells (Peng et al. 2010). TRPV2 is also expressed in endothelial cells (Fanntozzi et al. 2003) and in cardiomyocytes (Iwata et al. 2003).

In addition to the expression of TRPV2 in normal tissues, many studies have shown that TRPV2 is expressed in various types of tumor cells. In bladder tumors, the expression of TRPV2 is upregulated (Caprodossi et al. 2008; Yamada et al. 2010). In prostate cancer cells, the expression levels of TRPV2 are higher in metastatic tumors compared to nonmetastatic tumors (Monet et al. 2010).

TRPV2 is also expressed in hepatocellular carcinoma (Liu et al. 2010) and in a hepatoma cell line (Vriens et al. 2004). The expression of TRPV2 is also detected in gliomas and glioblastoma (Nabissi et al. 2010, 2013).

2 The Channel Protein/Interacting Proteins

TRPV2 shares approximately 50 % sequence identity with TRPV1. TRPV2 has a large N-terminal cytoplasmic domain containing approximately 390 amino acids, followed by six transmembrane segments (S1–S6) and a C-terminal cytoplasmic domain. The pore region is formed by a short hydrophobic stretch between S5 and S6. The S5–S6 segments is thought to define the pore and selective filter, whereas the S1–S4 segments together with the N- and C-terminal domains are thought to modulate the channel gating.

The ankyrin repeat domain (ARD) containing six ankyrin repeats is located in the middle of the long N-terminal cytoplasmic domain. Consequently, the N-terminal cytoplasmic domain is divided into three subdomains: the N-terminal region, the ARD, and the membrane proximal region. Deletion of 65 residues in the N-terminal region does not affect the function of TRPV2 significantly. In contrast, deletion of 83 residues alters the insertion of TRPV2 into the plasma membrane in HEK cells (Neeper et al. 2007). The sequence critical for insertion of TRPV2 to the plasma membrane may be located between residues 65 and 83.

The crystal structure of the ARD domain, which is located between residues 70 and 320, is determined in human and rat TRPV2 (McCleverty et al. 2006; Jin et al. 2006). The ARD is thought to be important for protein-protein interaction. TRPV2 ARD may be important for interaction with regulatory proteins rather than promoting tetrameric assembly (Jin et al. 2006). However, we only have limited information at present regarding the molecules the ARD of TRPV2 interacts with.

For example, a yeast two-hybrid screening using the N-terminal cytoplasmic domain as a bait resulted in a TRPV2-interacting protein recombinase gene activator (RGA) in a mast cell line RBL2H3 (Barnhill et al. 2004). Formation of the complex of RGA and TRPV2 is critical for the translocation of TRPV2 to the plasma membrane induced by cAMP (Stokes et al. 2005). In the case of TRPV1, the ARD binds to ATP and calmodulin (Lishko et al. 2007), which bind competitively to ARD. Indeed, mutation in the ARD alters the channel activity of TRPV1 (Phelps et al. 2007, 2010; Lishko et al. 2007). Similarly, mutation in the ARD of TRPV3 and TRPV4 also affects the activity of the channels (Phelps et al. 2010). However, unlike TRPV1, the ATP-binding domain is not conserved in ARD of TRPV2, and, accordingly, mutation in the ARD does not affect the channel activity of TRPV2 (Lishko et al. 2007; Phelps et al. 2010). It is likely that ARD of TRPV2 interacts with other molecules, which modify the function of TRPV2.

The domain between the ARD and the transmembrane domain is the membrane proximal region, which is comprised of 65 residues. The exact function of this highly conserved region has not been identified. Yao et al. (2011) prepared various types of chimeras between TRPV1 and TRPV2 and analyzed their functions. Their

results show that the membrane proximal domain is quite important for the temperature sensitivity.

There are six predicted transmembrane segments (S1-S6) following the membrane proximal domain. The pore-forming loop is thought to be located between S5 and S6. The sequence of the pore-forming loop is conserved in channels in the TRPV family. The divalent/monovalent cation permeability of TRPV2 is relatively lower compared to that of TRPV1 (Caterina et al. 1997). A subtle difference in the pore-forming domain may account for the difference in the selectivity of the TRPV channels.

In the transmembrane domain of TRPV2, there are one or two N-glycosylation sites depending on species. Interestingly, TRPV1 has an N-glycosylation site (N604) in the similar region (Wirkner et al. 2005). When the N604 is replaced by threonine, the V_{max} of the dose–response curve for capsaicin is depressed and EC_{50} is decreased. In addition, the dependence of the capsaicin effect on extracellular pH is altered. Interestingly, when TRPV2 is transducted in F11 DRG cells, transducted TRPV2 is expressed mostly in the plasma membrane as a glycosylated form, whereas endogenous TRPV2 remains largely inside the cells as a non-glycosylated form (Jahnel et al. 2003). These results suggest the importance of N-glycosylation in the trafficking of TRPV2.

The C-terminal cytoplasmic domain contains the TRP domain, which consists of approximately 20 amino acids. Also, binding sites for phosphatidylinositol 4, 5-bisphosphate (PIP_2) and calmodulin (Mercado et al. 2010; Holakovska et al. 2011) are located in this region. The TRP domain is thought to be important for oligomerization of the TRPV channels. Studies done in TRPV1 show that the C-terminal coiled-coil domain is critical for homotetramerization of the channel (Garicia-Sanz et al. 2004). This coiled-coil domain overlaps with the TRP domain. Thus, as in other members of the TRPV channels, the TRP domain is important for homotetramerization of TRPV2.

Like other members of the TRP channels, TRPV2 is thought to form a tetramer in the plasma membrane and function as a cation channel. It should be mentioned that heteromerization of TRPV2 with TRPV1 is shown when TRPV1 and TRPV2 are expressed ectopically in HEK cells (Hellwig et al. 2005; Rutter et al. 2005; Cheng et al. 2007). Furthermore, in dorsal root ganglion and cerebral cortex, TRPV2 and TRPV1 colocalize tightly, suggesting that heteromerization may also take place in vivo (Hellwig et al. 2005; Rutter et al. 2005; Liapi and Wood 2005).

Like TRPV1, TRPV2 undergoes Ca^{2+}-dependent desensitization. In the case of TRPV1, Ca^{2+}-dependent desensitization of TRPV1 is mediated by calmodulin, which binds to the N-terminal ARD (Rosenbaum et al. 2004) or distal C-terminal region (Numazaki et al. 2003). However, the ARD of TRPV2 does not bind to calmodulin (Lishko et al. 2007; Phelps et al. 2010). Moreover, the C-terminal cytoplasmic region does not have an apparent C-terminal calmodulin-binding domain. In this regard, Mercado et al. have shown that calmodulin binds to the C-terminal domain of TRPV2. Specifically, the calmodulin-binding site is located in the 654–683 region of human TRPV2, which overlaps with the TRP domain (Holakovska et al. 2011). Nevertheless, the calmodulin binding does not mediate

Ca^{2+}-dependent desensitization. Instead, Holakovska et al. (2011) have shown that the PIP_2-binding site located in the C-terminal region is important for Ca^{2+}-dependent desensitization of TRPV2.

3 A Biophysical Description of the Channel Function, Permeation, and Gating

TRPV2 forms homotetramer and functions as a calcium-permeable cation channel. When TRPV2 is heterologously expressed in *Xenopus* oocyte, it mediates cationic currents with a permeability sequence of $Ca^{2+} > Mg^{2+} > Na^{+} \sim Cs^{+} > K^{+}$. The relative permeability ratio P_{Ca2+}/P_{Na+} is 2.94 (Caterina et al. 1999). The current–voltage relationship demonstrates dual rectification, and the rectification is dominant in outward current. When TRPV2 is expressed in HEK293 cells, a considerable amount of TRPV2 protein is incorporated in the plasma membrane, and channel activity can be measured. The TRPV2 current was augmented by a temperature above 52 °C. This is in contrast to the fact that TRPV1 is activated by a temperature above 42 °C. TRPV2 is now nominated as a member of thermosensitive TRP channels or thermo-TRP (Benham et al. 2003; Tominaga and Caterina 2004). Although the regulation of TRPV2 by high temperature is observed in an in vitro system, the physiological role of TRPV2 in thermosensation in vivo is still uncertain.

When TRPV2 is expressed in CHO cells, the protein expression of TRPV2 is observed mostly inside the cells (Kanzaki et al. 1999). This is in contrast to the results obtained in HEK293 cells, where a considerable amount of TRPV2 is found in the plasma membrane. Therefore, the expression of heterologously transducted TRPV2 in the plasma membrane is different depending upon the cell type. In CHO cells incubated in serum-free medium, most of the transducted TRPV2 protein is localized in intracellular compartments. Consistent with this observation, the transmembrane TRPV2 current is low. When the cells are incubated with serum and several min pass, TRPV2 moves toward the plasma membrane and is then incorporated into the plasma membrane. In accordance with this, the transmembrane TRPV2 current is elevated. Upon removal of serum, the TRPV2 current is decreased gradually, and after 30 min of the removal of serum, most of the TRPV2 returns to the intracellular compartment. Concomitantly, the TRPV2 current returns to the basal level (Kanzaki et al. 1999). Hence, localization of TRPV2 in the cell is regulated by serum. Active components in serum are IGF-I, EGF, and PDGF, all of which are capable of inducing translocation of TRPV2 (Kanzaki et al. 1999). In unstimulated cells, a large portion of TRPV2 is located in the endoplasmic reticulum (ER) (Kanzaki et al. 1999; Nagasawa et al. 2007). It is not certain whether or not TRPV2 functions as a cation channel in the ER membrane. In this regard, TRPV2 localized in endosome is shown to function as a calcium-permeable cation channel (Wainszelbaum et al. 2006; Saito et al. 2007). It is thought that this calcium-permeable channel is important for the regulation of fusion of the early

endosome. If TRPV2 also functions as a calcium-permeable channel in ER, TRPV2 may be a major Ca^{2+} leak channel expressed in ER membrane.

As mentioned above, when cells are stimulated by IGF-I or other growth factors, a large portion of TRPV2 translocates to the plasma membrane. Presumably, TRPV2 is sorted into small vesicles by budding of the ER membrane, which is subsequently transported to the plasma membrane. Then, the TRPV2-containing vesicles perhaps fuse with the plasma membrane by exocytosis, and TRPV2 is exposed to the cell surface. Trafficking and exocytotic step are regulated by the signals generated by the IGF-I receptor. Among the intracellular signals evoked by IGF-I, activation of phosphatidylinositol (PI) 3-kinase is the most important. Thus, addition of a PI 3-kinase inhibitor, either wortmannin or LY294002, almost completely blocks translocation of TRPV2 and elevation of the transmembrane TRPV2 current (Kanzaki et al. 1999). With regard to downstream signaling molecules, subsequent activation of Rho GTPase is needed for translocation of TRPV2 (Nagasawa and Kojima 2012). Cytoskeletal proteins play important roles in the regulation of trafficking of various proteins and vesicles. In this regard, assembly of the actin filament is critical for translocation of TRPV2, and latrunculin A blocks translocation of TRPV2 (Nagasawa and Kojima 2012). In contrast, tubulin does not appear to be involved in the trafficking of TRPV2. When IGF-I is removed, the amount of TRPV2 in the plasma membrane decreases gradually. This is perhaps due to endocytosis of TRPV2-containing vesicles since dominant-negative dynamin blocks reduction of cell surface TRPV2 after the removal of IGF-I (Kojima and Nagasawa 2007). Dynamin is a large GTPase regulating endocytosis of clathrin-coated membrane vesicles (Urrutia et al. 1997). Specifically, hydrolysis of GTP on dynamin is critical for fission of clathrin-coated vesicles. Furthermore, reduction of the TRPV2 current after the removal of IGF-I is attenuated by D^k(62–85), a synthetic peptide derived from the α1 domain of the murine major histocompatibility complex class 1 antigen known to inhibit endocytosis of clathrin-coated vesicles (Shibata et al. 1995). Hence, TRPV2 moves to the early endosome and eventually returns to the ER membrane shortly after the removal of IGF-I. It should be mentioned that we have not morphologically identified the TRPV2-containing vesicles by electron microscopy (Nagasawa et al. 2007). However, this does not exclude the possibility that TRPV2 is transported on the vesicles since microvesicles transporting proteins secreted via the constitutive exocytotic pathway are usually difficult to detect morphologically. It should be mentioned that recycling of TRPV2-containing vesicles may take place even in the absence of ligand stimulation. This notion is supported by the observation that blocking endocytosis by D^k(62–85) under unstimulated condition leads to some increase in the TRPV2 current (Kojima and Nagasawa 2007). Collectively, trafficking of TRPV2 occurs even in the absence of ligand stimulation (Fig. 1a). In this condition, both exocytosis and endocytosis of TRPV2-containing vesicles take place slowly and are well balanced, so that the amount of TRPV2 channel expressed in the plasma membrane remains constant. When cells are stimulated by growth factors, for example, IGF-I, subsequent activation of PI 3-kinase facilitates recruitment and transport of the TRPV2-containing vesicles to the plasma membrane by a

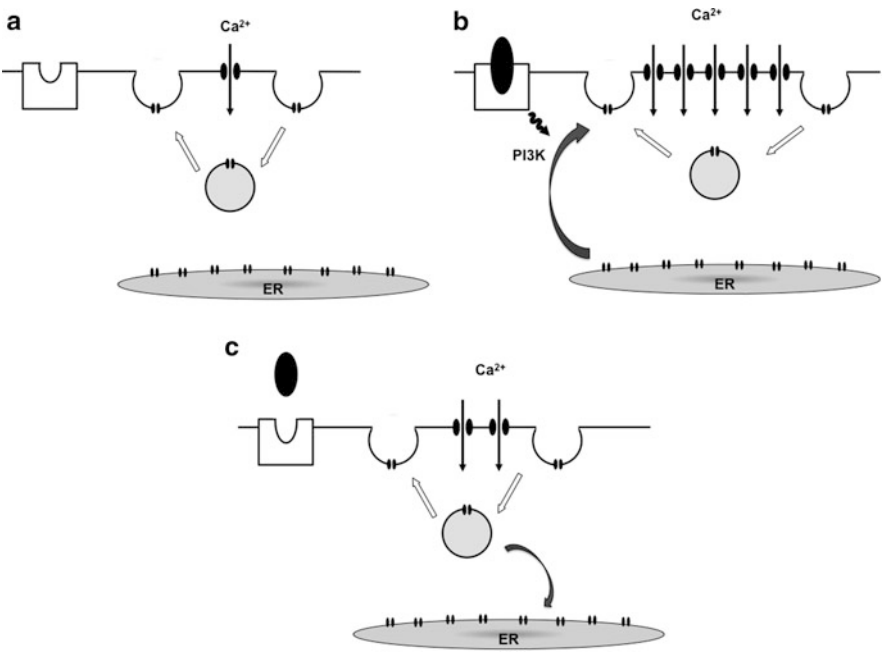

Fig. 1 (**a**) In an unstimulated condition, most of the TRPV2 is located in intracellular compartment, ER. A small portion of TRPV2 is in a recycling pathway, in which exocytosis and endocytosis are well balanced. Only a small amount of TRPV2 is exposed to the cell surface. (**b**) When cells are stimulated, for example, by IGF-I, a relatively large amount of TRPV2 is recruited to the plasma membrane by a PI 3-kinase-dependent mechanism. Even in this condition, some portion of TRPV2 in the plasma membrane is internalized to the endosome by endocytosis. (**c**) When the stimulation by ligand is terminated, TRPV2 in the plasma membrane is internalized gradually, and the amount of TRPV2 in the plasma membrane decreases gradually. Internalized TRPV2 returns to ER presumably via the endosome

Rho- and actin-dependent mechanism. Resultant increase in the TRPV2 exposed to the cell surface elevates the transmembrane TRPV2 current (Fig. 1b). Even in this condition, TRPV2-containing vesicles are internalized by endocytosis. Upon removal of IGF-I, recruitment and translocation of TRPV2-containing vesicles are slowed down, and amount of TRPV2 in the plasma membrane is decreased gradually because of endocytosis of the TRPV2-containing vesicles (Fig. 1c). Collectively, translocation is a unique and dynamic property of TRPV2, and various ligands including growth factors (Kanzaki et al. 1999), differentiation factors (Boels et al. 2001; Kajiya et al.; Kojima and Kodera, unpublished observation), cytokines (Nagasawa et al. 2007; Kajiya et al. 2010), lipid mediators (Monet et al. 2009), and hormones (Hisanaga et al. 2009; Aoyagi et al. 2010; Lin and Sun 2012) regulate trafficking of TRPV2. It should be mentioned that, when incorporated in the plasma membrane, TRPV2 does not distribute evenly. Rather than distributing diffusely, TRPV2 usually forms clusters in many occasions. TRPV2 is rich in adhesion apparatus in the plasma membrane including focal

adhesion and adhesion plaques (Kojima and Nagasawa, unpublished observation). An extreme example is the localization of TRPV2 in the podosome (Nagasawa and Kojima 2012). Podosome is a special form of adhesion apparatus in migrating cells such as endothelial cells, macrophages, neutrophils, and various types of cancer cells (Linder and Apfelbacher 2003) and is important for regulation of adhesion, migration, and, in the case of tumor cells, invasion. Various actin-binding proteins and signaling molecules such as gelsolin, paxillin, PI 3-kinase, Rho GTPases, and Pyk2 are associated with the podosome and regulate assembly of actin filaments. Given that TRPV2 is a calcium-permeable channel, localization of TRPV2 in adhesion apparatus may be advantageous to regulate cytoskeletal organization and motility of the cells (Nagasawa and Kojima 2012). It is reported that gating rather than translocation of TRPV2 is regulated by ligands (Penna et al. 2006). However, it is rather difficult to determine this possibility in the presence of dynamic changes in trafficking. For example, any changes in the amount of TRPV2 exposed to the cell surface would affect the apparent TRPV2 current. It becomes possible to analyze the effect on channel gating accurately if both exocytosis and endocytosis of TRPV2 are blocked, for example, by introducing dominant-negative Rho and dynamin. Alternately, analysis of single channel current makes it possible to determine the changes in channel gating because, by the single channel analysis, we can monitor the activity of particular channel molecule exposed to cell surface. However, this raises other concerns. Since TRPV2 is a mechanosensitive channel (see below), measurement of single channel current by cell-attached patch might not be accurate due to the presence of membrane stretch. Moreover, there is a possibility that membrane stretch induces translocation of TRPV2 (Iwata et al 2003). This means that formation of cell-attached patch would induce translocation. Establishment of giga-seal would also modify cytoskeletal architecture beneath the plasma membrane, which is critical for trafficking of TRPV2. These considerations raise concerns about analysis of gating of TRPV2 by a single channel recording.

Translocation of TRPV2 resembles in many respects translocation of the glucose transporter GLUT4 induced by insulin in adipocytes and skeletal muscle cells. In these cells, insulin induces translocation of GLUT4 from an intracellular retention pool to the plasma membrane (Suzuki and Kono 1980; Cushman and Wardzala 1980). It has been more than 30 years since translocation of GLUT4 was discovered. Yet, because of the technical difficulties, it is still not certain whether, other than induction of translocation, insulin increases the intrinsic activity of GLUT4 molecule.

In addition to regulation by heat and various ligands, many lines of evidence indicate that TRPV2 is regulated by mechanical stresses. Muraki et al. (2003) showed that TRPV2 is involved in the elevation of $[Ca^{2+}]_c$ induced by membrane stretch. In freshly dispersed vascular smooth muscle cells, exposure to hypotonic solution activates nonselective cation channel current and induces the elevation of $[Ca^{2+}]_c$, which is dependent on extracellular calcium. Likewise, application of membrane stretch also activates the cation channel and increases $[Ca^{2+}]_c$. These responses are blocked by adding ruthenium red, an inhibitor of TRPV channels, and

also by knocking down TRPV2 in vascular smooth muscle cells. Since TRPV2 is expressed in stretch-sensitive cells including vascular endothelial cells and cardiomyocytes, TRPV2 may act as a stretch-activated calcium-permeable channel in these cells. Consistent with this notion, Iwata et al. (2003) showed that application of membrane stretch activates TRPV2 in cardiomyocytes. More importantly, their results show that membrane stretch induces translocation of TRPV2 from the intracellular pool to the plasma membrane. Since membrane stretch activates PI 3-kinase (Kippenberger et al. 2005), it seems likely that membrane stretch increases the cell surface expression of TRPV2 by recruiting TRPV2 from the intracellular compartment via activation of PI 3-kinase.

4 Physiological Functions in Native Cells, Organs, and Organ Systems

To assess the physiological function of TRPV2, pharmacological activators and inhibitors would be useful. Unfortunately, most of them are not specific to TRPV2, and in addition, many of them demonstrate species-dependent effects (Vriends et al. 2009). Accordingly, caution must be taken when assessing the role of TRPV2 in vitro and in vivo using these pharmacological compounds.

2-Aminoethoxydiphenyl borate (2-APB) has been shown to be an activator of TRPV2, and EC_{50} is 129 μM in HEK293 cells expressing TRPV2 (Hu et al. 2004). 2-APB also activates TRPV1 and TRPV3, whereas TRPV4 is insensitive to 2-APB. A subsequent study revealed that human TRPV2 is not sensitive to 2-APB (Neeper et al. 2007; Juvin et al. 2007). Thus, the effects of 2-APB on TRPV2 are species dependent. 2-APB is also known to block inositol 1, 4, 5-trisphosphate receptor (Maruyama et al. 1997), store-operated channel (Dobrydneva and Blackmore 2001), and other types of channels (Lenonnier et al. 2004). Studies using various deletion mutants revealed that both N- and C-terminal cytoplasmic domains are important for the effect of 2-APB (Neeper et al. 2007). A related compound diphenylborinic anhydride (DPBA) is an activator of mouse TRPV2, but it is not effective on human TRPV2 (Juvin et al. 2007). Again, both N- and C-terminal cytoplasmic domains are important for the action of DPBA (Juvin et al. 2007).

The plant *Cannabis sativa* has been used as herbal medicine for pain relief. *Cannabis sativa* contains numerous compounds known as cannabinoids. Among many related compounds, (-)-trans-Δ^9-tetrahydrocannabidol (THC), cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabivarin (THCV) are well-characterized cannabinoids. Qin et al. (2008) showed that TRPV2 is activated by cannabinoids. It is not certain whether cannabinoids induce translocation of TRPV2. Although these compounds are not specific to TRPV2, they activate TRPV2 with EC_{50} of the micromolar range. The rank order of potency is THC > CBD > THCV > CBN in HEK293 cells expressing TRPV2 (De Petrocellis et al. 2011). Since these cannabinoids also activate other TRP channels including TRPV1, TRPA1, and TRPM8, again caution must be taken when interpreting the results obtained using these compounds.

Another interesting compound which activates TRPV2 is probenecid. This compound has been used for the treatment of gout (Robbin et al. 2012). It is a competitive inhibitor of organic anion transporter (OAT) (Burckhardt and Burckhardt 2003) and blocks uptake of uric acid from the urine. In HEK293T cells expressing TRPV2, probenecid increases the transmembrane TRPV2 current and elevated $[Ca^{2+}]_c$ (Bang et al. 2007). The EC_{50} is 31.9 μM. Other thermo-TRP channels, TRPV1, TRPV3, TRPV4, TRPM8, and TRPA1, were not activated. Analyses done in trigeminal neurons also indicate that probenecid activates TRPV2 among thermo-TRP channels (Bang et al. 2007). The results demonstrate that probenecid activates TRPV2 directly. Since probenecid is an inhibitor of OAT and induces morphological changes at least in some types of cells, a possibility still remains that probenecid modifies the activity of TRPV2 by causing membrane stretch. The absence of the effect of probenecid on TRPV4 seems to negate this possibility.

With regard to inhibitors of TRPV2, several compounds have been used. Unfortunately, none of them are specific to TRPV2, and interpretation of the results should be done carefully. An alternative approach to block TRPV2, for example, knockdown of TRPV2, may be needed to assess the involvement of TRPV2 in physiological processes.

1-(β-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKF96365) is an inhibitor of nonselective cation channel and also inhibits TRPV2 activated by (2-APB) (Juvin et al. 2007). Ruthenium red is an inhibitor of TRP channels and blocks TRPV2 as well (Hu et al. 2004; Leffler et al. 2007). We identified tranilast as an inhibitor of TRPV2 (Hisanaga et al. 2009). It should be noted that this compound was first identified as an inhibitor of IgE receptor-mediated calcium entry (Komatsu et al. 1988). Tranilast also inhibits calcium entry induced by PDGF (Nie et al. 1996) and IGF-I (Nie et al. 1997). Accordingly, the inhibitory effect of tranilast is not specific to TRPV2. In any event, because the action of the above-mentioned inhibitors is not specific to TRPV2, caution has to be taken in the interpretation of the results.

From an evolutionally point of view, *Caenorhabditis elegans* express OSM-9, osmotic avoidance abnormal family member 9. In fact, OSM-9 is the first identified member of the TRPV family involved in mechanosensation and olfaction (Colbert et al. 1997). *C. elegans* also express OCR-1 to OCR-4 (Osm-9/capsaicin receptor related), which belong to the TRPV family (Xiao and Xu 2011). Among 17 TRP channels encoded in the genome of *C. elegans*, five of them belong to the TRPV family. OSM-9 and OCR-2 are involved in osmosensation as well as mechanosensation. In *Drosophila melanogaster*, Nanchung and Inactive, two members of the TRPV family, are expressed in mechanosensitive chordotonal organs or Johnston's organ in the antenna and are involved in the sense of hearing (Kim et al. 2003). The role of these channels expressed in non-vertebrates suggests the essential function of members of the TRPV family.

Despite of the unique function and regulation in cells of various tissues and organs, the physiological role of TRPV2 has not been completely elucidated.

As mentioned below, TRPV2 knockout mice do not show apparent phenotypes (Link et al. 2010; Park et al. 2011a). Consequently, it is necessary to assess the role of TRPV2 channel in many biological processes carefully using multiple approaches.

4.1 Role of TRPV2 in the Nervous System

Since TRPV2 was first characterized as a heat-activated channel (Caterina et al. 1999), many studies have been carried out to assess the role of this channel in nociception. As mentioned below, mutant mice lacking TRPV2 do not show abnormalities in thermal sensing (Park et al. 2011a, b), providing no information as to the physiological role of TRPV2 in thermal sensing. Given that TRPV2 is expressed abundantly in DRG neurons (Caterina et al. 1999; Park et al. 2011b) and trigeminal ganglia (Ichikawa and Sugimoto 2001; Park et al. 2011a), it is reasonable to speculate that TRPV2 plays some role in nociception. Alternately, TRPV2 exerts some roles other than nociception in these neurons (Lewinter et al. 2004).

TRPV2 is expressed in neurons during development in the brain and in the spinal cord (Cahoy et al. 2008; Shibasaki et al. 2010; Nedungadi et al. 2012a). Shibasaki et al. (2010) showed that TRPV2 is expressed in growth cone and is activated by the membrane stretch. Calcium entry induced by membrane stretch may be important for the outgrowth of neurites.

The expression of TRPV2 is significant in the hypothalamus, especially in the supraoptic nucleus (SON) and paraventricular nucleus (PVN), which is critical for osmoregulation (Cahoy et al. 2008). Neurons in SON and PVN secrete vasopressin, and the expression of TRPV2 in these nuclei is upregulated in hepatic cirrhosis-induced hyponatremia (Nedungadi et al. 2012b). TRPV2 together with TRPV4 (Gao et al. 2003) may function as a volume-sensing channel in these nuclei.

TRPV2 is expressed in myenteric plexus. Some of the TRPV2-possitive neurons are also positive for calbindin D-28K, indicating that TRPV2 is expressed in intrinsic afferent neurons (Kashiba et al. 2004). In mice, intestinal intrinsic sensory and inhibitory motor neurons are positively stained with anti-TRPV2 antibody. In these neurons, activators of TRPV2 and mechanical stress activate TRPV2-like current and increase $[Ca^{2+}]_c$. In isolated intestine, activation of TRPV2 inhibits spontaneous contraction of circular muscle, and gastrointestinal transit is accelerated (Mihara et al. 2010). TRPV2 is involved in the regulation of intestinal motility.

4.2 Role of TRPV2 in the Immune System

TRPV2 is expressed in various types of cells regulating the immune system, and it is involved in both innate and adaptive immune responses (Santoni et al. 2013). It appears that the role of TRPV2 is prominent in the innate immune response.

TRPV2 is expressed abundantly in monocytes and macrophages (Kim et al. 2003; Nagasawa et al. 2007). Other members of the TRPV family are not expressed in macrophages (Nagasawa et al. 2007; Yamashiro et al. 2010). TRPV2 is activated by a chemotactic peptide fMet-Leu-Phe, which evokes sustained elevation of $[Ca^{2+}]_c$. fMet-Leu-Phe induces translocation of TRPV2 from ER to the plasma membrane by activating PI 3-kinase via a pertussis toxin-sensitive G protein (Nagasawa et al. 2007). When the translocation of TRPV2 is blocked by adding PI 3-kinase inhibitors or transduction of mutant TRPV2, which blocks translocation of TRPV2, translocation of TRPV2 is completely blocked, and the effect of fMet-Leu-Phe on $[Ca^{2+}]_c$ is markedly reduced. In this condition, fMet-Leu-Phe-induced migration is nearly completely blocked. Similarly, addition of ruthenium red or knockdown of TRPV2 by adding shTRPV2 markedly inhibited sustained elevation of $[Ca^{2+}]_c$ and migration of macrophages. Collectively, TRPV2 is critical for fMet-Leu-Phe-induced calcium entry and migration of macrophages.

The role of TRPV2 in phagocytosis of macrophages is demonstrated by Link et al. (2010) by using TRPV2 knockout mice. In the absence of TRPV2, phagocytosis of macrophages is attenuated. TRPV2 is also involved in the signaling pathway activated by Toll-like receptor 4 (TLR4). Thus, knockdown of TRPV2 attenuates LPS-induced expression of mRNA for tumor necrosis factor-α and interleukin-6, and it induces degradation of inhibitor of nuclear factor-κB-α (IκBα) (Yamashiro et al. 2010).

The expression of TRPV2 is also observed in granulocytes (Heiner et al. 2003). It is likely that TRPV2 is involved in the regulation of migration of granulocytes. TRPV2 is expressed in dendritic cells (Su et al. 2004), but its role is not determined to date. High expression of TRPV2 is observed in NK cells (Su et al. 2004). Although the role of TRPV2 in NK cells is not known at present, it is interesting that the administration of CBD greatly increases the number of NK cells (Ignatowska-Jankowska et al. 2009).

Cell surface expression and oligomerization of TRPV2 are observed in mast cells (Stokes et al. 2004), and TRPV2 is functionally coupled to degranulation of mast cells. Stokes et al. (2004) demonstrate a novel protein kinase A (PKA)-dependent signaling module containing PKA and a PKA adaptor protein, acyl CoA-binding protein 3. TRPV2 interacts with this signaling module and functionally couples proinflammatory degranulation. Mast cells also respond to various physical stimuli including high temperature, laser light of 640 nm, and light at 48 mW, all of which activate TRPV2 and eventually stimulate degranulation (Zhang et al. 2012).

In addition to the innate immune response, TRPV2 is also involved in adaptive immunity. In T lymphocytes, TRPV2 is expressed in both $CD4^+$ and $CD8^+$ T cells (Spinsanti et al. 2008). When T lymphocytes make contact with antigen-presenting cells, TRPV2 forms a cluster at the immunological synapse with Kv1.3, KCa3.1, STIM1, and Orai (Lioudyno et al. 2008; Cahalan and Chandy 2009). Interestingly, when TRPV2 is knocked down, calcium release from the store and subsequent activation of store-operated calcium entry are attenuated (Sauer and Jegla 2006). TRPV2 is also expressed in $CD19^+$ B lymphocytes (Su et al. 2004) and $CD138^+$

plasma cells (Boyd et al. 2009). The role of TRPV2 in B lymphocytes has not been determined. Given the functional similarity of TRPV2 and CD20, a non-TRP calcium-permeable channel (Bubien et al. 1993), it is possible that TRPV2 is involved in the regulation of growth of B lymphocytes.

In addition to peripheral blood cells, $CD34^+$ hematopoietic stem cells (HSC) express TRPV2 (Park et al. 2011b). Given that TRPV2 is expressed in various types of somatic stem cells, it is possible that TRPV2 is important for self-renewal of stem cells.

Osteoclast precursors are derived from HSC and differentiate into osteoclasts upon stimulation by macrophage colony-stimulating factor (M-CSF). M-CSF induces the expression of receptor activator of nuclear factor-κβ (RANK) (Negishi-Koga and Takayanagi 2009). Binding of RANK-ligand (RANKL) to RANK and activation of the immunoreceptor tyrosine-based activation motif-associated immunoglobulin-like receptor (Takayanagi et al. 2002; Koga et al. 2004) initiate osteoclast differentiation. PLCγ is activated by activation of the above-mentioned signaling pathway, and resultant increase in inositol (1, 4, 5)-triphosphate causes Ca^{2+} release from ER and eventually oscillation of $[Ca^{2+}]_c$. Calcium entry is required for the maintenance of $[Ca^{2+}]_c$ oscillation. TRPV2 is important for $[Ca^{2+}]_c$ oscillation found in osteoclasts (Kajiya et al. 2010) and thus critical for early osteoclastogenesis.

4.3 Role of TRPV2 in the Cardiovascular System

TRPV2 is expressed in vascular smooth muscle cells (Park et al. 2003; Inoue et al. 2006). TRPV2 is a calcium-permeable channel and functions as a mechanosensitive channel (Muraki et al. 2003). TRPV2 and TRPV4 may act as a mechanosensitive channel and are involved in the regulation of vascular tone (Dietrich et al. 2006). TRPV2 is also expressed in vascular endothelial cells (Fanntozzi et al. 2003). It seems likely that TRPV2 in endothelial cells senses shear stress and modulates the function of endothelial cells. TRPV2 is also expressed in cardiomyocytes. Koch et al. (2012) showed that probenecid increases cardiac contractility in vivo. This effect of probenecid is not observed in TRPV2 knockout mice and is not mediated by β-adrenergic receptor. Probenecid has a positive inotropic effect in isolated cardiomyocytes. These results indicate that TRPV2 expressed in cardiomyocytes regulates cardiac contractility. TRPV2 expressed in cardiomyocytes is also involved in pathophysiology. In cardiomyocytes derived from muscular dystrophic animals, the cell surface expression of TRPV2 is increased, which may cause calcium overload and eventually cell death of cardiomyocytes (Iwata et al. 2003). When translocation of TRPV2 to the plasma membrane is attenuated by introducing dominant-negative TRPV2, $[Ca^{2+}]_c$ increase in muscle fiber is reduced, and dystrophic pathology is ameliorated (Iwata et al. 2009). TRPV2 expressed in cardiomyocytes plays a significant role in the regulation of Ca^{2+} in cardiomyocytes.

4.4 Role of TRPV2 in the Endocrine System

TRPV2 is abundantly expressed in neuroendocrine cells of the gastrointestinal tract (Kowase et al. 2003). Also, it is expressed in pancreatic β-cells, which secrete insulin. In these cells, insulin induces translocation of TRPV2 to the plasma membrane and stimulates calcium influx (Hisanaga et al. 2009). Augmented calcium entry further promotes insulin secretion. In accordance with this notion, inhibition of TRPV2 action reduced not only glucose-induced insulin secretion but also insulin secretion induced by a depolarizing concentration of potassium (Hisanaga et al. 2009). TRPV2 participates significantly in the action of glucose on $[Ca^{2+}]_c$ (Aoyagi et al. 2010). Hence, TRPV2 plays a key role in the autocrine feed-forward action of insulin in pancreatic β-cells. In β-cells, overexpression of Klotho, an antiaging gene, enhances insulin secretion induced by glucose (Lin and Sun 2012). Interestingly, overexpression of Klotho increases the amount of TRPV2 in the plasma membrane and augments calcium entry. Conversely, knockdown of Klotho reduces the cell surface expression of TRPV2, calcium entry, and insulin secretion. Klotho appears to exert its action in β-cells by promoting the cell surface expression of TRPV2. In this regard, Klotho is shown to increase the cell surface expression of TRPV5 in renal epithelial cells (Chang et al. 2005; Cha et al. 2008). Klotho breaks the terminal sialic acid in the glycan structure of TRPV5 and promotes the binding of Galectin-1 to the glycan. Then, TRPV5 is anchored in the plasma membrane. A similar type of regulation may take place in β-cells, and TRPV2 is anchored in the plasma membrane of β-cells.

4.5 Role of TRPV2 in Oral and Urothelial Epithelium

TRPV2 is expressed in oral mucosa (Shimohira et al. 2009) and larynx (Hamamoto et al. 2008). These cells respond to hypotonicity and mechanical stresses. TRPV2 may function to sense these stresses. TRPV2 is also expressed in odontoblasts (Son et al. 2009). Application of hypotonic solution to odontoblasts activates the TRPV2 current and elevation of $[Ca^{2+}]_c$, which is inhibited by ruthenium red and tranilast. Probenid also elevated $[Ca^{2+}]_c$ (Sato et al. 2013). Odontoblasts are in dentin and possess elongated cellular processes. These cells sense many mechanical stresses including osmotic stress. TRPV2 functions as a mechanosensitive channel in these cells and is involved in the pathophysiology of tooth pain.

TRPV2 is also significantly expressed in the bladder urothelium, which is thought to be a sensory structure (Everaerts et al. 2009; Yu et al. 2011). In these cells, both TRPV2 and TRPV4 are expressed. Presumably, these two channels sense changes in the volume and osmolarity of the urine and are involved in the mechanosensing of the bladder (Anderson et al. 2010).

4.6 Role of TRPV2 in Cancer Cells

TRPV2 is overexpressed in certain types of cancer and cell lines of tumor cells (Lehen'kyi and Prevarskaya 2012). In some types of cancers, TRPV2 is thought to be involved in growth, cell death, or migration, but the role of TRPV2 in cancer cells still remains elucidated.

Significant expression of TRPV2 is found in bladder cancer. Caprodossi et al. (2008) showed that TRPV2 is expressed in the specimen of human bladder cancer. TRPV2 is expressed abundantly in cancer with higher grade of malignancy. Yamada et al. (2010) determined the expression of TRPV2 in bladder cancer cell lines and found that a poorly differentiated cell line expresses a larger amount of TRPV2 compared to that of a well-differentiated cancer cell line. They also showed that administration of CBD, an activator of TRPV2, induces apoptotic cell death in the poorly differentiated cell line. They interpret these data as TRPV2 being involved in cell death and postulate that TRPV2 is a potential therapeutic target. However, it is not surprising that the administration of the TRPV2 agonist to TRPV2-overexpressing cells results in cell death due Ca^{2+} overload. Their results therefore do not exclude the possibility that accelerated growth is causally related with overexpression of TRPV2. Experiments using knockdown of TRPV2 or administration of inhibitor of TRPV2 are required to assess the role of TRPV2 in the pathobiology of cancer cells. In this regard, inhibition of calcium entry by tranilast markedly reduces growth of breast cancer cell line MCF-7 (Nie et al. 1997). It seems likely that overexpression of TRPV2 in poorly differentiated cancer cells is at least partly responsible for accelerated growth in these cells.

TRPV2 is expressed in prostate cancer, and its expression levels are high in patients with metastatic cancer (Monet et al. 2010). In other words, the expression of TRPV2 positively correlates with the malignancy of the cancer cells. The same authors (Monet et al. 2009) showed that, in prostate cancer cells, lysophospholipids including lysophosphatidylcholine and lysophosphatidylinositol induce translocation of TRPV2 to the plasma membrane and increase $[Ca^{2+}]_c$. Activation of TRPV2 induces migration of prostate cancer cells, and conversely, blocking translocation of TRPV2 attenuates migration. They postulated that TRPV2 is important for migration of prostate cancer cells. It should be mentioned that knockdown of TRPV2 reduces growth speed of cancer cells (Monet et al. 2010). Collectively, TRPV2 is important for growth and migration of prostatic cancer, and the expression levels of TRPV2 affect growth and metastasis (Gkika and Prevarskaya 2011).

Liu et al. (2010) investigated the expression of TRPV2 in normal liver, cirrhotic liver, and hepatocellular carcinoma tissue. Their results show that the expression of TRPV2 is increased in cirrhotic liver. The expression level of TRPV2 is also high in moderately and well-differentiated hepatocellular carcinoma but is rather reduced in poorly differentiated carcinoma. The expression levels of TRPV2 may be a prognostic marker, but its role in hepatocellular carcinogenesis is not certain at present.

In glioma cells, the expression of TRPV2 appears to be negatively correlated with proliferation of tumor cells (Nabissi et al. 2010). The expression level of

TRPV2 is reduced in glioma cells compared to that of normal astrocytes, but is further reduced in glioblastoma multiforme (GBM), the most malignant form of gliomas (Nabissi et al. 2010). When TRPV2 is blocked or knocked down, proliferation of glioma cell line is accelerated. Conversely, overexpression of TRPV2 reduces proliferation and promotes differentiation (Morelli et al. 2012). Recently, the same authors show that a cannabinoid CBD, an agonist for TRPV2, upregulates the expression of TRPV2 and the TRPV2 current in GBM cells. Furthermore, CBD accelerates the uptake of chemotherapeutic compounds and promotes apoptotic cell death (Nabissi et al. 2013). TRPV2 can be a new therapeutic target to treat GBM.

5 Lessons from Knockouts

Mutant mice lacking TRPV2 were generated and analyzed by Link et al. (2010). TRPV2 knockout mice do not show abnormal phenotypes except that activations of macrophages are impaired. In the absence of TRPV2, phagocytosis of macrophages is attenuated. Upon binding of zymosan, TRPV2 is recruited to the phagosome by a PI 3-kinase-dependent mechanism. This depolarizes the plasma membrane, which in turn stimulates synthesis of PI 4, 5-bisphosphate and subsequent actin depolymerization. Similarly, IgG- and complement-mediated particle binding and phagocytosis are attenuated in TRPV2-deficient macrophages. Chemotaxis is also impaired in TRPV2-deficient macrophages. In vivo, organ bacterial load is increased and mortality worsens in TRPV2-deficient mice when challenged with *Listeria monocytogenes*. The innate immunity is impaired in TRPV2-deficient mice. This may be a reason for increased perinatal lethality observed in TRPV2 null mice.

Since TRPV2 was first characterized as a heat-activated channel, its role in nociception is investigated in TRPV2 knockout mice (Park et al. 2011a). In TRPV2-deficient mice, no abnormality is detected in thermosensation. Specifically, acute nociception and hyperalgesia in response to thermal stimulation are not altered. This is in contrast to mice lacking TRPV1, whose thermal sensation is significantly impaired (Caterina 2000). In addition, a behavioral study performed in TRPV1/TRPV2 double knockout mice reveals that these double knockout mice are not different from TRPV1 single knockout mice. Furthermore, deletion of TRPV2 does not affect C-fiber response to heat and C- and Aδ-fiber responses to noxious stimuli in the skin (Park et al. 2011a). Collectively, the in vivo role of TRPV2 in thermosensing is not demonstrated.

Although the abnormal phenotypes described in TRPV2 is not many, this does not exclude the possibility that TRPV2 is involved in physiological regulation in many systems. One of the reasons could be redundant regulation by other members of the TRPV family, for example, TRPV4 and TRPV1, and compensation by other types of TRP channels. For example, growth factors such as EGF, PDGF, and IGF-I stimulate calcium entry by activating TRPV2 (Kanzaki et al. 1999). Growth factors also activate other types of TRP channels such as TRPC4 (Odell et al. 2005) and other non-TRP channels (Schwartz et al. 1993; Peppelenbosch et al. 1996; Kanzaki

et al. 1997). Such complex regulation of calcium entry by growth factors is difficult to be assessed by employing single or double knockout mice.

6 Role in the Hereditary and Acquired Diseases

To date, there is no report showing that mutation or deletion of the TRPV2 gene causes a hereditary disease. It should be mentioned that TRPV2 is involved in pathophysiology of some hereditary diseases. For example, in patients with various types of muscular dystrophy, skeletal muscle cells and cardiomyocytes are thought to be damaged by calcium overload. Iwata et al. (2003) showed that the amount of TRPV2 in the sarcolemma is markedly increased in dystrophin-deficient skeletal and cardiac muscle. The elevation of TRPV2 in sarcolemma is observed both in patients with muscular dystrophy and in an animal model of the disease. TRPV2 acts as a mechanosensitive channel and membrane stretch causes translocation of TRPV2 to the sarcolemma (Iwata et al. 2003). When dystrophin-glycoprotein complex is disrupted by genetic mutation of dystrophin or sarcoglycans, amount of TRPV2 in sarcolemma is increased. In other words, TRPV2 is translocated without the application of membrane stretch. This results in calcium overload and causes cell damages. It is also shown that transgenic overexpression of TRPV2 in the heart causes calcium overload and damages of cardiomyocytes. When dominant-negative TRPV2 is transducted in muscles of the animal model of muscular dystrophy, derangements in cardiac and skeletal muscle are rescued (Iwata et al. 2009). These results indicate that trafficking of TRPV2 is impaired in dystrophic muscle, and resultant overexpression of TRPV2 in sarcolemma induces serious damages in muscle cells.

7 Perspectives

In this article, we described recent progresses in our understanding of the structure, regulation, and function of TRPV2. Although many aspects of TRPV2 have been described, many uncertainties still remain in this field. For example, one of the unique properties of TRPV2 is that this channel is quite active in terms of trafficking. Besides targeting to the plasma membrane following synthesis and modification in Golgi apparatus, the trafficking of TRPV2 is actively regulated by various stimuli. Intracellular trafficking of TRPV2 may be quite complex, and there remain many issues to be solved. The vesicles carrying TRPV2 and the mechanism for exocytosis and endocytosis need to be identified. In addition, cell surface distribution of TRPV2 in various types of cells should be determined. It also remains unsolved whether TRPV2 is constitutively active or whether channel opening is regulated by various stimuli. Given that TRPV2 is actively moving inside the cell, this question is rather difficult to answer. Obviously, approaches employing new techniques are needed to solve this issue. It should be mentioned that translocation of TRPV2 is not detected in some experimental conditions (Penna et al. 2006). For

example, in HEK293 cells, most of the transfected TRPV2 is translated, and TRPV2 protein is expressed in the cell surface (Penna et al. 2006, Nagasawa and Kojima, unpublished observation). The mechanism responsible for the inability of active trafficking of TRPV2 in these cells still needs to be identified. In any event, active trafficking of TRPV2 seems to be a reasonable way to prevent cells from Ca^{2+} overload since TRPV2 is stimulated by long-acting ligands such as growth factors or differentiation factors. Unless the localization and activity are tuned finely, cells become sick due to Ca^{2+} overload as seen in HEK293 cells, in which TRPV2 is continuously exposed to the cell surface.

There are many uncertainties as to the physiological functions of TRPV2. This is partly due to the subtle abnormal phenotypes in TRPV2 knockout mice (Link et al. 2010; Park et al. 2011a). However, this does not exclude the possibility that TRPV2 plays an important role in physiological settings. The role of TRPV2 in many biological systems employing multiple approaches needs to be examined. In this regard, the absence of selective inhibitor makes it rather hard to easily assess the significance of TRPV2 in many systems. Development of a new compound modifying the activity of TRPV2 selectively is desired. In this regard, short-form TRPV2, which acts as a dominant-negative variant of TRPV2, can be used to block the function of TRPV2 in a rather specific manner (Nagasawa et al. 2007). In any case, the role of TRPV2 in many biological systems using variety of approaches needs to be assessed.

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TRPV3

Pu Yang and Michael X. Zhu

Contents

Abstract

Transient receptor potential vanilloid-3 (TRPV3) is a Ca^{2+}-permeable nonselective cation channel widely expressed in skin keratinocytes, as well as oral and nasal epithelia. TRPV3 is activated by innocuous warm as well as noxious hot temperatures. Activation of TRPV3 in skin keratinocytes causes release of multiple substances, which in turn regulate diverse functions including skin barrier formation, hair growth, wound healing, temperature sensing, and itch and pain perceptions. While several natural and synthetic ligands have been described for TRPV3, only one of them, farnesyl pyrophosphate, is naturally produced in animal cells. Together with the use of genetic mouse models,

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applications of these compounds have revealed not only the physiological functions but also regulatory mechanisms of TRPV3 channel by extracellular Ca^{2+}, Mg^{2+}, and protons as well as intracellular Ca^{2+}-calmodulin, ATP, phosphatidylinositol 4,5-bisphosphate, polyunsaturated fatty acids, protons, and Mg^{2+}. Gain-of-function genetic mutations of TRPV3 in rodents and humans have been instrumental in unveiling the critical role of this channel in skin health and disease.

Keywords
TRPV • Thermosensation • Skin disorders • TRP channels • Olmsted syndrome • Channelopathy

1 Gene

In humans, the gene for TRPV3, also called vanilloid receptor-like 3 (VRL3 or VRL-3), is located in chromosome 17p13, immediately next to the gene for TRPV1 (separated by 7.45 kbs) and in the same transcriptional orientation. The entire gene spans approximately 47.5 kbs and contains 18 exons. Similarly, the gene for mouse TRPV3 is also very close to that for mouse TRPV1 (separated by about 7 kbs) on chromosome 11B4, with the same transcriptional orientation. The mouse gene is approximately 30 kbs with 18 exons (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002). The close proximity between genes for TRPV1 and TRPV3 appears to be conserved among all animals that have the *Trpv3* gene, which seem to include just amphibians, reptiles, birds, and mammals because *Trpv3* is not found in fish (Saito et al. 2011).

Only a small number of splice variants have been identified for human *Trpv3* based on mRNA sequences. In one case, the last exon, exon 18, is missing (GenBank: AY118267, protein: AAM80558.1), giving rise to a shorter C-terminal tail, GTVAVR, after Thr-759 instead of the usual 32 residues encoded by exon 18. In another case, an alternative splice acceptor site is used by exon 18, causing the deletion of Ala-760 in the protein product (GenBank: AJ487035.2, AF514998.1, protein: CAD31711.2, AAM54027.1) (Smith et al. 2002; Xu et al. 2002). Interestingly, an equivalent splice variant has also been reported in a cDNA clone for mouse *Trpv3* (GenBank: BC108984.1, protein: AAI08985.1). Finally, an extra exon (exon 2′) was found after the usual exon 2 in a human cDNA clone (GenBank: BC143299.1, protein: AAI43300.1), coding for a short N-terminal sequence (MSHHTWPSPALNLDFATYLLRDLG-), excluding the usual 40 residues encoded by exon 2 because of frame shift and stop codon. To date, the functional consequences and expression patterns of these splice forms remain undetermined.

2 Expression

It was shown by in situ hybridization and RT-PCR that *Trpv3* is highly expressed in the brain, spinal cord, dorsal root ganglia (DRG), skin, and testis (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002). Lower expression was also seen in the stomach, tongue, trachea, small intestine, and placenta (Xu et al. 2002). In monkey, *Trpv3* mRNA was found throughout the cortex, thalamus, and striatum of the brain, in multiple areas of the spinal cord, and in most sensory neurons in the DRG and trigeminal ganglia (Xu et al. 2002).

However, the most prominent tissue and cell types for *Trpv3* expression appear to be at the epithelial layer of the skin, oral cavity, and gastrointestinal tract. In fact, a Northern blot analysis performed on rat tissue samples revealed *Trpv3* mRNA expression only in the skin, but not in the brain, DRG, and spinal cord, which contrasted the quite predominant expression of *Trpv1* in the DRG sample (Peier et al. 2002). *Trpv3* mRNA was also detected in cells surrounding hair follicles of human and mouse skin (Xu et al. 2002; Peier et al. 2002), as well as the mouth and nose of mice, especially in the epithelium of the palate, tongue, and nose (Xu et al. 2006). While retrograde labeling did not find *Trpv3* mRNA in mouse vagal afferent neurons that innervate stomach, the mRNA was detected in muscle and mucosa of stomach and small intestine (Zhang et al. 2004) as well as the superficial epithelium of distal colon (Ueda et al. 2009).

Immunocytochemical staining also revealed the presence of TRPV3 protein in most keratinocytes at the epidermal layer and in hair follicles from newborn and adult rodent tissues (Peier et al. 2002). These include the basal keratinocytes and some of the more differentiated suprabasal layers of the epidermis (Peier et al. 2002). The expression decreases in the surface keratinized layer and becomes sparse in the stratum corneum, the outermost and completely keratinized layer of the epithelium (Xu et al. 2006). TRPV3 immunoreactivity is also rich in the epithelial layers of the dorsal tongue and palate, including the epithelia of filiform papillae as well as in the epithelia surrounding taste buds of circumvallate papillae, but with little or no staining in the lamina propria (Xu et al. 2006). In mouse nose, TRPV3 immunoreactivity is high in the epithelial lining of nasal cavities (Ahmed et al. 2009; Xu et al. 2006), with similar expression levels across all cells in the olfactory epithelium (Ahmed et al. 2009; Xu et al. 2006). In the respiratory epithelium, TRPV3 was observed in both the epithelial layer and the lamina propria (Ahmed et al. 2009). Furthermore, TRPV3 protein expression has been shown in superficial epithelial cells in mouse distal colon (Ueda et al. 2009).

For sensory nerves, although TRPV3 immunoreactivity has been shown in some small diameter neurons (mostly $\leq$50 μm in diameter) of human DRG sections, which coexpress TRPV1 (Smith et al. 2002), to date no TRPV3-like activity has been detected from DRG neurons.

3 TRPV3 Protein and Structure

The complete open reading frame of TRPV3 for either human or mouse is 2,373 bp, coding for a polypeptide of 791 amino acids. The original reports that described human TRPV3 functions used the cDNA clones that encoded the Ala-760 deleted version of 790 amino acids (Smith et al. 2002; Xu et al. 2002). Human TRPV3 protein is 38 %, 38 %, 32 %, 30 %, and 12 % identical to that of TRPV1, TRPV4, TRPV2, TRPV5 or TRPV6, and TRPM8, respectively (Smith et al. 2002; Xu et al. 2002). Mouse TRPV3 protein has 43 % identity to TRPV1 and TRPV4, 41 % to TRPV2, and about 20 % to TRPV5 and TRPV6. Like other TRP channels, the predicted protein structure for TRPV3 encompasses cytoplasmic amino and carboxyl termini, ankyrin repeats and coiled-coil domains at the amino terminus, six transmembrane segments, and a reentrant pore loop, along with several putative phosphorylation sites (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002).

The N-terminal ankyrin repeats are thought to be involved in protein-protein interactions. Analyses of the primary amino acid sequences only predicted three to four ankyrin repeats for TRPV3. However, based on the crystal structures of ankyrin repeat domains of other TRPVs (McCleverty et al. 2006; Lishko et al. 2007; Inada et al. 2012), TRPV3 is believed to have six ankyrin repeats, despite an insertion in repeat 1 and two short deletions in repeats 4 and 5, as compared to the ankyrin repeat domains of TRPV1 and TRPV4 (Phelps et al. 2010). Like that of TRPV1 and TRPV4, the ankyrin repeat domain of TRPV3 binds ATP and calmodulin (CaM), although the functional consequences of ATP and CaM binding to TRPV3 appear to be different from that to TRPV1 and TRPV4 (Phelps et al. 2010).

4 Interacting Proteins

Unlike some other TRP channels, there have been only limited studies on TRPV3 interacting proteins. Well-documented interaction partners include TRPV1 (Smith et al. 2002; Cheng et al. 2012), CaM (Xiao et al. 2008a; Phelps et al. 2010), A-kinase anchor protein (AKAP)-5 (AKAP79/150) (Zhang et al. 2008), and epidermal growth factor receptor (EGFR) (Cheng et al. 2010) (see http://www.trpchannel.org/summaries/TRPV3 for a summary and links to some original studies).

It was brought up very early on that TRPV1 and TRPV3 might form heterotetrameric channels with properties unique from homomeric channels formed by each subtype alone (Smith et al. 2002). Native TRPV1 and TRPV3 proteins were shown to coexpress in a fraction of human DRG neurons by immunocytochemistry in serial tissue sections, and reciprocal co-immunoprecipitation experiments were performed in HEK293 cells overexpressing TRPV1 and TRPV3 to demonstrate physical association between the two proteins. It was also shown that coexpression of TRPV3 enhanced capsaicin- and proton-evoked intracellular Ca^{2+} concentration rise as compared to expression of TRPV1 alone (Smith et al. 2002). However,

another group challenged the close association between TRPV1 and TRPV3 by showing poor colocalization, low fluorescence resonance energy transfer, and lack of or very weak co-immunoprecipitation between the two proteins heterologously expressed in HEK293 cells (Hellwig et al. 2005). Despite these, the more recent data show that TRPV1 and TRPV3 do form functional heteromeric channels with distinct temperature sensitivity, heat activation kinetics, and response patterns to repeated heating. The TRPV1–TRPV3 heteromers formed by expressing a concatemeric construct with TRPV3 fused to the C-terminus of TRPV1 displayed different ligand sensitivities from TRPV1 homomers and had an intermediate single channel conductance as compared to TRPV1 and TRPV3 homomers (Cheng et al. 2012). More importantly, coexpression of a TRPV3 mutant (D641N at the outer mouth of the conducting pore) with wild-type TRPV1 markedly reduced the sensitivity of capsaicin-elicited response to ruthenium red by about 20-fold (Cheng et al. 2012). In this case, the tetrameric channel must contain at least one wild-type TRPV1 subunit to gain capsaicin response and at least one $TRPV3^{D641N}$ mutant, which strongly lowers its affinity to ruthenium red (Chung et al. 2005) in order to have reduced sensitivity to the blocker. This elegantly designed experiment, therefore, clearly demonstrated that heterologously coexpressed TRPV1 and $TRPV3^{D641N}$ must share the same pore (Cheng et al. 2012).

Calmodulin (CaM) has been shown to directly bind to TRPV3 N-terminus in a Ca^{2+}-dependent manner and inhibit channel activation, which appeared typically by slowing down channel sensitization to repeated stimulation (Xiao et al. 2008a; Phelps et al. 2010). However, the CaM-binding sites determined by the two studies are different. In one case, the critical binding site was mapped using an in vitro pull-down assay to residues Ala-108 to Gly-130, which encompasses the beginning of the first ankyrin repeat (starting from Lys-117) (Xiao et al. 2008a), while in the second case, key residues in the second ankyrin repeat, Lys-169 and Lys-174, were tested because of homology with TRPV1 and shown to be critical for Ca^{2+}-CaM binding and channel regulation (Phelps et al. 2010). Interestingly, the same lysine residues are also required for the binding and channel inhibition by ATP (Phelps et al. 2010). However, although the CaM- and ATP-binding sites are conserved in TRPV1 and TRPV3, functional effects appear opposite (see later and also Nilius et al. 2014).

TRPV3 has also been shown to be associated with AKAP79/150 by co-immunoprecipitation. However, this association is much weaker than that between TRPV1 and AKAP79/150 (Zhang et al. 2008). The functional effect of this association is not known, but could facilitate localized regulation as in the case of TRPV1 and AKAP79/150 interaction (Efendiev et al. 2013).

The presence of EGFR and TRPV3 in the same signaling complex was demonstrated by co-immunoprecipitation in both heterologous and native systems, in support of a functional interplay between the two (Cheng et al. 2010). In this case, stimulation of EGFR increases TRPV3 channel activity and in turn enhances the release of transforming growth factor-α (TGF-α) and/or other EGFR ligands, promoting transglutaminase activities, a process critical for hair morphogenesis and skin barrier formation. The close physical association is also thought to facilitate

tyrosine phosphorylation of TRPV3 in response to EGFR activation by TGF-α (Cheng et al. 2010).

5 Functional Regulation, Permeation, and Gating

5.1 Activation

5.1.1 Temperature

TRPV3 belongs to the warm-sensitive TRP channels with reported temperature thresholds of activation that ranged from 31 to 39 °C (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002) and no saturation at up to 60 °C (Yao et al. 2011), indicative of activities from innocuous warm to noxious hot temperatures. The temperature sensitivity is affected by voltage as activation was detected at positive potentials at as low as 22.6 ± 0.1 °C (Xu et al. 2002). The rate and direction of temperature changes also profoundly affect the level of TRPV3 activation, with faster temperature rise being more effective and giving larger currents than slower temperature rise and even small temperature drops causing far more rapid deactivation than the progressive activation evoked by temperature rises (Xu et al. 2002). Furthermore, the unitary single channel conductance of TRPV3 increases with the temperature rise (Chung et al. 2004a; Yao et al. 2009). The slope conductance at negative potentials increased from 201 pS at 25 °C to 337 pS at 39 °C; the conductance at positive potentials was ~75 % of that at negative potentials and similarly affected by the temperature rise (Chung et al. 2004a). The unitary conductance of TRPV3 is by far the largest among TRPV channels.

In most cases, the heat-evoked TRPV3 activation is rather weak, as indicated by the strong outward rectification of the current–voltage (I–V) relationships and large fluctuations of the heat-evoked whole-cell currents (Peier et al. 2002; Smith et al. 2002; Xu et al. 2002; Chung et al. 2004b). This might due largely to the relatively low stimulating temperatures (40–45 °C) applied and the slow rate of temperature rise of the heating apparatus. With the application of superfast temperature jumps using the infrared diode laser, pronounced TRPV3 whole-cell current was only evoked at >54 °C (Yao et al. 2011). Additionally, TRPV3 whole-cell currents become progressively larger with repeated heat stimulation (Xu et al. 2002; Chung et al. 2004b). Sensitization of TRPV3 to repetitive stimulation has been considered a unique feature that distinguishes this channel from other TRPVs (Chung et al. 2004b; Xiao et al. 2008a; Phelps et al. 2010).

Five single point mutations (N643S, I644S, N647Y, L657I, and Y661C) have been shown to specifically abolish heat activation of TRPV3 without affecting activation by chemical activators or modulation by voltage. All five mutations are located in the putative sixth transmembrane helix and the adjacent extracellular loop in the pore region of mouse TRPV3 (Grandl et al. 2008). More recently, two nearby residues, I652 and L655, when mutated to cysteines, were shown to undergo a temperature-dependent change in accessibility by cysteine reactive compounds (Kim et al. 2013), demonstrating that heat activation of TRPV3 involves a structural

change in the pore region. However, studies of TRPV chimeras also suggested that the membrane proximal region between ankyrin repeats and the first transmembrane segment is crucial for temperature sensing of TRPV channels (Yao et al. 2011).

5.1.2 Chemical Ligands

A number of chemical activators for TRPV3 have been described, but all of them are nonspecific drugs and have to be used at relatively high concentrations. Among them, 2-aminoethoxydiphenyl borate (2-APB) is the most frequently used (Hu et al. 2004; Chung et al. 2004a). 2-APB was first reported as an inhibitor of IP_3 receptors (Maruyama et al. 1997) and then shown to also block store-operated Ca^{2+} channels and a number of other channels and transporters (Bootman et al. 2002). It has been, at one time, considered a general inhibitor of TRP channels (Clapham et al. 2001). However, with the report that 2-APB activates TRPV1, V2, and V3 (Hu et al. 2004), as well as Orai1, TRPA1, and TRPM6 (Prakriya and Lewis 2001; Hinman et al. 2006; Li et al. 2006), the complexity of 2-APB action on ion channels has been clearly recognized. Indeed, at higher concentrations (>300 μM), 2-APB could also cause an inhibition of TRPV3 (Chung et al. 2005). A small number of 2-APB analogues have also been shown to either activate or inhibit TRPV3 (Chung et al. 2005).

2-APB also sensitizes TRPV3 to activation by heat, even at subthreshold concentrations (Chung et al. 2004a; Hu et al. 2004). In primary mouse keratinocytes, whereas heat alone activated TRPV3-like currents only in a small proportion of cells, in the presence of 2-APB, heat-induced activation was observed in the majority of these cells (Chung et al. 2004a). Synergy between chemical ligand and heat as well as that between structurally different chemical ligands is commonly observed for thermosensitive TRPV channels. This phenomenon has been described in detail and discussed in a recent review (Nilius et al. 2014).

Two amino acid residues (His-426 and Arg-696) have been reported to be specifically required for the 2-APB sensitivity of TRPV3 (Hu et al. 2009). The N-terminal mutation H426N in mouse, humans, dog, and frog TRPV3 largely reduced the sensitivity to 2-APB without altering that to heat and camphor, whereas the Asn to His substitution in chicken TRPV3 at the equivalent position (N427H) greatly enhanced the affinity to 2-APB by at least 25-fold. Different from the N-terminal site, the C-terminal mutation, R696K in the TRP box, displayed the loss of 2-APB sensitivity only in the presence of extracellular Ca^{2+}, suggesting a strong enhancement of Ca^{2+}-dependent desensitization. The R696K mutation also affected the activation by heat but not that by camphor (Hu et al. 2009).

Other reported TRPV3 activators include camphor (Moqrich et al. 2005; Xu et al. 2005), carvacrol, eugenol and thymol (Xu et al. 2006), and citral (Stotz et al. 2008). These plant extracts are commonly known as flavoring ingredient, skin sensitizers, and/or allergens (Corsini et al. 1998; Scholl and Jensen-Jarolim 2004). Camphor is also an active ingredient of Tiger Balm, a cooling ointment used for pain relief and insect bites, and other traditional remedies because of the moderate local anesthetic, anti-itching, and antimicrobial actions. Ironically, the

other main ingredient of the ointment, menthol, also activates TRPV3 (Macpherson et al. 2006). Menthol is best known for its activation of the cool sensor TRPM8 (McKemy et al. 2002) and also the cold sensor TRPA1 (Karashima et al. 2007). Another TRPA1 agonist, cinnamaldehyde, also activates TRPV3 (Macpherson et al. 2006). The sensitivity of TRPV3 to these flavor enhancers, together with the finding of high expression of this channel in the tongue and oral and nasal epithelia, strongly supports a function of TRPV3 in chemesthesis (Xu et al. 2006). Notably, these substances also activate other TRP channels, such as TRPV1 and TRPA1 (Xu et al. 2005, 2006; Stotz et al. 2008). Incensole acetate, a component of Boswellia resin of which the incense has strong psychoactive effects, has been shown to directly activate TRPV3 and cause anxiolytic-like and antidepressive-like behaviors in mice, which were absent after deletion of the TRPV3 gene (Moussaieff et al. 2008). Activation of TRPV3 by incensole acetate in the mouse brain also contributed to the protective effect of this compound on ischemic brain injury (Moussaieff et al. 2012).

Several cannabinoids, including cannabidiol and tetrahydrocannabivarin, have been shown to activate rat TRPV3 stably expressed in HEK293 cells in the Ca^{2+} assay (De Petrocellis et al. 2012). These results require further confirmation by electrophysiology.

5.1.3 Endogenous Activator and Modulators

While the endogenous TRPV3 activators remain largely undefined, farnesyl pyrophosphate (FPP), an intermediate in the biosynthetic pathway of steroid hormones, has been shown to potently, with an EC_{50} of ~130 nM, and specifically activate TRPV3, but not other thermosensitive TRP channels (Bang et al. 2010). Interestingly, its upstream metabolite, isopentenyl pyrophosphate, was later shown to inhibit TRPV3 and TRPA1 by the same group of investigators (Bang et al. 2011).

Although not directly activated by unsaturated fatty acids, the 2-APB-evoked TRPV3 activity was strongly potentiated by these lipids, especially the polyunsaturated fatty acids, including arachidonic acid (20:4 n6), 5,8,11-eicosatrienoic acid (20:3 n9), 5,8,11,14,17-eicosapentaenoic acid (20:5 n3), and linolenic acids (18:3 n3 and 18:3 n6). This action of fatty acids was not dependent on oxidation or metabolism and was mimicked by non-metabolizable analogues of arachidonic acid (Hu et al. 2006). The potentiation by fatty acids is not mediated through protein kinase C (PKC) even though activation of PKC also causes an increase of TRPV3 activity (Hu et al. 2006).

Unlike TRPV1, TRPV3 is not activated by extracellular protons (Smith et al. 2002; Ryu et al. 2007). However, it was recently shown that TRPV3 can be activated by intracellular acidification resulting from extracellularly applied glycolic acid as well as direct application of the low pH solution to the cytoplasmic side of excised inside-out patches (Cao et al. 2012). A limited survey of protonable residues at the cytoplasmic side revealed that H426, which had been shown to be critical for TRPV3 activation by 2-APB (Hu et al. 2009), might also be involved in sensing intracellular protons. Nonetheless, the H426N mutation only displayed a partial loss of the proton stimulation, indicating that additional proton sensing site

(s) could still exist. Furthermore, the same study also revealed an inhibitory effect of acid on TRPV3, but the mechanism remained unexplored (Cao et al. 2012).

It has also suggested that TRPV3 may be directly activated by nitric oxide (NO) via cysteine S-nitrosylation. NO donors caused a slow increase in intracellular Ca^{2+} level in HEK293 cells that expressed TRPV3 (Yoshida et al. 2006). However, this result needs confirmation by electrophysiological studies.

5.2 Permeation

The relative permeability of human TRPV3 has been determined to be $Ca^{2+} > Na^{+} = K^{+} = Cs^{+}$, consistent with it being a Ca^{2+}-permeable nonselective cation channel (Xu et al. 2002). Based on reversal potential measurement, the permeability ratio of Ca^{2+}/Na^{+} for TRPV3 was estimated to >10 (Gees et al. 2010). Fractional Ca^{2+} current measurements also gave an estimate of >15 % for TRPV3 (L. Zheng, M. X. Zhu, and Z. Zhou, unpublished result). It is likely that similar to TRPV1, TRPV3 undergoes pore dilation during persistent and/or repetitive stimulation (Chung et al 2008), as reflected by changes in permeability to NMDG and inhibition by extracellular ruthenium red and Ca^{2+} (Chung et al. 2005; Xiao et al. 2008a). As described later, Mg^{2+}, at millimolar concentrations, decreases the single channel conductance by about 60 % (Luo et al. 2012).

5.3 Regulation/Gating

5.3.1 Ca^{2+} and Voltage Dependence

Unlike TRPV1 and TRPM8, when unstimulated, the expressed TRPV3 channels do not show voltage-dependent activation at high positive potentials. Repetitive stimulations by 2-APB or other stimulus gradually shift the voltage dependence to less positive potentials for more than 100 mV (Xiao et al. 2008a). However, the voltage dependence of TRPV3, shown as doubly rectifying I-V relationship, appears to largely depend on Ca^{2+} binding to key residues at the pore. One of these is Asp-641, which is equivalent to Asp-646 of rat TRPV1 (Asp-647 for mouse TRPV1) and is critical for the blocking effect of ruthenium red, a hexavalent cationic dye, on these channels (Garcia-Martinez et al. 2000; Voets et al. 2002; Xiao et al. 2008a). Upon complete removal of extracellular Ca^{2+}, or mutation of Asp-641 to an Asn (D641N) and extracellular Ca^{2+} maintained at <10 μM, the 2-APB-evoked TRPV3 current became linear. Measurement of the Ca^{2+}-dependent inhibition of TRPV3 current revealed two affinity states, with the high affinity in the range of 1–2 μM and low affinity being around 1–3 mM. The D641N mutant only exhibited the low-affinity but not high-affinity inhibition by extracellular Ca^{2+}, suggesting that Asp-641 is critical for the high-affinity inhibition of TRPV3 by Ca^{2+} (Xiao et al. 2008a).

5.3.2 Ca^{2+}-CaM

TRPV3 is unique among TRP channels in that its activity is sensitized by repetitive stimulations (Xu et al. 2002; Chung et al. 2004b; Xiao et al. 2008a; Phelps et al. 2010). The sensitization is in part due to a decrease in high-affinity inhibition by extracellular Ca^{2+}. The IC_{50} of Ca^{2+} for the high-affinity inhibition changed from ~1.5 μM to ~9 μM upon repetitive stimulations by 2APB (Xiao et al. 2008a). On the other hand, the sensitization of TRPV3 is also dependent on the strength of intracellular Ca^{2+} buffer. When dialyzed intracellularly with 10 mM BAPTA, instead of 1 or 10 mM EGTA, TRPV3 appeared to be pre-sensitized, exhibiting a relatively large response even to the first application of 2-APB and little sensitization to subsequent drug applications (Xiao et al. 2008a). BAPTA is a much faster Ca^{2+} chelator than EGTA and therefore can prevent Ca^{2+} fluctuations, which commonly occur with the opening of Ca^{2+} permeable channels. Consistent with the cytoplasmic side of Ca^{2+} action, with the free Ca^{2+} concentration clamped to 1.6 μM by 10 mM BAPTA in the patch pipette solution, the initial response to 2-APB was dramatically reduced and sensitization to repetitive stimulation reemerged. More importantly, the small response to initial stimulation and sensitization to subsequent repetitive stimulations were largely abolished by the treatment with CaM inhibitors, ophiobolin A, W-7, and calmidazolium, suggesting a role for Ca^{2+}-CaM in maintaining the low activity of TRPV3, and such an inhibitory effect was drastically reduced upon repetitive stimulations (Xiao et al. 2008a). A Ca^{2+}-CaM-binding site has been identified at the N-terminus of the TRPV3 protein (amino acids 108–130), which upon mutation abolished the difference in the sensitization processes between EGTA- and BAPTA-based internal solutions (Xiao et al. 2008a).

More recently, an alternative explanation for the effect of BAPTA on TRPV3 sensitization has been suggested. In this case, BAPTA was thought to potentiate TRPV3 function independently of its binding to Ca^{2+} (Liu et al. 2011). However, such an action of BAPTA could not explain the inhibitory effect of 1.6 μM free Ca^{2+} clamped by 10 mM BAPTA and its reversal by CaM inhibitors (Xiao et al. 2008a).

5.3.3 ATP

Based on findings from ankyrin repeat domain of TRPV1 (Lishko et al. 2007), two conserved lysine residues, Lys-169 and Lys-174, were tested and shown to be critical for interaction of TRPV3 ankyrin repeat domain to ATP and Ca^{2+}-CaM (Phelps et al. 2010). The binding by ATP reduced the sensitivity of the channel to TRPV3 agonists and abolished the sensitization of TRPV3 to repeated stimulation by 2-APB (Phelps et al. 2010). This effect is opposite to the effect of ATP on TRPV1, which attenuated desensitization (Lishko et al. 2007). In the same study, depleting CaM from TRPV3-expressing cells using an antibody against CaM strongly enhanced the response to first application of 2-APB and abolished further sensitization by repetitive stimulations, an effect that was also seen by substituting Lys-169 with an alanine (Phelps et al. 2010). The net effect of depleting CaM appears to be similar on TRPV1 and TRPV3, rendering the channels incapable of either sensitization or desensitization to repeated activation (Lishko et al. 2007;

Phelps et al. 2010). It was concluded that these lysine residues in the ankyrin repeat domain were essential for TRPV3 channel inhibition by both ATP and Ca^{2+}-CaM. However, it cannot be excluded that, perhaps, ATP binding to this site has a dominant effect on TRPV3 gating, such that in the absence of ATP binding, CaM no longer inhibits the channel function, even if the critical site of Ca^{2+}-CaM regulation lies somewhere else, e.g., that identified by Xiao et al. (2008a).

5.3.4 Mg^{2+}

Mg^{2+} inhibits TRPV3 function from both intracellular and extracellular sides of the plasma membrane by reducing the unitary conductance without affecting open probability of single channels. It was determined that Asp-641 constituted the extracellular site of Mg^{2+} action, whereas two glutamates at the beginning of the C-terminus immediately connected with the S6 transmembrane segment mediated the inhibition by intracellular Mg^{2+} (Luo et al. 2012).

5.3.5 $PI(4,5)P_2$

Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) is a universal modulator of ion channels on the plasma membrane. The phospholipid was reported to inhibit TRPV3 channel function in a manner that depended on basic residues at the channel's TRP domain Arg-696 and Lys-705 (Doerner et al. 2011). Breakdown of $PI(4,5)P_2$ by the activation of receptors that stimulate phospholipase C caused a large shift (~60–100 mV) of the activation voltage of TRPV3 to more physiological membrane potentials (Doerner et al. 2011), which helps explain the potentiating effect on TRPV3 of activating $G_{q/11}$-mediated signaling pathways (Xu et al. 2006; Doerner et al. 2011).

6 Physiological Functions in Native Cells, Organs, and Organ Systems

The most frequently discussed and examined functions of TRPV3 are related to its role in temperature sensing and its pronounced distribution in skin keratinocytes as well as oral and nasal epithelia. Because TRPV3 is activated at warm to noxious heat temperatures and its close relationship with TRPV1, earlier studies on physiological implications of TRPV3 had focused nearly extensively on temperature sensing and nociception. However, unlike TRPV1, the expression levels of TRPV3 in sensory neurons, at least in rodents, are not very high (Peier et al. 2002). Instead temperature sensing was thought to occur via TRPV3 expressed in skin keratinocytes, which then release substances to activate sensory nerves that innervate the skin. The substances have been shown to include prostaglandin E_2 (Huang et al. 2008) and ATP (Mandadi et al. 2009). In cultured keratinocytes, stimulation of TRPV3 with eugenol increased the release of cytokines, such as interleukin-1α (Xu et al. 2006). Likewise, treatment of normal human epidermal keratinocytes with TRPV3 agonists, 2-APB, and carvacrol also enhanced the release of TGF-α (Cheng et al. 2010). Furthermore, TRPV3 regulates

intracutaneous NO synthesis, through nitrite reduction, independent of NO synthase. This function was shown to be important for wound healing and thermosensory behaviors of experimental animals (Miyamoto et al. 2011). More details on the function of TRPV3 in skin physiology can be found in excellent reviews by Oláh et al. (2012) and Nilius and Bíró (2013).

The role of TRPV3 in sensing innocuous and noxious heat has been investigated using TRPV3 knockout mice (see below) as well as the TRPV3 activator, FPP (Bang et al. 2010). When injected intradermally into mouse hind paws, FPP increased the heat sensitivity in a TRPV3-dependent manner, and furthermore, this treatment enhanced the nocifensive behaviors under inflamed conditions (Bang et al. 2010). These effects were abolished by 17(R)-resolvin D1, which was shown to inhibit TRPV3 (Bang et al. 2012). As a TRPV3 antagonist, 17(R)-resolvin D1 also reduced the heat sensitivity under inflamed but not normal conditions (Bang et al. 2012).

Based on the stimulatory action of incensole acetate on TRPV3, the channel is proposed to exert psychoactive effects and a neuroprotective function in the brain (Moussaieff et al. 2008, 2012). Studying the effect of carvacrol also suggested a role of TRPV3 in endothelium-dependent vasodilation (Earley et al. 2010).

7 Lessons from Knockouts

Much of what we know about the physiological functions of TRPV3 came out from studies using TRPV3 knockout mice, with thermosensation being the major focus of earlier studies, and then broader aspects of physiological functions followed. The first report on the TRPV3 knockout mice showed strong deficits in responses to innocuous and noxious heat but not in other sensory modalities (Moqrich et al. 2005). Heat-induced ATP release from keratinocytes was also found to be defective in TRPV3 knockout mice (Mandadi et al. 2009). However, a later study showed that the effect of TRPV3 knockout on thermosensing was dependent on the background strain used and neither TRPV3 nor TRPV4 appeared to be involved in noxious heat sensation (Huang et al. 2011). Nevertheless, in the study that linked nitrite-dependent NO synthesis to TRPV3, both nitrite and TRPV3 were shown to be required for normal innocuous thermosensory behaviors, which was diminished in the TRPV3 knockout mice (Miyamoto et al. 2011). The NO released from TRPV3-expressing keratinocytes promotes keratinocyte migration and wound healing and, together with other factors released because of TRPV3 activity, such as ATP and prostaglandin E_2, can induce pain, likely through activation of TRPV1 and TRPA1 in the cutaneous sensory termini. It is possible that functional redundancy among TRPV channels may exclude the possibility of detecting the defect in thermosensation in the TRPV3 knockout mice (Nilius and Bíró 2013).

The role of TRPV3 in pruritus (itch) has also been studied using TRPV3 knockout mice (Yamamoto-Kasai et al. 2012). Daily application of an acetone/ether/water mixture to the rostral back of mice resulted in spontaneous scratching in a histamine-independent manner. This treatment caused dry skin in both wild-type

and TRPV3 knockout mice, but only the wild-type mice exhibited an increase in spontaneous scratching directed toward the treated area, suggesting an essential role for TRPV3 in itch sensation with dry skin in mice (Yamamoto-Kasai et al. 2012).

The TRPV3-deficient mice also exhibit curly whiskers and wavy hair (Cheng et al. 2010). The curly whiskers were evident at birth, allowing newborn TRPV3 knockout mice to be easily identified from the whisker morphology. Importantly, curly whiskers also occur in mice with conditional TRPV3 knockouts specifically in keratinocytes. Wavy hair also grows throughout the dorsal and ventral coat fur, as well as the tail in both conventional and conditioned TRPV3 knockout mice beginning at 1 week after birth. Mechanistically, it was shown that TRPV3 forms a signaling complex with TGF-α and EGFR and functional interactions among them regulate transglutaminases, a family of Ca^{2+}-dependent cross-linking enzymes, which in turn affect hair morphogenesis and skin barrier formation (Cheng et al. 2010). Activation of EGFR enhances TRPV3 activity, which results in production, shedding, and/or release of TGF-α and other EGFR ligands, causing further increase of the EGFR function. The lack of TRPV3 is thought to impair this signaling pathway and reduce transglutaminase activity, leading to defects in epidermal barrier structure (Cheng et al. 2010).

The use of TRPV3-deficient mice also revealed its function in the brain. The psychoactive effects induced by incensole acetate, including anxiolytic-like and antidepressive-like behaviors, are absent in the TRPV3 knockout mice (Moussaieff et al. 2008). Intraperitoneal injection of incensole acetate in mice also protected against brain damage induced by ischemia/reperfusion. This effect was partially attenuated by the knockout of TRPV3 gene (Moussaieff et al. 2012). More recently, it was shown that TRPV3 knockout mice failed to develop long-term depression at excitatory synapses on s. radiatum hippocampal interneurons. As a result, the excess release of GABA attenuated tetanus-induced long-term potentiation in CA1 pyramidal cells (Brown et al. 2013). These effects of TRPV3 knockout are similar to that of TRPV1 gene deletion, which could suggest that TRPV1 and TRPV3 function as obligate heteromultimers in hippocampal interneurons, although several other possibilities are also suggested (Brown et al. 2013).

8 Role in Hereditary and Acquired Diseases

The most striking discovery on TRPV3 function in diseases emerged from the studies of two hairless strains of rodents, which were then followed by, incidentally, demonstration of exactly the same mutations in a rare hereditary human disease named Olmsted syndrome. Mapping the genomic mutation associated with the autosomal dominant hairless phenotype of the DS-Nh (no-hair) mice revealed a substitution of Gly-573 by a serine (G573S) in the S4-S5 linker of the TRPV3 (Asakawa et al. 2006). Strikingly, the WBN/Kob-Ht rats, which also show spontaneous hair loss in an autosomal dominant fashion, contain a mutation at exactly the same site, but with the substitution by a cysteine (G573C) (Asakawa et al. 2006).

Even more amazingly, when genetic mutations were identified for six unrelated patients with Olmsted syndrome in China, five carried either the G573S or G573C mutation and one had a mutation at the TRP domain, W692G (Lin et al. 2012). The G573S mutation was also found in an Indian patient (Lai-Cheong et al. 2012) and a new G573A mutant has also been reported for a patient from Belgium (Danso-Abeam et al. 2013). Thus, the apparent sporadic cases of the Olmsted syndrome often share mutations at a common amino acid of TRPV3 (Gly-573), which also occurred independently in a mouse and a rat strain with autosomal dominant hairless phenotypes. It was shown that both G573S and G573C mutations, as well as the W692G substitution, cause constitutive activation of TRPV3 (Xiao et al. 2008b; Lin et al. 2012). The enhanced basal activity was detectable with coexpression of the mutant construct with the wild-type TRPV3, suggesting that heteromers between the mutant and wild-type TRPV3 are still constitutively active. This explains the autosomal dominant inheritance of the DS-Nh mice and WBN/Kob-Ht rats (Asakawa et al. 2006).

The rarity and lack of a strong apparent inheritance of Olmsted syndrome in humans are most likely because of the severe skin defects exhibited by affected individuals. These include hair loss, skin inflammation, dermatitis, and keratoderma of palms and soles, with flexion deformity of the digits. As such, the patient may not find a mate to reproduce. The gain-of-function TRPV3 mutants resulted in hair loss in both rodent species, suggesting a pivotal role of TRPV3 in hair growth/hair cycle (Asakawa et al. 2006). This is consistent with the enrichment of TRPV3 mRNA and protein keratinocytes at and around hair follicles (Xu et al. 2002; Peier et al. 2002). TRPV3 function has also been demonstrated in cultured human hair follicle-derived outer root sheath keratinocytes, and inhibition of such function and the accompanied intracellular Ca^{2+} concentration rise decreased cell growth and triggered cell death (Borbíró et al. 2011). Olmsted syndrome patients also exhibit profuse alopecia. Therefore, TRPV3 exerts a negative regulatory role in the hair growth.

The TRPV3 mutations also cause skin inflammation and dermatitis. In addition, the mutant animals exhibit excessive scratching behavior, implicating a role of TRPV3 in pruritus (Asakawa et al. 2006; Imura et al. 2007; Yoshioka et al. 2009), which mirrors the results from using the TRPV3 knockout mice (Yamamoto-Kasai et al. 2012). Consistent with findings from rodent models, Olmsted syndrome is also known as “mutilating palmoplantar keratoderma with periorificial keratotic plaques” or “polykeratosis of Touraine.” Patients show periorificial, keratotic plaques, bilateral palmoplantar and periorificial keratodermas, as well as severe itching (Lin et al. 2012). Therefore, the gain-of-function mutants of TRPV3 result in multiple problems of skin health, arguing for the potential value of TRPV3 drugs in the treatment of skin disease, hair loss, and alopecia (Oláh et al. 2012; Nilius and Bíró 2013).

In addition to skin disorders associated with the gain-of-function TRPV3 mutations, TRPV3 has also been linked to gastrointestinal disease. In a study analyzing association between 392 single-nucleotide polymorphisms in 43 fatty acid metabolism-related genes and the risk for colorectal cancer involving a cohort

of 1,225 patients with cancer and 2,032 non-cancer controls, TRPV3 was shown to be associated with a high risk for development of colorectal cancer (Hoeft et al. 2010). In a separate study associating genetic variations to congenital hyperinsulinism, the L776F substitution of TRPV3 was shown to be a potential causative change that might lead to dysregulation of insulin secretion (Proverbio et al. 2013). These findings are consistent with the broad expression of TRPV3 in gastrointestinal epithelium. However, the physiological significance of TRPV3 in nutrient sensing and digestion and its role in gastrointestinal disorders remain largely unexplored (Nilius and Bíró 2013).

Concluding Remarks

Although being less studied as compared to its closest relatives, TRPV1 and TRPV4, TRPV3 has turned out to be a very important channel for skin health. A properly balanced function of TRPV3 appears to be critical for skin barrier formation, hair growth, wound healing, keratinocyte maturation, and cutaneous pain, itch, and temperature sensations. Naturally occurring genetic mutations with augmented TRPV3 function lead to hair loss, skin inflammation, severe itchiness, and dermatitis, suggesting a potential use of TRPV3 blockers in the treatment of skin disease.

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The TRPV4 Channel

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Abstract

The widely distributed TRPV4 cationic channel participates in the transduction of both physical (osmotic, mechanical, and heat) and chemical (endogenous, plant-derived, and synthetic ligands) stimuli. In this chapter we will review TRPV4 expression, biophysics, structure, regulation, and interacting partners as well as physiological and pathological insights obtained in TRPV4 animal models and human genetic studies.

Keywords

TRPV4 • genetics • knock-out • pathophysiology • biophysics

1 Gene

The TRPV4 channel was first described in 2000 (Liedtke et al. 2000; Strotmann et al. 2000; Wissenbach et al. 2000) and received several different names before the current nomenclature was accepted: OTRPC4 (osmosensitive transient receptor potential channel), VR-OAC (vanilloid receptor-related osmotically activated channel), VRL-2 (vanilloid receptor-like), and TRP-12. The human TRPV4 gene is found in chromosome 12q23-q24.1 and presents 15 exons. Five splice variants (TRPV4-A-E) have been identified. Variants B, C, and E involve deletions in the N-terminal ankyrin repeat domains (ANK) that result in protein retention in the endoplasmic reticulum, defective oligomerization, and lack of channel activity (Arniges et al. 2006; Vazquez and Valverde 2006).

Compared to the vast knowledge obtained about TRPV4 channel regulation, little is known about the control of TRPV4 transcription. Progesterone receptor mediates repression of TRPV4 transcription in epithelial and vascular smooth muscle cells (Jung et al. 2009). Downregulation of TRPV4 expression by microRNA 203 in condylar cartilage of the temporomandibular joint (Hu et al. 2012) and by probiotic bacteria strains in the colon (Distrutti et al. 2013) has also been reported. Inflammatory signals such as interleukin 1β and interleukin 17 increase

TRPV4 mRNA levels in dorsal root ganglia (DRG) neurons (Segond von Banchet et al. 2013), and nerve growth factor (NGF) increases TRPV4 expression in the urothelium (Girard et al. 2013). Hypoxia/ischemia increases TRPV4 expression and function in astrocytes (Butenko et al. 2012) and in pulmonary arterial smooth muscle cells of mice exposed to chronic hypoxia-induced pulmonary hypertension (Xia et al. 2013).

2 Expression

TRPV4 is broadly expressed in heart, arteries, lung, skin, bone, brain, urinary bladder, kidney, intestine, liver, pancreas, and female reproductive tract [for a review see Everaerts et al. (2010a)]. TRPV4 is commonly found in the epithelial cells of the cornea (Mergler et al. 2010; Pan et al. 2008), bronchi (Fernandez-Fernandez et al. 2002, 2008; Li et al. 2011), trachea (Arniges et al. 2004; Lorenzo et al. 2008), intestine (d'Aldebert et al. 2011), urothelium (Everaerts et al. 2010b), larynx (Hamamoto et al. 2008), oviduct (Andrade et al. 2005), bile duct (Gradilone et al. 2007), epidermis (Sokabe et al. 2010), mammary gland (Jung et al. 2009), and endolymphatic sac (Kumagami et al. 2009). TRPV4 is also found in the endothelium (Watanabe et al. 2002b), smooth (Earley et al. 2005; Jia et al. 2004) and skeletal muscle (Kruger et al. 2008), sensorial and brain neurons (Alessandri-Haber et al. 2003; Li et al. 2013; Shibasaki et al. 2007), glia (Benfenati et al. 2007; Konno et al. 2012), immune cells (Kim et al. 2010), osteoclasts (Masuyama et al. 2008), osteoblasts and chondrocytes (Muramatsu et al. 2007), and pancreatic islets (Casas et al. 2008).

3 Protein

The TRPV4 protein consists of 871 amino acids (aa) with 6 transmembrane (TM) domains presenting both N- and C-terminal cytoplasmic tails (Fig. 1). The pore of the channel (aa 663–686) is found in the loop between TM5 and TM6. The 12 central amino acids of the pore are identical to those of TRPV1 and 2, the closest relatives of TRPV4 (Voets et al. 2002). Two key amino acids have been shown to regulate TRPV4 permeability: D672 and D682. Neutralization of both D672 and D682 greatly reduces permeability for calcium and rectification and increases monovalent permeation, suggesting that these two negatively charged residues are important for binding calcium ions inside the pore (Voets et al. 2002). D682 also participates in ruthenium red block. M680 residue strongly affects Ca^{2+} permeation; K675 does not contribute significantly to the properties of the pore. Glycosylation of N651 is involved in the trafficking of TRPV4 (Xu et al. 2006). Mutation of E797 renders the channel constitutively opened (Watanabe et al. 2003a).

The long N-terminal tail (aa 1–465) accounts for more than 50 % of total TRPV4 length and contains 6 ANK (Phelps et al. 2010) that participate in channel oligomerization (Arniges et al. 2006). The N-terminal tail plays a prominent role in channel regulation, having a phosphoinositide-binding site (PIBS, aa 121–125)

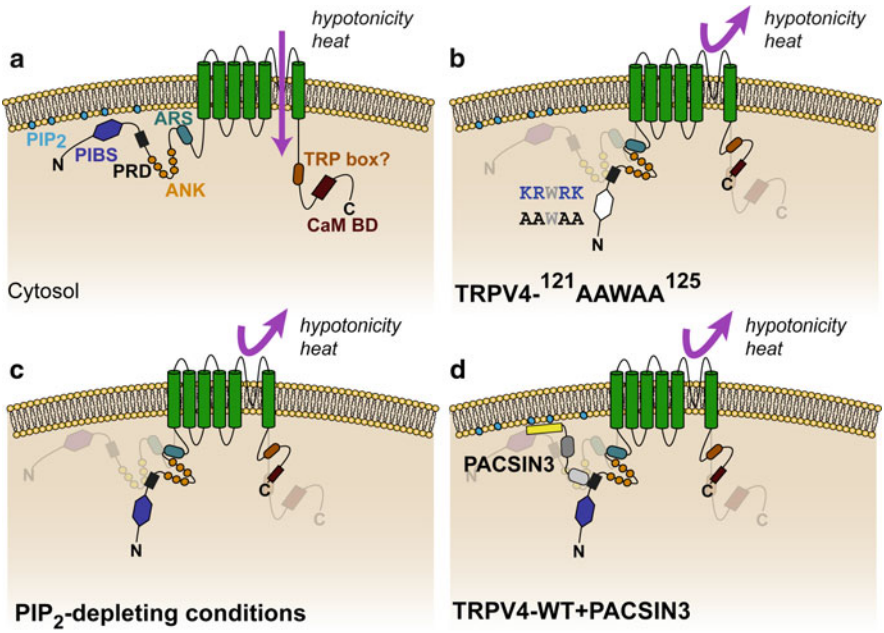

Fig. 1 Domain structure and PIP_2-dependent functional rearrangement of TRPV4. (**a**) Cartoon of a single TRPV4 protein in its expanded conformation due to interaction of the N-tail with PIP_2. Phosphoinositide-binding site (PIBS), proline-rich domain (PRD), ankyrin domains (ANK), arachidonate-like recognition sequence (ARS), the six transmembrane domains, a questioned TRP box, and the CaM-binding domain (CaM-BD). Intracellular tail rearranges into a more compacted form upon neutralization of positive charges in the PIBS (**b**), depleting PIP_2 from the plasma membrane (**c**), or coexpression of TRPV4 with PACSIN3 (**d**). The expanded conformation is required for TRPV4 response to hypotonic and heat stimuli

required for channel activation by physiological stimuli, hypotonicity and heat (Garcia-Elias et al. 2013); a proline-rich domain (PRD) (aa 132–144) used for binding of and regulation by kinase C and casein kinase substrate in neurons 3 (PACSIN3) (Cuajungco et al. 2006), and an arachidonate-like recognition sequence (ARS-L) (aa 402–408) (Nilius et al. 2003). In addition, complete deletion of PRD (Garcia-Elias et al. 2008), aa 1–130 or 100–130, renders the channel insensitive to all stimuli, including the synthetic activator 4α-phorbol 12,13 didecanoate (4α-PDD), suggesting an important role of the N-tail in the gating of TRPV4 (Fig. 2). The C-terminal tail presents a calmodulin-binding domain (CaM-BD) (812–831 aa) (Strotmann et al. 2003), an oligomerization domain (Becker et al. 2008), and a PDZ-like domain (Garcia-Elias et al. 2008; van de Graaf et al. 2006). The existence of a TRP box in the C-terminal tail has been proposed for TRPV1 (Garcia-Sanz et al. 2007) although its existence in TRPV4 has not been thoroughly studied. Heteromerization of TRPV4 with other TRP channels is discussed in Sect. 4.4.

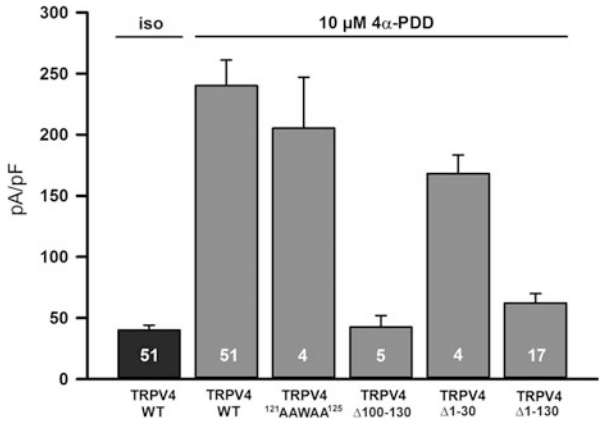

Fig. 2 Response to 4α-PDD of N-terminal truncations and mutations of TRPV4. Mean current responses to 4α-PDD (10 μM) stimulation in cells transfected with TRPV4-WT, TRPV4-^{121}AAWAA^{125}, TRPV4-Δ1-30, TRPV4-Δ1-130, and TRPV4-Δ100-130. The number of cells recorded is shown for each condition. *Iso* isotonic

4 Interacting and Regulatory Proteins

A detailed database of TRPV4 channel protein–protein interactions (Chun et al. 2014; Shin et al. 2012b) is available at http://trpchannel.org/summaries/TRPV4.

4.1 Proteins Modifying TRPV4 Location on the Plasma Membrane

In addition to being triggered by activating stimuli, TRPV4 channel activity in the plasma membrane is affected at several different levels: targeting of the channel protein to its final destination, posttranslational modification, and lysosomal degradation.

TRPV4 location on the plasma membrane and channel response to hypotonicity and warm temperature is regulated by PACSIN3 (Cuajungco et al. 2006; D'hoedt et al. 2008). PACSIN3 belongs to a family of three proteins with the Bin-amphiphysin-Rvs (BAR) domain required to penetrate and remodel the plasma membrane and to participate in endocytic processes, neurotransmission, and cell morphology and motility (Plomann et al. 2009). All members of the PACSIN family bind to the PRD of TRPV4 through their SRC homology 3 (SH3) domain; however, only PACSIN3 appears to regulate TRPV4.

A few more proteins affect the presence of TRPV4 at the plasma membrane. OS-9, a ubiquitous protein found in the cytoplasmic site of the endoplasmic reticulum (ER), plays a role in selecting substrates for degradation. It interacts

with TRPV4 monomers (aa 438–468 at the N-tail) retaining the monomers in the ER and reducing the amount of channel in the membrane, thereby protecting TRPV4 from polyubiquitination and premature proteasomal degradation (Wang et al. 2007). The ubiquitin ligase AIP4 binds TRPV4 (presumably to its N-tail) and promotes its endocytosis (Wegierski et al. 2006). Intimately linked to TRPV4 ubiquitination in vascular smooth muscle is the complex formed by the G protein-coupled angiotensin receptor, β-arrestin and TRPV4 (Shukla et al. 2010). In the absence of angiotensin, β-arrestin (an adaptor between AIP4 and TRPV4) does not bind TRPV4 and no AIP4-dependent internalization occurs. Another protein binding to and modulating TRPV4 presence and function at the plasma membrane is caveolin-1 (Saliez et al. 2008). TRPV4 location to lipid rafts containing caveolin-1 favors nitric oxide (NO) and endothelium-derived hyperpolarizing factor-dependent vasodilatation. Annexin 2A, a calcium-dependent membrane-binding protein that is linked to vesicular trafficking and endosome formation, also binds TRPV4 in dorsal root ganglia (Huai et al. 2012), although the functional relevance of this interaction is presently unknown.

4.2 Signaling Molecules

Early studies on TRPV4 reported its activation and/or modulation by phorbol esters and protein kinase C (PKC) (Watanabe et al. 2002a; Xu et al. 2003a) as well as by activators of protein kinase A (PKA) (Alessandri-Haber et al. 2006). Subsequent studies (Fan et al. 2009) identified the residues involved in PKA (S824)- and PKC (S162, T175, and S189)-mediated modulation of TRPV4 and the role of the A Kinase Anchoring Protein (AKAP79) in the optimization of TRPV4 phosphorylation by PKA and PKC.

TRPV4 regulation by tyrosine kinases is controversial. The proposed role of Y253 in the hypotonicity-mediated activation of TRPV4 (Xu et al. 2003b) was not observed by others (Vriens et al. 2004). Y110 has been shown to participate in the sensitization of TRPV4 response to heat and hypotonicity but not to 4α-PDD (Wegierski et al. 2009). However, preincubation with the tyrosine kinase inhibitors PP1 (Vriens et al. 2004) or PP2 (Fig. 3) does not affect TRPV4-WT activation by hypotonicity.

The "with no lysine" (WNK) kinases WNK1 and WNK4 downregulate TRPV4 membrane location; hypertension-causing WNK mutants are not able to exert this effect. Experiments deleting the TRPV4 N-tail suggested the participation of this region in TRPV4 interaction with WNK kinases, without providing evidence for a direct interaction between WNK proteins and TRPV4 (Fu et al. 2006).

Calmodulin (CaM) binding to TRPV4 has been identified within the second ANK domain (a binding site shared with ATP) (Phelps et al. 2010) and at the C-tail (aa 812–831) (Strotmann et al. 2003), a site also used for binding to the inositol 1,4,5-trisphosphate (IP3) receptor (IP_3R) (Fernandes et al. 2008; Garcia-Elias et al. 2008) and phosphorylation by the serum glucocorticoid-induced protein kinase-1 (SGK-1) (Shin et al. 2012a). However, the reported effects of CaM on

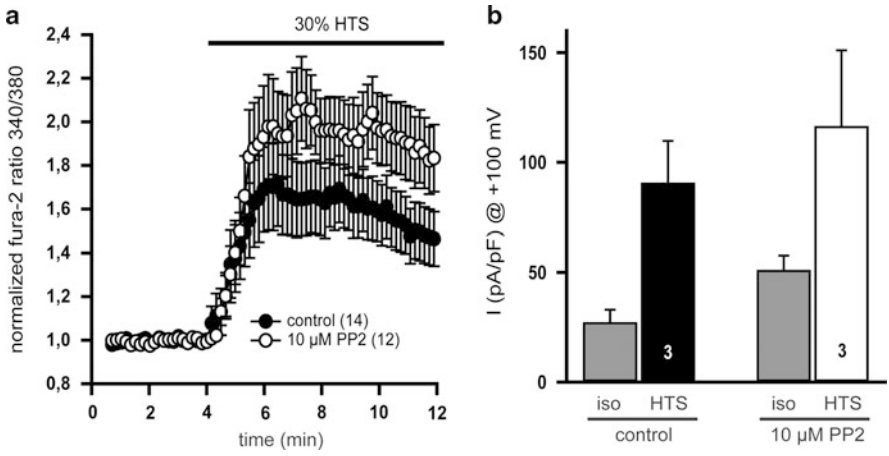

Fig. 3 Tyrosine phosphorylation and TRPV4 activation. Calcium signals (**a**) and whole-cell currents (**b**) obtained in HeLa cells transfected with TRPV4-WT and exposed to vehicle (control) or the tyrosine kinase inhibitor PP2 (10 μM). *Iso* isotonic, *HTS* 30 % hypotonic solution

TRPV4 activity range from a positive modulation (Strotmann et al. 2003) to an inhibitory effect (Phelps et al. 2010). The other molecules interacting with these sites, ATP and IP_3R, are positive modulators of TRPV4 channel activity.

4.3 Cytoskeletal Proteins

The first reported link between TRPV4 and the cytoskeleton came with the observation that microtubule-associated protein 7 (MAP7), which also interacts with actin microfilaments, enhances TRPV4 presence at the plasma membrane and, thereby, increases TRPV4 activity (Suzuki et al. 2003a). The binding of MAP7 to TRPV4 was proposed to be at the channel C-tail.

TRPV4 interacts directly with actin and tubulin (Becker et al. 2009; Goswami et al. 2010). The interaction between TRPV4 and F-actin is essential to support channel activation following cell swelling (Becker et al. 2009), and tubulin competes with actin for binding to the TRPV4 C-tail. The interplay between these three molecules exerts a two-way modulation of cytoskeletal dynamics and TRPV4 activity (Fiorio et al. 2012; Goswami et al. 2010) that may contribute to the mechanical allodynia reported in mice models of neuropathic pain (Huai et al. 2012; Wei et al. 2013). Key molecules that connect the actin cytoskeleton with structures that maintain the barrier function in epithelia also interact with TRPV4. Both β-catenin and E-cadherin, the major components of the tight junctions in keratinocytes, interact with the proximal TRPV4 N-tail to maintain the integrity of the skin barrier (Sokabe et al. 2010). Another study showed coimmunoprecipitation of TRPV4 with α-catenin in urothelial cells but not with

β-catenin (Janssen et al. 2011). TRPV4 also interacts with and is regulated by non-muscle myosin IIa (Masuyama et al. 2012).

TRPV4 coimmunoprecipitates with α2 integrin and Lyn kinase in rat dorsal root ganglion (DRG) neurons (Alessandri-Haber et al. 2008) and participates in mechanical activation of β1 integrin (Thodeti et al. 2009). Moreover, mechanical forces applied to β1 integrin activate TRPV4 at focal adhesions (Matthews et al. 2010), another illustration of the cross talk between TRPV4 and cytoskeletal structures involved in mechanotransduction.

4.4 Channel Proteins

Heteromeric channels are formed by TRPV4 interacting with TRPP2 (Kottgen et al. 2008), aquaporin 4 (Benfenati et al. 2011), aquaporin 2 (Galizia et al. 2012), TRPC1 (Ma et al. 2010), or calcium-activated potassium channel ($K_{Ca}2.3$ cells) (Ma et al. 2013). IP_3R3 interacts with and modulates TRPV4 response, particularly under conditions of low-level stimulation (Fernandes et al. 2008; Garcia-Elias et al. 2008).

5 TRPV4 Biophysics and Activation

5.1 Basic Biophysical Properties

TRPV4 is a nonselective cationic channel with higher permeability to Ca^{2+} and Mg^{2+} than to Na^+ cations, which generates an influx of Ca^{2+} following its activation under normal physiological conditions (Voets et al. 2002). Although TRPV4 also permeates monovalent cations in the absence of divalent ions, it discriminates very poorly between them. The sequence of permeation is $K^+ > Cs^+ > Rb^+ > Na^+ > Li^+$ (Nilius et al. 2001).

Single-channel conductance of TRPV4 is larger at positive (80–100 pS) than at negative potentials (30–60 pS), and the current–voltage relationship of TRPV4 whole-cell currents presents outward rectification (with a slight inward rectification at very negative voltages). This process depends on extracellular Ca^{2+} ions that at the same time permeate and block TRPV4 (Everaerts et al. 2010a; Nilius et al. 2004; Voets et al. 2002; Watanabe et al. 2002a).

5.2 Activation by Osmotic and Mechanical Stimuli

TRPV4 responds to osmotic changes in the cell environment by increasing or decreasing its activity in hypotonic and hypertonic solutions, respectively (Liedtke et al. 2000; Strotmann et al. 2000; Wissenbach et al. 2000), thereby contributing to cellular (Arniges et al. 2004; Fernandez-Fernandez et al. 2008) and systemic volume homeostasis (Liedtke and Friedman 2003; Mizuno et al. 2003). TRPV4

also responds to mechanical stimuli such as shear stress (Gao et al. 2003; Kohler et al. 2006) or high viscous loading (Andrade et al. 2005). Its osmotic (Vriens et al. 2004) and mechanical (Andrade et al. 2005; Fernandes et al. 2008) sensitivity depends on phospholipase A_2 activation and the subsequent production of the arachidonic acid (AA) metabolites, epoxyeicosatrienoic acids (EET), by the cytochrome P450. A recent report has also claimed a direct and potent activation of TRPV4 by AA (Zheng et al. 2013). To date, however, it is not known how EETs mediate channel opening. In any case, whether it binds TRPV4 or is related to changes in the lipid environment, EET-mediated activation of TRPV4 requires the binding of PIP_2 to a PIBS at the N-tail (Garcia-Elias et al. 2013). Alternatively, EET-independent mechanisms have also been reported: TRPV4 is activated by membrane stretch in excised patches from oocytes (Loukin et al. 2010), in apparent contradiction with early reports (Strotmann et al. 2000), and responds to hypotonic stimuli in yeast, which do not contain AA (Loukin et al. 2009).

5.3 Activation by Temperature

Moderate heat (24–38 °C) activates TRPV4 (Q_{10} between 10 and 20) in heterologous expression systems and native tissues (Garcia-Elias et al. 2013; Guler et al. 2002; Watanabe et al. 2002b), although other studies claimed no role of TRPV4 in mouse thermosensation (Huang et al. 2011). Early reports (Guler et al. 2002; Watanabe et al. 2002b) showed no channel response to heat in excised patches, but it has recently been demonstrated that the reported lack of activation is fully recovered in the presence of PIP_2, which suggests that TRPV4 is a bona fide thermosensitive channel (Garcia-Elias et al. 2013). Mutation of the PIBS (Garcia-Elias et al. 2013) or Y556 (Vriens et al. 2004, 2007) impairs TRPV4 activation by heat.

5.4 Activation by Chemicals

The non-PKC-activating, synthetic phorbol ester 4α-PDD (EC_{50} ~ 400 nM) (Watanabe et al. 2002a) is widely used as a TRPV4 activator. 4α-PDD binds to a pocket formed between TM 3 and TM4. Mutations of Y556, L584, W586, and M587 affect 4α-PDD-mediated responses (Klausen et al. 2009; Vriens et al. 2007). Another potent channel activator is GSK1016790A (EC_{50} ~ 10 nM) (Dunn et al. 2013; Thorneloe et al. 2008). However, it has been recently reported no activation of TRPV4 by GSK1016790A and TRPV4-independent, 4-α-PDD-mediated Ca^{2+} responses in DRG neurons (Alexander et al. 2013).

TRPV4 is activated by bisandrographolide A (BBA, EC_{50} ~ 800 nM) extracted from *Andrographis paniculata*, a plant commonly used in Chinese traditional medicine, and mutation of L584 and W586, but not of Y556, prevents TRPV4 activation by BBA (Smith et al. 2006; Vriens et al. 2007). Apigenin, a plant-derived flavone, activates TRPV4 (EC_{50} ~ 10 μM) in heterologous systems as well as in

cultured mesenteric artery endothelial cells (Ma et al. 2012). Plant cannabinoids also activate TRPV4 (EC_{50} ~ 1–6 μM) (De et al. 2012).

Two endogenous activators of TRPV4 have been identified. The endocannabinoid anandamide produces a robust TRPV4 activation via its metabolite AA and the formation of 5,6-EET (Watanabe et al. 2003b), and dimethylallyl pyrophosphate (DMAPP), a metabolite of the mevalonate pathway, activates TRPV4 (EC_{50} ~ 5 μM) in heterologous expression systems, cultured sensory neurons, and keratinocytes (Bang et al. 2012b).

5.5 Regulation by Calcium

Calcium-dependent regulation of TRPV4 is complex. Extracellular Ca^{2+} is responsible for the rectification of the whole-cell TRPV4 currents and intracellular Ca^{2+}, depending on its concentration, inhibits or potentiates TRPV4 channel activity. Intracellular Ca^{2+}-dependent inactivation (IC_{50} ~ 400 nM) mediates the transient response of TRPV4 to many stimuli (Watanabe et al. 2002a, 2003a). Although the exact mechanisms of Ca^{2+}-dependent inactivation are not fully characterized, F707 in TM 6 is involved in the extracellular Ca^{2+}-dependent inactivation (Watanabe et al. 2003a). Positive modulation of TRPV4 by Ca^{2+} via a CaM-dependent mechanism has also been proposed (Strotmann et al. 2003).

5.6 Modulation by PIP_2 and the Phospholipase C (PLC)-IP_3R Pathway

Modulation of TRPV4 by the purinergic receptor (P2Y2)-PLC-IP_3R pathway was first described in ciliated epithelial cells and heterologous expression systems (Fernandes et al. 2008; Garcia-Elias et al. 2008; Lorenzo et al. 2008) and later in kidney cells (Mamenko et al. 2011) and astrocytes (Dunn et al. 2013). TRPV4 and many other TRP channels are regulated by PIP_2. The interaction of the N-tail PIBS with plasma membrane PIP_2 favors an expanded conformation of the intracellular tails as well as channel activation by hypotonicity and heat (Garcia-Elias et al. 2013). Conditions such as mutations in the PIBS, coexpression with PACSIN3, or reduced PIP_2 levels interfere the interaction of TRPV4 with PIP_2 and promote a compacted tail conformation and prevent channel activation. Following the activation of P2Y2 receptor, the sensitization of TRPV4 activity to low mechanical/osmotic stimulation may be counteracted by depletion of PIP_2 due to PLC activation. The meaning of this dual and antagonistic TRPV4 regulation by the PIP_2-PLC-IP_3R pathway remains unclear at present.

5.7 TRPV4 Antagonists

Three blockers have been classically used for inhibition of TRP channels, although none of them are specific: ruthenium red, gadolinium, and lanthanum (Nilius et al. 2004). Citral, a bioactive component of lemongrass commonly used as a taste enhancer and insect repellent, is a transient TRPV4 antagonist (Stotz et al. 2008). HC-067047 (IC_{50} ~ 50–120 nM) has been shown to be a potent and reversible TRPV4 inhibitor that improved bladder function in animal models of cystitis but inhibited TRPM8 at higher concentrations (Everaerts et al. 2010b). GSK2193874 (IC_{50} ~ 2–100 nM) has been identified as a TRPV4 inhibitor with therapeutic potential against pulmonary edema (Thorneloe et al. 2012).

Butamben (n-butyl-p-aminobenzoic acid), a local anesthetic for topical use known to affect voltage-gated channels, blocks TRPV4 (IC_{50} ~ 20 μM) and TRPA1 (IC_{50} ~ 70 μM) (Bang et al. 2012a). RN-1747 and RN-1734 have both agonist (EC_{50} = 700 nM) and antagonist activity (IC_{50} ~ 2–6 μM), respectively (Vincent et al. 2009). Both compounds affect TRPV1, TRPV3, and TRPM8 channels at higher concentrations (Vincent et al. 2009).

6 Physiological Functions of TRPV4

6.1 Osmoregulation and Mechanotransduction

When exposed to hypotonic solutions, cells rapidly swell. The regulatory response to this increase in cell volume is called regulatory volume decrease (RVD), which is normally associated with changes in intracellular Ca^{2+} concentrations, particularly in epithelial cells, and typically activates K^+ and Cl^- channels, permitting the passive loss of inorganic ions and osmotically obliged water [(Arniges et al. 2004) and references within]. TRPV4 provides the Ca^{2+} signal required to activate Ca^{2+}-dependent potassium channels and the subsequent RVD in epithelial cells (Arniges et al. 2004; Fernandez-Fernandez et al. 2002). TRPV4 also acts in astrocyte RVD (Benfenati et al. 2011) and participates in the maintenance of systemic osmoregulation (Liedtke and Friedman 2003; Mizuno et al. 2003). TRPV4 is expressed in primary osmosensory neurons in the brains' organum vasculosum (Liedtke et al. 2000) and kidney epithelium (Berrout et al. 2012; Tian et al. 2004), although the exact mechanism by which TRPV4 participates in systemic osmoregulation is not yet known.

Mechanical and osmotic activation of TRPV4 triggers ATP release from many different epithelial cells (Gevaert et al. 2007; Seminario-Vidal et al. 2011; Ueda et al. 2011) and increases ciliary beat frequency (CBF) in ciliated epithelia (Andrade et al. 2005). ATP-induced increase in Ca^{2+} and CBF acceleration are also favored by TRPV4 (Lorenzo et al. 2008), which may generate a positive feedback mechanism between ATP- and TRPV4-mediated responses.

TRPV4 channels activated by AA, EET, and shear stress are coupled to the calcium-dependent potassium channels in the endothelium (Bagher et al. 2012;

Kohler et al. 2006; Sonkusare et al. 2012; Vriens et al. 2005) and in vascular smooth muscle (Earley et al. 2005), thereby favoring vasodilation. TRPV4 is essential to endothelial reorientation in response to mechanical forces, which is required to shape vascular growth and development (Thodeti et al. 2009). Excessive activation of TRPV4 also bears harmful vasculature consequences due to increased endothelial permeability and circulatory collapse (Thorneloe et al. 2012; Willette et al. 2008). For a recent review on TRPV4 and the control of vascular tone, see Filosa et al. (2013).

TRPV4 is highly expressed in the kidneys, particularly in the apical water-impermeant regions of the nephron (Delany et al. 2001; Strotmann et al. 2000), although discrepancies exist on its polarized membrane location (Berrout et al. 2012; Tian et al. 2004). It also has functions in the sensing of flow and osmolality (Wu et al. 2007), RVD (Galizia et al. 2012), ATP release (Silva and Garvin 2008), and, more importantly, in flow-dependent salt reabsorption and potassium secretion (Taniguchi et al. 2006). A heteromeric TRPV4-TRPP2 channel in the primary cilium of collecting duct cells is required for the calcium cascade involved in flow sensing (Du et al. 2012; Kottgen et al. 2008).

TRPV4 is also highly expressed in the bladder urothelium where it participates in the sensing of intravesical mechanical pressure (bladder filling) and ATP release (Birder et al. 2007; Everaerts et al. 2010b; Gevaert et al. 2007). TRPV4-KO mice manifest an incontinent phenotype with a lower frequency of voiding contractions (Gevaert et al. 2007).

6.2 Thermoregulation

TRPV4 activates at normal body temperatures (see Sect. 5.3), thereby participating in cell functions ranging from regulating neuronal excitability (Shibasaki et al. 2007) and possibly thermogenesis (Guler et al. 2002) to maintaining epithelial barrier function (Sokabe and Tominaga 2010) and vasodilation (Earley et al. 2005; Watanabe et al. 2002b). However, direct evidence of TRPV4-mediated vasodilation in response to heat is lacking. Peripheral temperature sensing at the level of keratinocytes, corneal epithelium, and sensory neurons has been also associated with TRPV4 (Chung et al. 2003; Lee et al. 2005; Mergler et al. 2010) but challenged by other studies (Huang et al. 2011).

6.3 Nociception and Neuroinflammation

TRPV4 is expressed in peripheral nociceptive neurons and has been involved in hyperalgesia. Hypotonic stimuli trigger pain-related behavior by activating TRPV4 channels in dorsal root ganglion neurons (Alessandri-Haber et al. 2003), and TRPV4-KO mice have a lower sensitivity to harmful pressure on the tail (Suzuki et al. 2003b). TRPV4 is sensitized by PKC, PKA, and Src phosphorylation (Alessandri-Haber et al. 2008); proteases (Grant et al. 2007); and serotonin,

histamine, and neurogenic inflammation (Cenac et al. 2010; Vergnolle et al. 2010). This can lead to hypersensitivity.

6.4 TRPV4 in the Central Nervous System

In the brain, TRPV4 expression and function has been seen in both neurons and glial cells. Activation of microglia by lipopolysaccharide (LPS) is suppressed following activation of TRPV4 (Konno et al. 2012). TRPV4-mediated Ca^{2+} entry into astrocytic end feet leads to parenchymal arteriole dilation (Dunn et al. 2013) and in hippocampal CA1 pyramidal neurons potentiates NMDA response and the excitotoxicity associated with cerebral ischemia (Li et al. 2013). Together with TRPV1, TRPV4 is involved in the glucocorticoid-mediated regulation of feeding-related neuroendocrine cells (Boychuk et al. 2013).

6.5 TRPV4 in Cell Migration and Motility

In recent years, somewhat contradictory reports on the role of TRPV4 in cell migration have appeared. TRPV4 activation reduces migration of neuroendocrine cells (Zaninetti et al. 2011) but mediates migration of pulmonary artery smooth muscle (Martin et al. 2012) and AA-induced migration of endothelial cells (Fiorio et al. 2012). At present no clear explanation exists for these apparent discrepancies.

7 Lessons from Knockout Mice

Many different studies have made use of TRPV4 knockout models ($Trpv4^{-/-}$). In this section we focus on studies offering novel insights into the physiological role of TRPV4 that have not been introduced in other sections or reporting results that conflict with previous cell-based experiments. Two different $Trpv4^{-/-}$ mice models have been generated through neo-replacement of exon 4 (Mizuno et al. 2003) and lox-cre-mediated excision of exon 12 (Liedtke and Friedman 2003), a fact to be considered in view of contradictory information when comparing functional responses between the two $Trpv4^{-/-}$ models.

7.1 Thermosensation

Initial studies with $Trpv4^{-/-}$ mice revealed the contribution of TRPV4 in detecting warm temperatures (Lee et al. 2005; Todaka et al. 2004) and chemically induced hyperalgesia (Todaka et al. 2004). However, more recent studies from the same laboratories showed no thermal response differences between $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice (Huang et al. 2011).

7.2 Systemic Osmoregulation

In vivo analysis of *Trpv4*$^{-/-}$ mice has produced conflicting results, showing increased (Liedtke and Friedman 2003) or unaffected serum osmolarity (Mizuno et al. 2003). In other reports, *Trpv4*$^{-/-}$ mice have no defect in the response to tonicity or mechanical stimulation by the primary osmosensory neurons in the organum vasculosum lamina terminalis (Ciura et al. 2011) but defective responses in peripheral osmosensory neurons (Lechner et al. 2011).

7.3 Epithelia

Mechanically induced ATP release and bladder function are strongly impaired in *Trpv4*$^{-/-}$ mice (Gevaert et al. 2007). Moreover, the development of cystitis-induced bladder dysfunction is lessened in *Trpv4*$^{-/-}$ mice (Everaerts et al. 2010b). TRPV4 activity and ATP release from esophageal keratinocytes are also reduced in *Trpv4*$^{-/-}$ mice (Mihara et al. 2011). In *Trpv4*$^{-/-}$ mice, the response to different TRPV4-activating stimuli in tracheal ciliated cells displays a reduced Ca^{2+} entry and CBF (Lorenzo et al. 2008). Activation of TRPV4 disrupts the alveolar barrier and activates macrophages, both leading to acute lung injury (Alvarez et al. 2006; Hamanaka et al. 2010).

7.4 Osteoarticular and Muscular Systems

Bone resorption defects due to disrupted osteoclast function have been reported for *Trpv4*$^{-/-}$ mice (Masuyama et al. 2008; Mizoguchi et al. 2008). Normal cartilage physiology also depends greatly on TRPV4 function. Chondrocyte differentiation requires TRPV4 (Muramatsu et al. 2007) and responses to hypotonic and 4α-PDD are reduced in *Trpv4*$^{-/-}$ mice (Clark et al. 2010).

7.5 Metabolism

Knockout of *Trvp4* induces compensatory increases in TRPC3 and TRPC6, elevation of calcineurin activity affecting energy metabolism in skeletal muscle, and protection from diet-induced obesity in mice (Kusudo et al. 2012). *Trpv4*$^{-/-}$ mice have elevated thermogenesis and protection from diet-induced obesity, adipose inflammation, and insulin resistance, highlighting the role of TRPV4 in metabolic disorders (Ye et al. 2012).

7.6 Vascular Function

The development of pulmonary hypertension, right heart hypertrophy, and vascular remodeling was significantly delayed and suppressed in hypoxic *Trpv4*$^{-/-}$ mice, suggesting that TRPV4 serves as a signal pathway crucial for the development of hypoxia-induced pulmonary hypertension (Yang et al. 2012). TRPV4 plays also a role in blood pressure control. Although portal osmolality decreases after water ingestion in both wild-type and *Trpv4*$^{-/-}$ mice, only the wild-type animals show a pressure response (McHugh et al. 2010).

8 TRPV4 in Hereditary and Acquired Diseases

The participation of TRPV4 in disease has been documented at different levels ranging from disease-causing mutations (Fig. 4) and single nucleotide polymorphisms (SNP) to abnormal responses to pathological stimuli. Further research is required to address the intriguing questions that remain.

8.1 Causal Mutations

A puzzling question about the pathophysiological consequences of TRPV4 dysfunction is why the clinically relevant TRPV4 mutations mainly affect osteoarticular and peripheral nervous systems despite wide tissue distribution of TRPV4. Also surprising is the very mild phenotype of *Trpv4*$^{-/-}$ mice under normal conditions. Together, these observations may indicate that the cellular environment is essential to determining TRPV4 function and regulation. Cells from different tissues most likely present different protein networks that modulate the final outcome of TRPV4 functions.

8.1.1 Osteoarticular Disorders

The first disease-causing TRPV4 mutations were identified in patients with autosomal dominant brachyolmia (ADB), a rather mild type of skeletal dysplasia (Rock et al. 2008). TRPV4-R616Q and TRPV4-V620I were identified as causal gain-of-function mutants, and 33 other TRPV4 mutations have been linked to different skeletal dysplasias. Due to space restrictions, we cannot cite all original studies on TRPV4-causing mutations and, instead, refer the reader to excellent reviews (Dai et al. 2010; Nilius and Voets 2013). All these skeletal dysplasias form part of a heterogeneous group of bone disorders ranging from mild to lethal. Patients may present abnormalities in vertebrae and tubular bones as well as cartilage, resulting in severe scoliosis, short trunk, and extremities and craniofacial defects. Although phenotypes may differ widely, they all share defects in bone ossification. Furthermore, the same mutation may be found in patients presenting widely different phenotypes.

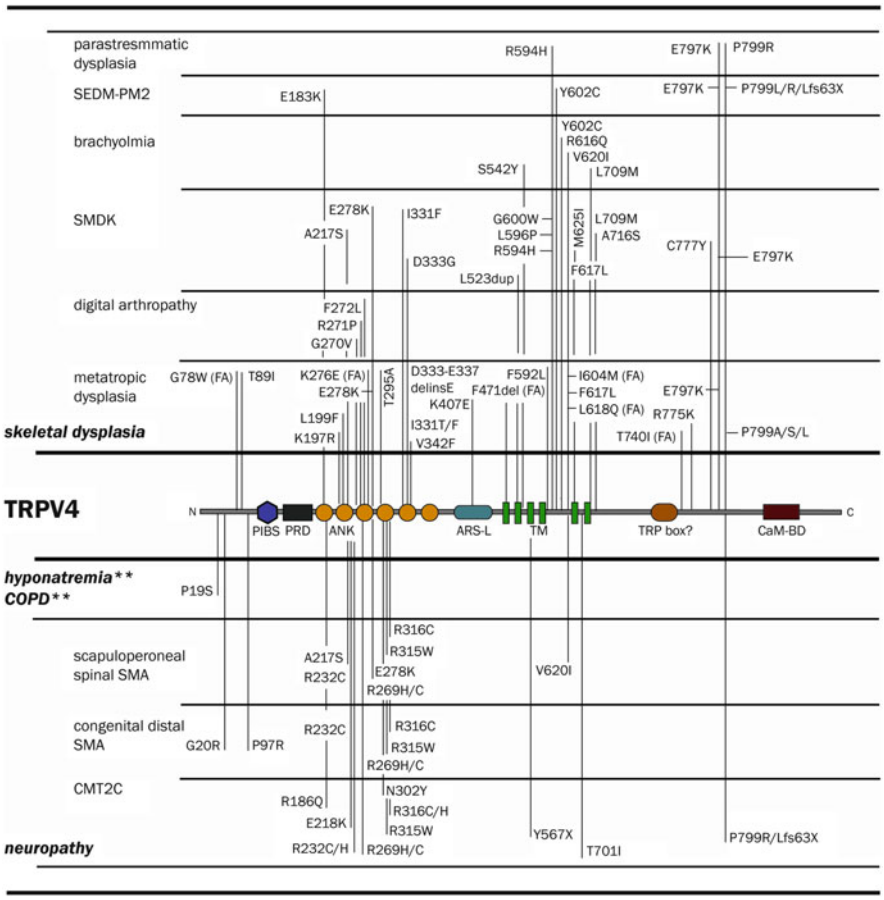

Fig. 4 TRPV4 mutations related to human diseases. TRPV4 mutations and SNPs associated to different skeletal dysplasias, neuropathies, hyponatremia, and COPD are shown. Each mutation is positioned over the schematic representation of the channel. **TRPV4-P19S SNP, although not causal, has been associated with hyponatremia and COPD. *PRD* proline-rich domain, *ANK* ankyrin repeats, *ARS-L* arachidonate recognition sequence like, *TM* transmembrane segments, *CaM-BD* calmodulin-binding domain, *CMT2C* hereditary motor and sensory neuropathy 2C (Charcot-Marie-Tooth 2C disease), *SMA* spinal muscular atrophy, *COPD* chronic obstructive pulmonary disease, *SMDK* spondylometaphyseal dysplasia Kozlowski type, *SEDM-PM2* spondyloepimetaphyseal dysplasia Maroteaux pseudo-Morquio type 2. Adapted from Dai et al. (2010)

Three TRPV4 mutations have been found in familial digital arthropathy-brachydactyly (FDAB), an inherited arthropathy in hands and feet with a related severe osteoarthritis (OA) (Lamande et al. 2011). These three mutations presented increased baseline but decreased stimuli-dependent channel activity. The mechanism by which these mutations lead to OA is not known. Previous studies in animal models had shown that TRPV4 was responsible for the hypotonic responses seen in articular chondrocytes and that TRPV4 KO mice had an age- and sex-dependent

progression to OA (Clark et al. 2010). Still unanswered is the question of how TRPV4 mutations lead to osteoarticular pathology: Is the cause of the disease related to changes in TRPV4 channel activity and/or TRPV4 interaction with other proteins?

8.1.2 Neuropathies

TRPV4-linked neuropathies were first described in 2010 (Auer-Grumbach et al. 2010; Deng et al. 2010; Landoure et al. 2010). Although very heterogeneous, all of the disorders lead to a degeneration of peripheral nerves. They may occur alone or with sensory-associated phenotypes such as vocal cord paresis (Chen et al. 2010) or hearing defects (Zimon et al. 2010). Wide variability in phenotype or in age at onset is observed, even between families that share the same causal mutation. Genetic and environmental factors are likely responsible for such variation, but further studies should clarify this point. As seen with the skeletal disorders, most of the neuropathy-related TRPV4 mutations generate gain of function, and the same mutation can produce different phenotypes (Nilius and Voets 2013). Single-channel analysis of skeletal (Loukin et al. 2011) and neuronal (Fecto et al. 2011) disease-causing mutations revealed increased basal open probability of mutant channels.

8.1.3 Mixed Skeletal and Neuromuscular Disorders

A few TRPV4 mutations have been associated with both skeletal and neuromuscular disorders. Patients with motor neuropathies have a short trunk (Chen et al. 2010; Cho et al. 2012), although patients with the mild forms of skeletal dysplasias rarely have any neuropathy except in metatropic dysplasia with fetal akinesia (Unger et al. 2011).

8.2 Single Nucleotide Polymorphisms and Abnormal TRPV4 Responses Associated with Disease

The rs3742030 polymorphism (P19S) generates a TRPV4 channel with reduced response to mild hypotonic shocks and is associated with higher risk of hyponatremia (Tian et al. 2009) and chronic obstructive pulmonary disease and forced expiratory volume in 1 s (FEV_1) (Zhu et al. 2009) but not with cough in asthmatic children (Cantero-Recasens et al. 2010) or healthy/asthmatic adults (Smit et al. 2012). Dysregulation of TRPV4 has been described in cystic fibrosis epithelium (Arniges et al. 2004). TRPV4 mRNA and protein are increased in sinus mucosal biopsies from chronic rhinosinusitis patients (Bhargave et al. 2008). TRPV4 participates in the inflammatory signaling pathways leading to neurogenic inflammation and pancreatitis (Ceppa et al. 2010; Zhang et al. 2013), intestinal chronic inflammation (d'Aldebert et al. 2011; Fichna et al. 2012), and mastication-associated pain at the temporomandibular joint (Chen et al. 2013).

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TRPV5: A Ca^{2+} Channel for the Fine-Tuning of Ca^{2+} Reabsorption

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Abstract

TRPV5 is one of the two channels in the TRPV family that exhibit high selectivity to Ca^{2+} ions. TRPV5 mediates Ca^{2+} influx into cells as the first step to transport Ca^{2+} across epithelia. The specialized distribution in the distal tubule of the kidney positions TRPV5 as a key player in Ca^{2+} reabsorption. The responsiveness in expression and/or activity of TRPV5 to hormones such as 1,25-dihydroxyvitamin D_3, parathyroid hormone, estrogen, and testosterone makes TRPV5 suitable for its role in the fine-tuning of Ca^{2+} reabsorption. This role is further optimized by the modulation of TRPV5 trafficking and activity via its binding partners; co-expressed proteins; tubular factors such as calbindin-D_{28k}, calmodulin, klotho, uromodulin, and plasmin; extracellular and intracellular factors such as proton, Mg^{2+}, Ca^{2+}, and phosphatidylinositol-4,5-bisphosphate; and fluid flow. These regulations allow TRPV5 to adjust its overall activity in response to the body's demand for Ca^{2+} and to prevent kidney stone formation. A point mutation in mouse *Trpv5* gene leads to hypercalciuria similar to *Trpv5* knockout mice, suggesting a possible role of TRPV5 in hypercalciuric disorders in humans. In addition, the single nucleotide polymorphisms in *Trpv5* gene prevalently present in African descents may contribute to the efficient renal Ca^{2+} reabsorption among African descendants. TRPV5 represents a potential therapeutic target for disorders with altered Ca^{2+} homeostasis.

Keywords

TRPV5 • TRPV6 • Gene duplication • Calcium channel • Calcium transport • Calcium reabsorption • Distal convoluted tubule • Hypercalciuria • Vitamin D • Protein–protein interaction • Single nucleotide polymorphisms • African American

1 Introduction

Ca^{2+} serves as an important intracellular and extracellular messenger, and it is indispensable for many physiological activities, such as muscle contraction, neuron excitability, and blood coagulation (Brown et al. 1995; Clapham 1995). For this reason, the plasma Ca^{2+} concentration is monitored by a Ca^{2+}-sensing receptor, which controls the secretion of parathyroid hormone (PTH) (Brown 1991). PTH regulates the synthesis of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (Henry 1985; Yang et al. 1999). PTH and $1,25(OH)_2D_3$ regulate Ca^{2+} homeostasis via intestinal

absorption and renal reabsorption, as well as deposition into and mobilization from the bone. In the intestine and kidney, Ca^{2+} is transported across epithelia through the tight junction between cells (paracellular pathway), or across the apical and basolateral membranes of epithelial cells (transcellular pathway) (Bronner 1998; Wasserman and Fullmer 1995). Only the transcellular pathway allows Ca^{2+} to be transported against an electrical and concentration gradient. The Ca^{2+}-transporting epithelial cells in the intestinal and the kidney express proteins critical in the transcellular Ca^{2+} transport pathway. Ca^{2+} channels TRPV5 and TRPV6 in the apical membrane mediate Ca^{2+} entering into the cell (Hoenderop et al. 1999; Peng et al. 1999; Zhuang et al. 2002). The steep Ca^{2+} concentration gradient (approximately 1 mM extracellular vs. 0.1 μM intracellular) and the transmembrane potential (typically −60 mV inside the cell) allow Ca^{2+} to enter the cells via channels. Ca^{2+} exits the cell via high-affinity Ca^{2+}–ATPase (PMCA) and/or Na^{+}–Ca^{2+} exchanger 1 (NCX1) at the expense of energy stored in ATP or in Na^{+} concentration gradient (Wasserman et al. 1992). Ca^{2+}-binding proteins (e.g., calbindins) are also expressed to keep the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) low so that the driving force for Ca^{2+} entry is maintained and $[Ca^{2+}]_i$ will not rise to a level which is toxic to cells (Christakos et al. 1992; Lambers et al. 2006; Zheng et al. 2004). Although TRPV5 and TRPV6 were identified at a much later date compared to calbindins, PMCA, and NCX, their specific roles in the transcellular Ca^{2+} transport pathway energized the studies of Ca^{2+} absorption and reabsorption at molecular level since the cloning of TRPV5 and TRPV6. Compared to the broader distribution of TRPV6, TRPV5 is relatively confined to the kidney, where it is responsive to physiological signals to regulate Ca^{2+}excretion into the urine.

2 Gene

TRPV5 was cloned as a result of searching for genes involved in Ca^{2+} reabsorption in kidney cells (Hoenderop et al. 1999). Using an expression cloning approach, Hoenderop and Bindels identified a cDNA from primary cells of rabbit connecting tubule (CNT) and cortical collecting duct (CCD) that stimulates $^{45}Ca^{2+}$ uptake in *Xenopus laevis* oocytes. The cDNA encodes a protein of 730 amino acid residues and was initially named as epithelial Ca^{2+} channel (ECaC) (Hoenderop et al. 1999). At the time of cloning, the only mammalian protein similar to TRPV5 was rat vanilloid receptor VR1 (TRPV1). Human, rat, and mouse TRPV5 share high levels of amino acid identity to rabbit TRPV5 (Peng et al. 2000a, 2001). The protein most similar to TRPV5 is TRPV6 (75 % amino acid identity), which was independently identified from rat duodenum using similar approach (Peng et al. 1999). TRPV5 shares approximately 40–45 % amino acid identity with other members of the TRPV family, including TRPV1 to TRPV4.

Trpv5 gene is located in human chromosome 7q35 side by side with *TRPV6* gene in 7q33–34 (Muller et al. 2000; Peng et al. 2001). The human *Trpv5* gene comprises 15 exons. Except for the first and last exons, exons 2–14 of *Trpv5* and *Trpv6* genes are identical in size (Peng et al. 2001). In fact, all genes of the TRPV family share a

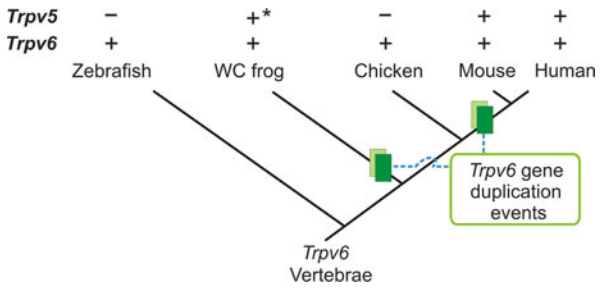

Fig. 1 *Trpv5* gene was likely produced by the duplication of *Trpv6* gene during the process of evolution. Gene duplication events are indicated on the respective branches. WC frog, Western clawed frog (*Xenopus tropicalis*). *Plus symbol* indicates present, *minus symbol* indicates absent, and *asterisk* indicates that there are five genes similar to TRPV6 in Western clawed frogs

conserved gene structure (Peng et al. 2001). In addition to *Trpv5* and *Trpv6* in 7q34–35, genes for *Trpv1*, *Trpv2*, and *Trpv3* are located in 17q11.2 to 17q13.2, suggesting recent gene duplication events in this subfamily. Fish and birds have only one *Trpv6*-like gene (Qiu and Hogstrand 2004; Yang et al. 2011), whereas mammalians have both *Trpv5* and *Trpv6* genes. Based on the Joint Genome Institute (JGI; http://genome.jgi-psf.org/) genome database for *Xenopus tropicalis* (Western clawed frog), five *Trpv6*-like genes are present. This is not restricted to *Trpv6*, as 6 *Trpv4*-like genes are present in *X. tropicalis* (Saito and Shingai 2006). Thus, it is likely that duplication events occurred in *Trpv6* gene when amphibians and mammals were evolved, respectively (Fig. 1). The physiological roles of the *Trpv6*-like genes in *X. tropicalis* are unclear. In mammals, the duplicated gene became *Trpv5* as it gained the ability for specialized expression in the distal tubule of kidney and properties for a finer regulation of channel function and trafficking.

3 Tissue Distribution

In contrast to the broader tissue distribution of TRPV6, TRPV5 is rather specifically distributed to the kidney. Due to the high sequence similarity between TRPV5 and TRPV6, initial studies showed strong signals in Northern blot analyses with TRPV5 cDNA probe in rabbit and rat duodenum (Peng et al. 2000a; Hoenderop et al. 1999), but this is likely due to cross hybridization with TRPV6 mRNA, which is highly expressed in the duodenum (Peng et al. 2000a). Similarly, although TRPV5 is also expressed in the placenta, the mRNA abundance of TRPV5 is much lower than that of TRPV6 in human placenta (Peng et al. 2001).

Based on Expressed Sequence Tag (EST) database from the National Center for Biotechnology Information (NCBI), among 45 human organs listed, TRPV5 is detected in only two of them (numbers in brackets are tag/million): blood [8] and lung [2]. In contrast, TRPV6 is present in 13 organs/tissues: bladder [33], blood [16], brain [4], cervix [20], eye [9], intestine [8], lung [2], mammary gland [6],

pancreas [4], placenta [215], prostate [47], testis [6], and trachea [19]. Both TRPV5 and TRPV6 are not detected in human kidney, indicating their number of EST is lower than 1/million. In mouse, among 47 organs/tissues listed, TRPV5 is present in 2, including embryonic tissue [1] and kidney [24], whereas TRPV6 is present in 9 of them including brain [10], embryonic tissue [2], extraembryonic tissue [120], lung [30], mammary gland [69], pancreas [37], salivary gland [103], skin [8], and thymus [8]. These EST profiles may not be accurate due to various reasons; however, they provide an unbiased overview of the gene expression. The expression profile of TRPV5 in mouse suggests a specific role of TRPV5 in the kidney.

In rabbit kidney, TRPV5 protein is expressed in the apical membrane of distal convoluted tubule (DCT), CNT, and CCD, where it co-localizes with calbindin-D_{28k} (Hoenderop et al. 1999). In rats, TRPV5 mRNA exhibits similar expression pattern as NCX1 and calbindin-D_{28k} (Peng et al. 2000a). In mouse kidney, TRPV5 is expressed in the late segment of DCT and CNT (Loffing et al. 2001).

In mice and rats, TRPV5 mRNA is much more abundant than that of TRPV6 in the kidney (Song et al. 2003; Van Cromphaut et al. 2007). TRPV5 mRNA level is approximately 10–20 times higher than that of TRPV6 in mouse kidney (Song et al. 2003). In contrast, we have observed much higher mRNA level of TRPV6 than TRPV5 in the human kidney (Peng et al. 2001). Our unpublished observations also indicated a higher mRNA level of TRPV6 in outer medulla than cortex of human kidney, opposite to that of TRPV5. Similarly, TRPV6 is also much abundant than TRPV5 in horse kidney (Rourke et al. 2010). Thus, TRPV6 may play more significant roles in some species such as humans and horses.

Even though TRPV5 is mainly expressed in the kidney, TRPV5 mRNA or protein has also been detected in other organs/tissues. TRPV5 is detected in mouse testis (Jang et al. 2012) and inner ear (Takumida et al. 2009) and rat testis, spermatogenic cells, sperm (Li et al. 2010), cochlea (Yamauchi et al. 2010), and osteoclast-like cells (Yan et al. 2011). In horse, TRPV5 mRNA is expressed in the duodenum and proximal jejunum (Rourke et al. 2010), and TRPV5 protein is detected in chondrocytes from the superficial zone of articular cartilage (Hdud et al. 2012). In human, TRPV5 protein and mRNA are present in placenta (Bernucci et al. 2006), osteoclasts (van der Eerden et al. 2005), retinal pigment epithelium (Kennedy et al. 2010), lymphocytes, Jurkat leukemia T cells (Vassilieva et al. 2013), and leukemia K562 cells (Semenova et al. 2009).

4 Structural Aspects

The mammalian TRPV5 proteins comprise around 730 amino acid residues (729, 731, 730, and 723 amino acids in human, horse, rabbit, rat, and mouse, respectively). Mammalian TRPV5 proteins consist of an intracellular N- and C-termini, six transmembrane domains, and a pore region between the last two transmembrane domains. In the intracellular portions of TRPV5, six ankyrin repeats are present in the N-terminal region, and a PDZ-binding motif is located in the C terminus (Phelps et al. 2008; de Groot et al. 2011b; Palmada et al. 2005;

Jing et al. 2011). An *N*-linked glycosylation site is located in the first extracellular loop, and asparagine 358 (N358) is indispensable for the *N*-linked glycosylation of TRPV5 (Chang et al. 2005; Jiang et al. 2008). Putative protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites and calmodulin-binding sites are present within the N- and C-terminal regions (Hoenderop et al. 1999; Kovalevskaya et al. 2012). Some of these sites have already been shown to be important in regulating TRPV5.

Four TRPV5 molecules form a homotetramer with a single pore in the middle (Nilius et al. 2001b; Hoenderop et al. 2003b). Two critical motifs at positions 64–77 (part of the first ankyrin repeat) in the N terminus and at 596–601 in the C terminus of TRPV5 are needed for the formation of the functional channel complex (Chang et al. 2004). The N-terminal ankyrin repeats appear to be important for channel assembly. The first ankyrin repeat does not appear to be critical to the formation of TRPV5 tetramer; however, it is likely essential for correct folding of TRPV5 into a functional channel complex (de Groot et al. 2011b). In contrast, the third ankyrin repeat initiates a zippering process through the fifth ankyrin repeat, and they form an anchor for channel assembly (Erler et al. 2004). The tetramerization of TRPV5 allows the four aspartate 542 (D542) residues in pore-forming loop between transmembrane domain 5 and 6 to form a Ca^{2+}-selective ring, which is also involved in Mg^{2+} blockade (Nilius et al. 2001b; Voets et al. 2004; Lee et al. 2005).

TRPV5 and TRPV6 appeared to form heterotetramers when four pieces of TRPV5 and TRPV6 were linked together and heterologously expressed in *X. laevis* oocytes (Hoenderop et al. 2003b). The more TRPV6 subunits presented in the heterotetramer, the more TRPV6 characteristics were exhibited (Hoenderop et al. 2003b). Other TRPV members preferentially form homomeric assembly; however, TRPV5 and TRPV6 can form heteromeric complex in HEK293 cells (Hellwig et al. 2005). This is likely due to the high degree of similarity between TRPV5 and TRPV6. Unlike in transfected cells, TRPV5 is likely outnumbered by TRPV6 in most cells. It is unclear whether heteromeric TRPV5 and TRPV6 complexes play a significant physiological role if they exist in vivo. In addition, TRPV5 and TRPV6 share certain structure–function similarities with TRPML3 (Grimm et al. 2007). They can form heteromers which display different features than the respective homomers (Guo et al. 2013). TRPML3 and TRPV5 are expressed in the kidney and inner ear (Takumida et al. 2009; Castiglioni et al. 2011); however, it is yet to be determined whether TRPML3 and TRPV5 form heteromeric complexes in native cells.

5 Biophysical Properties

5.1 Ca^{2+} Selectivity

TRPV5 is a Ca^{2+} permeant ion channel as it was identified as a result of increasing $^{45}Ca^{2+}$ uptake activity when expressed in *Xenopus* oocytes (Hoenderop et al. 1999). The most distinctive feature of TRPV5 is its Ca^{2+} selectivity. The permeability ratio

between Ca^{2+} and Na^+ (P_{Ca}:P_{Na}) is over 100 (Vennekens et al. 2000). The divalent cation selectivity profile of TRPV5 is $Ca^{2+} > Mn^{2+} > Ba^{2+} \approx Sr^{2+}$ (Vennekens et al. 2000). In the absence of divalent cation in the extracellular solution, TRPV5 allows monovalent cations to pass through. The permeation sequence for monovalent cations through TRPV5 is $Na^+ > Li^+ > K^+ > Cs^+ > NMDG^+$ (Nilius et al. 2000). The Na^+ current of TRPV5 correlates with the amplitude of Ca^{2+} uptake and is often used as a measure of TRPV5 channel activity. Some trivalent and divalent cations block current through TRPV5, and the sequence of block is $Pb^{2+} = Cu^{2+} = Gd^{3+} > Cd^{2+} > Zn^{2+} > La^{3+} > Co^{2+} > Fe^{2+} > Fe^{3+}$ (Nilius et al. 2001a). TRPV5 is also sensitive to ruthenium red and econazole (Nilius et al. 2001a). TRPV5 and TRPV6 show difference in ruthenium red sensitivity: the IC_{50} of ruthenium red for TRPV5 is around 121 nM, which is nearly100-fold lower than that for TRPV6 (Hoenderop et al. 2001b). Extracellular and intracellular Mg^{2+} blocks TRPV5 voltage dependently (Voets et al. 2001; Lee et al. 2005; Hoenderop et al. 2001b). D542 is the key residue to form the selectivity filter of TRPV5 and determines Ca^{2+} permeation and Mg^{2+} blockade (Nilius et al. 2001b; Lee et al. 2005; Dodier et al. 2007). The nonsynonymous single nucleotide polymorphism (SNP) A563T variation, close to D542, increases TRPV5 sensitivity to extracellular Mg^{2+}, resulting in suppressed Na^+ permeation through TRPV5 (Na et al. 2009). In clinical trials, urinary Ca^{2+} excretion is proportional to changes in magnesium excretion, and this is likely related to the blockade of TRPV5 by Mg^{2+} (Bonny et al. 2008).

TRPV5 currents exhibit strong inward rectification (Vennekens et al. 2000). Using TRPV6 as a model, Voets et al. demonstrated that intracellular Mg^{2+} acts as a permeant pore blocker and contributes to strong inward rectification of the channel (Voets et al. 2003). In addition, TRPV6 also exhibits intrinsic Mg^{2+}-independent inward rectification for which the mechanism is not fully understood (Voets et al. 2003). Similar mechanisms are likely applicable to TRPV5 due to the high degree of similarity in structure and function between TRPV5 and TRPV6. Unitary channel activity of TRPV5 could be detected in the absence of extracellular divalent cations (Nilius et al. 2000). The single-channel conductance is 77.5 pS for rabbit TRPV5 using Na^+ as a charge carrier (Nilius et al. 2000). Single-channel activity of TRPV5 was also detected using K^+ as a charge carrier (Vassilev et al. 2001). In addition, subconductance state (partial channel opening) of TRPV5 was also observed (subconductance at 29 pS vs. full conductance at 59 pS at intracellular pH 7.4) (Cha et al. 2007).

5.2 pH Sensitivity

Another feature of TRPV5 is its pH sensitivity. In the initial characterization, TRPV5-mediated Ca^{2+} uptake was significantly inhibited when the pH value in the extracellular medium was lowered to 5.9 (Hoenderop et al. 1999). At single-channel level, both full and subconductance and their open probabilities were reduced by lower intracellular pH (Cha et al. 2007). On the other hand, high pH

stimulates TRPV5-mediated Ca^{2+} uptake (Peng et al. 2000a). Low pH increases the blockage of monovalent cation current by extracellular Mg^{2+} (Vennekens et al. 2001). Glutamate 522 (E522) of rabbit TRPV5 acts as the extracellular pH sensor, and extracellular protons decrease the estimated diameter of channel pore to inhibit TRPV5 (Yeh et al. 2003, 2005). Glutamate 535 (E535) is also involved; however, its effect depends on E522 (Yeh et al. 2006). Meanwhile, intracellular protons also regulate TRPV5 (Yeh et al. 2005). Intracellular acidification promotes proton binding to lysine 607, intracellular pH sensor, and induces rotation of the pore helix, leading to decreases in the pore diameter, open probability, and single-channel conductance (Yeh et al. 2005; Cha et al. 2007).

In addition to channel property, pH may also affect the level of TRPV5 protein at the cell surface. Extracellular alkalization induces the recruitment of TRPV5 proteins to the cell surface from TRPV5-containing vesicles, and extracellular acidification leads to the retrieval of TRPV5 from plasma membrane (Lambers et al. 2007). Thus, TRPV5 could be regulated by pH in both channel activity and plasma membrane expression. Metabolic acidosis and alkalosis affect Ca^{2+} transport in the kidney (Sutton et al. 1979). Clinical trial data indicate that changes in urinary Ca^{2+} excretion and urine pH are inversely related (Bonny et al. 2008). The pH sensitivity of TRPV5 may contribute to the dysregulation of Ca^{2+} reabsorption under disturbance in acid–base balance.

5.3 Ca^{2+}-Dependent Inactivation

High level of free Ca^{2+} is toxic to cells. The Ca^{2+}-dependent inactivation limits the amount of Ca^{2+} ions that enter the cell through TRPV5 and prevents the toxic impact of Ca^{2+} overload in the cell. Calbindin-D_{28k} binds Ca^{2+} beneath the plasma membrane and therefore relieves the Ca^{2+}-dependent inactivation of TRPV5 to some extent; it may also buffer free Ca^{2+} to limit the toxic effect (Lambers et al. 2006). TRPV5 exhibits Ca^{2+}-dependent autoregulatory mechanisms, including fast inactivation and slow rundown (Vennekens et al. 2000). The C-terminal sequences in rabbit TRPV5 701–730 and 650–653 are critical determinants of Ca^{2+}-dependent inactivation (Nilius et al. 2003). Compared to TRPV6, TRPV5 exhibit a much slower initial inactivation phase (Nilius et al. 2002). The first intracellular loop is critical to the kinetics of the initial phase in the inactivation process; three residues at positions 409, 411, and 412 in TRPV5 are critical for the delayed inactivation (Nilius et al. 2002). In addition, Q579 following the last transmembrane domain of TRPV5 is also important to the fast Ca^{2+}-dependent inactivation kinetics of TRPV5 (Suzuki et al. 2002). The first intracellular loop of TRPV5 is capable of binding to CaM (Kovalevskaya et al. 2012), so is the C terminus of TRPV5 (de Groot et al. 2011a; Kovalevskaya et al. 2012). TRPV5 mutant deficient in interacting with CaM at C terminus (residues 696–729) exhibits diminished Ca^{2+}-dependent inactivation (de Groot et al. 2011a). However, it is yet to be clarified what roles the other CaM-binding sites of TRPV5 play in the Ca^{2+}-dependent inactivation mechanism. In addition, a helix-breaking mutation

(M490P) in transmembrane domain 5 reduces Ca^{2+}-dependent inactivation of TRPV5 and induces apoptosis due to Ca^{2+} overload in cells expressing $TRPV5^{M490P}$ (Lee et al. 2010). Corresponding mutation in TRPML3 (A419P) leads to constitutive channel activity, which likely results in hair cell death in the inner ear and profound deafness and other abnormalities in mice homozygous in $TRPML3^{A419P}$ (Grimm et al. 2007).

Although TRPV5 works constitutively in the kidney, its activity depends on the availability of phosphatidylinositol-4,5-bisphosphate (PIP_2). As the level of PIP_2 decreases, so does the activity of TRPV5 (Lee et al. 2005). PIP_2 activates TRPV5 in part by reducing conformational change-induced Mg^{2+} binding (Lee et al. 2005). Arginine 599 in mouse or rat TRPV5 (corresponding to R606 in rabbit and human TRPV5) in the "TRP domain" is likely the PIP_2-binding site (Rohacs et al. 2005). Elevation of $[Ca^{2+}]_i$ due to Ca^{2+} influx through TRPV5 may activate phospholipase C (PLC), which depletes PIP_2. Thus, removal of PIP_2 is an important Ca^{2+}-dependent inactivation mechanism for TRPV5 as well as TRPV6 (Thyagarajan et al. 2008).

5.4 Responsiveness to Fluid Flow

Flow-stimulated K^+ secretion in the distal nephron involves Ca^{2+}-activated K^+ channels. TRPV5 is expressed in the apical membrane in the distal nephron and therefore may provide a Ca^{2+} influx pathway to activate K^+ channels. Recent study indicates that both TRPV5 and TRPV6 expressed in HEK cells are activated by shear force generated by fluid flow in physiological range (Cha et al. 2013). Flow-induced surge in TRPV5 or TRPV6 activity leads to the activation of co-expressed Slo1 maxi-K^+ channel without affecting ROMK channel (Cha et al. 2013). Activation of maxi-K^+ channel may counteract the depolarization due to Ca^{2+} influx and cause hyperpolarization which in turn would improve Ca^{2+} influx through TRPV5. *N*-linked glycosylation of the channels is involved in the responsiveness to fluid flow (Cha et al. 2013). The responsiveness of TRPV5 and TRPV6 to flow may have implications not only in flow-stimulated K^+ secretion but also in thiazide-induced Ca^{2+} reabsorption.

6 Regulation by Interacting, Co-expressed, and Tubular Proteins

A number of proteins have been found to regulate TRPV5. These include TRPV5-interacting proteins, proteins co-expressed with TRPV5, and proteins in the tubular fluid (Table 1). Regulation of TRPV5 by some of the proteins is briefly summarized in the following.

Table 1 Proteins that regulate TRPV5

Protein name	Binding motif in TRPV5	Effect/mechanism	References
TRPV5-interacting proteins affecting trafficking/plasma membrane expression			
S100A10	^{598}VATTV^{602a}	Associates with annexin 2 to regulate the plasma membrane localization of TRPV5; upregulated by 1,25$(OH)_2D_3$	Lewit-Bentley et al. (2000)
Rab11a	^{603}MLERK^{607}	Plasma membrane localization of TRPV5	van de Graaf et al. (2006a)
NHERF2	^{726}VYHF^{729}	Trafficking and stability at plasma membrane	Embark et al. (2004), Palmada et al. (2005), Jing et al. (2011)
NHERF4	603–624	Unknown	van de Graaf et al. (2006b)
TRPV5-interacting proteins regulating channel function/activity			
Calbindin-D_{28k}	N- and C-termini	Buffers $[Ca^{2+}]_i$ and counteracts inactivation of TRPV5	Lambers et al., (2006)
Calmodulin	133–154, 310–330, 401–428, 591–612, 696–712	Regulates channel function such as in feedback inhibition by Ca^{2+}	Holakovska et al., (2011), de Groot et al. (2011a), Kovalevskaya et al. (2012)
80K-H	598–608	Intracellular Ca^{2+} sensor to regulate TRPV5 activity	Gkika et al. (2004)
BSPRY	C terminus	Inhibits TRPV5 activity. Inversely regulated by 1,25 $(OH)_2D_3$	van de Graaf et al. (2006c)
FKBP52	Full length	Enzymatic activity is involved in its inhibitory effect	Gkika et al. (2006c)
TRPML3	Unknown	Form heteromeric channel complex with TRPV5 in vitro	Guo et al. (2013)
Proteins in co-expressed cells or in tubular fluid that regulate TRPV5			
Klotho		Expressed in DCT and enhances the stability of TRPV5 on plasma membrane	Cha et al. (2008a), Chang et al. (2005), Lu et al. (2008), Leunissen et al. (2013)
Tissue transglutaminase		Promotes channel aggregation, leading to reduction in pore diameter	Boros et al. (2012)
Tissue kallikrein		Delays channel retrieval from plasma membrane	Gkika et al. (2006b)
Nedd4-2/Nedd4		Increases the degradation of TRPV5 protein	Zhang et al. (2010)
Ca^{2+}-sensing receptor		Stimulates TRPV5 via PKC	Topala et al. (2009)
SGK1/3		Enhances channel stability and trafficking	Embark et al. (2004), Sandulache et al. (2006), Palmada et al. (2005)

(continued)

Table 1 (continued)

Protein name	Binding motif in TRPV5	Effect/mechanism	References
WNK3		Increases TRPV5 delivery to the plasma membrane	Zhang et al. (2008)
WNK4		Increases forward trafficking or enhances endocytosis	Jiang et al. (2007, 2008, Jing et al. (2011), Cha and Huang (2010)
Plasmin		Suppresses TRPV5 by activating PAR-1	Tudpor et al. (2012)
Uromodulin		Secreted in the thick ascending limb and decreases endocytosis of TRPV5	Wolf et al. (2013)

[a]Human TRPV5 numbering throughout this table

6.1 Regulation by Interacting Proteins

Some TRPV5-interacting proteins, such as S100A10, Rab11a, and NHERF2, regulate the trafficking and plasma membrane abundance of TRPV5. On the other hand, some TRPV5-interacting proteins, such as calbindin-D_{28k}, CaM, 80H-K, BSPRY, and FKBP52, regulate TRPV5 activity. It is worth noting that the C terminus of TRPV5 interacts with most of its binding partners, and the region from 598 to 608 in TRPV5 within the conserved "TRP domain" binds to S100A10, Rab11a, 80K-H, NHERF4, and CaM (Fig. 2). This region also contains the PIP_2-binding site (R606) and a pH sensor (K607). It is also not far away from histidine 711 (corresponding to H712 in rabbit TRPV5), a critical residue involved in the constitutive internalization of TRPV5 (de Groot et al. 2010). Thus, it is plausible that TRPV5-binding partners may affect TRPV5 function, membrane stability, and trafficking. However, it is not likely that these TRPV5-binding proteins regulate TRPV5 at the same time. It is possible that some of the proteins only regulate TRPV5 at a certain cell type, at a specific developmental stage, or at a state being stimulated by a hormone or other physiological cues. Most TRPV5-interacting proteins also interact with TRPV6 due to the conserved binding sites in the two channels. Thus, it is likely some of the TRPV5-interacting proteins may be more involved in regulating TRPV6 than TRPV5. Lastly, although overlapping expression with TRPV5 was found for most of the TRPV5-interacting proteins, they do express in cells that do not express TRPV5; they all have functions other than regulating TRPV5.

6.1.1 S100A10

S100A10 binds to a conserved motif of five residues (^{598}VATTV^{602}) in the C terminus of TRPV5 (van de Graaf et al. 2003). S100A10 is a member of the EF-hand containing S100 protein family, and it forms heterotetramer with annexin 2, a Ca^{2+}, and phospholipid-binding protein in association with cytoskeleton underneath cell membrane (Gerke and Moss 2002). S100A10–annexin 2 complex is

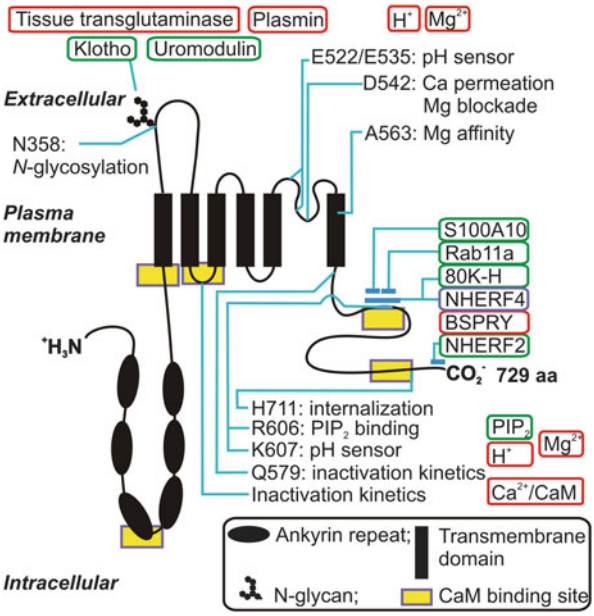

Fig. 2 Functional domains/sites in human TRPV5 (GenBank #: AF304464.1). Key residues, domains, and interacting motifs/sites for partners are indicated in the predicted structure of TRPV5. Factors with positive and negative effects on TRPV5 are labeled with *green* and *red rectangles*, respectively. See text for details

important for the plasma membrane localization of TRPV5 (van de Graaf et al. 2003). In addition, this complex may be involved in $1,25(OH)_2D_3$-induced Ca^{2+} reabsorption as S100A10 is upregulated by $1,25(OH)_2D_3$ (van de Graaf et al. 2003).

6.1.2 Rab11a

The small GTPase Rab11a is another TRPV5-interacting protein that regulates its trafficking to the plasma membrane (van de Graaf et al. 2006a). It binds to ^{596}MLERK^{600} of mouse TRPV5 (603–607 in human TRPV5), following the S100A10-binding motif (van de Graaf et al. 2006a). Glycine substitution in the MLERK motif disrupts the binding of Rab11a to TRPV5 and the plasma membrane expression of TRPV5 (van de Graaf et al. 2006a). TRPV5 is one of a few cargo proteins that bind directly to the GDP-bound Rab proteins.

6.1.3 PDZ Proteins

The last four amino acids of TRPV5 and TRPV6 are PDZ (postsynaptic density-95, Drosophila discs-large protein, zonula occludens protein 1)-binding motifs. TRPV5 interacts with Na^+/H^+ exchanger regulating factors 2 (NHERF2) (Palmada et al. 2005). Removal of the last three amino acids of TRPV5 abolishes the interaction (van de Graaf et al. 2006b). TRPV5 also interacts with NHERF4; however, the interacting motif is located between residues 596 and 617 of mouse TRPV5 (603–624 of human TRPV5) (van de Graaf et al. 2006b). In contrast, NHERF4 interact with the last three amino acid residues of TRPV6 (Kim et al. 2007), suggesting it is likely TRPV6 is the real binding partner of NHERF4.

The 4th PDZ domain of NHERF4 and the second PDZ domain of NHERF2 are essential for their interactions with TRPV5 (Kim et al. 2007; Palmada et al. 2005). NHERF2 is important to the effects of SGK1 and WNK4 on TRPV5 (Embark et al. 2004; Jing et al. 2011).

6.1.4 Calbindin-D_{28k}

Calbindins are Ca^{2+}-binding proteins that are well known for their responsiveness to vitamin D and possible roles in Ca^{2+} absorption and reabsorption (Christakos et al. 1992). Calbindin-D_{28k} is co-expressed with TRPV5 in DCT and CNT (Loffing et al. 2001). Both N- and C-termini of TRPV5 interact with calbindin-D_{28k} in the absence but not in the presence of Ca^{2+} ions (Lambers et al. 2006). When $[Ca^{2+}]_i$ is low, calbindin-D_{28k} translocates toward the plasma membrane and associates with TRPV5. Calbindin-D_{28k} buffers Ca^{2+} close to the vicinity of the channel opening, thereby reducing local accumulation of free Ca^{2+} ions. After binding to Ca^{2+}, calbindin-D_{28k} disassociates from TRPV5 and facilitates the diffusion of Ca^{2+} to the basolateral membrane (Lambers et al. 2006). Calbindin-D_{28k} expression in the kidney is greatly reduced in mice lacking TRPV5 (Hoenderop et al. 2003a). Mice lacking calbindin-D_{28k} do not show hypercalciuria, and mice lacking both TRPV5 and calbindin-D_{28k} do not exhibit more severe phenotype in Ca^{2+} homeostasis than mice lacking TRPV5 alone (Gkika et al. 2006a). These results indicate TRPV5 but not calbindin-D_{28k} is critical to Ca^{2+} reabsorption. Calbindin-D_{28k} acts as a dynamic Ca^{2+} buffer to avoid a sudden elevation of $[Ca^{2+}]_i$; however, it appears that its role could be compensated by other vitamin D-regulated Ca^{2+}-binding proteins. The role of calbindin-D_{28k} in Ca^{2+} homeostasis could only be observed in the absence of the effects of vitamin D: mice lacking vitamin D receptor (VDR) and calbindin-D_{28k} display more severe hypercalciuria and secondary hyperparathyroidism than mice lacking VDR alone (Zheng et al. 2004).

6.1.5 Calmodulin

Being an intracellular Ca^{2+} sensor, calmodulin (CaM) is expected to play a role in modulating TRPV5 function. However, CaM antagonists calmidazolium R24571 and trifluoperazine only modestly inhibit TRPV5 at high concentrations (Nilius et al. 2001a). In addition, Ca^{2+}-insensitive CaM mutants significantly reduced Na^+ and Ca^{2+} currents of TRPV6 but not those of TRPV5 (Lambers et al. 2004). Yet, CaM is capable of binding to TRPV5. So far five CaM-binding sites in TRPV5 have been identified, including residues 696–712 (de Groot et al. 2011a; Kovalevskaya et al. 2012) and 591–612 (Kovalevskaya et al. 2012; Holakovska et al. 2011) in the C terminus, 401–428 in the first intracellular loop (Kovalevskaya et al. 2012), and 310–330 and 133–154 in the N-terminal region (Kovalevskaya et al. 2012). These CaM-binding sites display diversity in binding mode, stoichiometry, and affinity in interaction with CaM in vitro (Kovalevskaya et al. 2012). The first intracellular loop of TRPV5/6 plays a critical role in the fast and slow inactivation kinetics (Nilius et al. 2002), but it is unclear to what extent Ca^{2+}/CaM is involved in this process. CaM negatively modulates TRPV5 activity by binding to the residues 696–729, and PTH-mediated PKA phosphorylation of the CaM-binding site reverses this action

(de Groot et al. 2009, 2011a). In addition, phosphorylation of TRPV5 by PKC at serine 144 (S144) in the N-terminal CaM-binding site (133–154) results in decreased pore size and open probability in TRPV5 (Tudpor et al. 2012). This regulation is important in mediating the action of plasmin on TRPV5 (Tudpor et al. 2012).

6.1.6 80K-H

80K-H is a Ca^{2+}-binding protein and a PKC substrate (Sakai et al. 1989). It was identified as a potential TRPV5-interacting protein because it was downregulated in $1,25(OH)_2D_3$-deficient mice and was upregulated by dietary Ca^{2+} (Gkika et al. 2004). 80K-H-binding site in TRPV5 is located between residues 598 and 608 (Gkika et al. 2004). Co-expression of 80K-H did not alter the level of TRPV5 in the cell surface, but increased the sensitivity of TRPV5 to $[Ca^{2+}]_i$ (Gkika et al. 2004). Thus, 80K-H acts as a Ca^{2+} sensor to control the activity of TRPV5 in addition to CaM.

6.1.7 BSPRY

BSPRY (B-box and SPRY-domain-containing protein), a protein with unknown function, interacts with TRPV5 C terminus (van de Graaf et al. 2006c). It inhibits Ca^{2+} transport activity of TRPV5 without altering its surface level. BSPRY co-localized with TRPV5 in the DCT and CNT, and it is inversely regulated by $1,25(OH)_2D_3$ (van de Graaf et al. 2006c). Thus, BSPRY adds a layer of regulation of TRPV5 by $1,25(OH)_2D_3$.

6.1.8 FKBP52

Immunophilin FKBP52, one of the downstream targets of FK-506, co-localizes with TRPV5 in the DCT and CNT and inhibits TRPV5 activity in vitro (Gkika et al. 2006c). The peptidyl-propyl *cis-trans* isomerase activity of FKBP52 is essential to its inhibitory effect on TRPV5, and the inhibitory effect is reversed by the administration of FK-506. FKBP52 interacts with full-length TRPV5, but not its N- or C terminus (Gkika et al. 2006c). FKBP52 expression is decreased in *Trpv5* KO mice, indicating a link between the two proteins (Gkika et al. 2006c). Since FK-506 induces hypercalciuria and causes a reduction in calbindin-D_{28k} and TRPV5 expression in the kidney (Nijenhuis et al. 2004; Lee et al. 2011; Aicher et al. 1997), further investigation is necessary to clarify the role of interaction between FKBP52 and TRPV5 in FK-506-induced hypercalciuria.

6.2 Regulation of TRPV5 by Co-expressed Proteins

In addition to the proteins that interact directly with TRPV5, proteins co-expressed with TRPV5 may also regulate TRPV5. These TRPV5-regulating proteins were studied because of physiological relevance, not as results of searching for TRPV5-binding partners. Transient protein interaction may or may not be involved in these regulations.

6.2.1 Klotho

Klotho functions to suppress aging process (Kuro-o et al. 1997). Klotho exists in membrane bound and circulating forms; it converts fibroblast growth factor (FGF) receptor FGFR1(IIIc) into a specific receptor for FGF 23 by binding with it (Urakawa et al. 2006). In the kidney, klotho is expressed in the DCT where it co-localizes with TRPV5 and increases TRPV5 activity in vitro (Chang et al. 2005; Lu et al. 2008; Cha et al. 2008a). The mechanism by which klotho regulates TRPV5 is not well understood. Because klotho exhibits β-glucuronidase activity (Tohyama et al. 2004), it was thought that klotho modifies TRPV5 glycan through this activity (Chang et al. 2005). Like klotho, β-glucuronidase also activates TRPV5 and TRPV6 but not related TRP channels TRPV4 and TRPM6 (Lu et al. 2008). Alternatively, klotho removes terminal sialic acids from their glycan chains of TRPV5 and exposes disaccharide galactose-*N*-acetylglucosamine, which binds to galactoside-binding lectin galectin-1 (Cha et al. 2008a). The galectin-1-linked TRPV5 proteins are likely resistant to endocytosis. However, complete removal of *N*-glycan by endoglycosidase-F also increases TRPV5 activity (Lu et al. 2008). However, galectin-3 but not galactin-1 co-localizes with TRPV5 in the DCT (Leunissen et al. 2013). Sialidase appears to increase TRPV5 activity by inhibiting lipid-raft-mediated endocytosis, and it does not discriminate *N*-glycan-deficient N358Q mutant and wild-type TRPV5 (Leunissen et al. 2013). Thus, klotho regulates TRPV5 in an *N*-glycan-dependent manner, whereas sialidase regulates TRPV5 in an *N*-glycan-independent and lipid-raft-mediated endocytosis-dependent manner (Leunissen et al. 2013). Although the mechanisms are still to be clarified, the regulation of TRPV5 by klotho represents a novel area of ion channel regulation. The dysregulation of TRPV5 may be responsible for the increased excretion of Ca^{2+} in *Kl* (klotho gene)-deficient mice, in which PTH-stimulated Ca^{2+} reabsorption in the CNT is impaired (Tsuruoka et al. 2006). Lower plasma klotho concentration is associated with older age and lower serum Ca^{2+} level and is an independent risk factor for mortality in community-dwelling adults of 65 years or older (Semba et al. 2011). Presumably reduced TRPV5 activity due to the lower klotho level likely contributes to the lower serum Ca^{2+} and may play a role in the aging process. Although klotho is considered a hormone, its action on TRPV5 from the luminal side is distinct from its hormonal action from interstitium. However, it could be considered as a factor in the tubular fluid similar to plasmin and uromodulin.

6.2.2 Tissue Transglutaminase

Tissue transglutaminase (tTG) catalyzes Ca^{2+}-dependent covalent cross-linking of specific lysine and glutamine residues of substrate proteins (Lorand and Graham 2003). Calbindin-D_{28K} and S100A10, which regulate TRPV5, are substrates of tTG (Vig et al. 2007; van de Graaf et al. 2003; Ruse et al. 2001; Lambers et al. 2006). In addition, activity of TRPV5 is inhibited by extracellular tTG treatment in HEK-293 and in rabbit CNT/CCD cells (Boros et al. 2012). This is caused by the reduction in channel pore diameter in aggregated TRPV5 in the plasma membrane as a result of tTG activity. *N*-glycosylation-deficient TRPV5 mutant is insensitive to tTG (Boros

et al. 2012). Klotho and tTG both require the presence of *N*-glycan of TRPV5 for their action, but they direct TRPV5 activity to opposite directions.

6.2.3 Tissue Kallikrein

Tissue kallikrein (TK) is a serine protease expressed in CNT (Figueroa et al. 1988). TK knockout (KO) mice exhibit hypercalciuria (Picard et al. 2005). Consistent with this, TRPV5 was shown to be regulated by TK via PLC/diacylglycerol/PKC pathway (Gkika et al. 2006b). TK enhances TRPV5-mediated Ca^{2+} influx by delaying its retrieval from the plasma membrane (Gkika et al. 2006b). This effect was abolished by S299A and S654A mutations, which may disrupt phosphorylation of TRPV5 by PKC (Gkika et al. 2006b), which increases TRPV5 activity by inhibiting its endocytosis from plasma membrane (Cha et al. 2008b).

6.2.4 SGK1/3

Co-expression of NHERF2 and serum/glucocorticoid regulated kinase 1 or 3 (SGK1/3) with TRPV5 enhances TRPV5 activity in *X. laevis* oocytes (Embark et al. 2004). However, NHERF2 or SGK1/3 alone does not alter TRPV5 activity (Embark et al. 2004). The second PDZ domain in NHERF2 is required for the stimulatory effect of SGK1/NHERF2 on TRPV5 (Palmada et al. 2005). In the SGK1 KO mice, TRPV5 protein abundance in the CNT was reduced; however, urinary Ca^{2+} excretion was also reduced (Sandulache et al. 2006). The increased Ca^{2+} reabsorption is likely due to a compensatory increase in the function of thick ascending limb in response to the salt loss in the aldosterone-sensitive distal nephron. The reduced expression of TRPV5 in the CNT of SGK1 KO mice is possibly a compensatory effect due to increased Ca^{2+} reabsorption in the thick ascending limb, not necessarily a result due to the lack of positive regulation of TRPV5 by SGK1 (Sandulache et al. 2006).

6.2.5 WNK Kinases

Point mutations in with-no-lysine (K) kinase 4 (WNK4) result in pseudohypoaldosteronism type II (PHAII, also known as familial hyperkalemia and hypertension or Gordon's syndrome) (Wilson et al. 2001). Hypercalciuria was observed in PHAII patients with $WNK4^{Q565E}$ mutation, leaving the possibility that WNK4 may regulate a Ca^{2+} channel or transporter in the kidney (Mayan et al. 2004). We found WNK4 increases the activity of TRPV5 channel by increasing the forward trafficking of the channel to the plasma membrane via the secretory pathway in *X. laevis* oocytes (Jiang et al. 2007, 2008). The positive effect of WNK4 on TRPV5 was greatly reduced when NCC was co-expressed (Jiang et al. 2007). Similar to WNK4, WNK3 also increases Ca^{2+} influx mediated by TRPV5 via a kinase-dependent pathway (Zhang et al. 2008). The effect of WNK4 on TRPV5 is enhanced and stabilized by NHERF2 (Jing et al. 2011). When the last two amino acid residues of TRPV5 were replaced by those of TRPV6, the effect of NHERF2 on TRPV5 was abolished (Jing et al. 2011). Thus, WNK4 and NHERF2 increase TRPV5 forward trafficking and membrane stability synergistically, leading to additive enhancement in TRPV5-mediated Ca^{2+} transport. On the other hand, Cha et al. found that WNK4

kinase enhances the endocytosis and decreases the plasma membrane abundance of TRPV5 in HEK293 cells (Cha and Huang 2010). The reason for the difference in WNK4-mediated regulation on TRPV5 is unclear.

6.2.6 Ubiquitin E3 Ligases

Nedd4-2 is an archetypal member of the ubiquitin E3 ligase family regulating cell surface stability of membrane proteins (Staub and Rotin 2006). Nedd4-2 is expressed in the DCT (Verrey et al. 2003) and CCD (Flores et al. 2005) in the kidney where TRPV5 is functionally expressed. When expressed in *Xenopus* oocytes, TRPV5/6-mediated Ca^{2+} uptake and Na^{+} current were decreased by Nedd4-2 and Nedd4 due to the reduction in TRPV5 protein level (Zhang et al. 2010). In all cases, Nedd4-2 exhibited stronger inhibitory effects than Nedd4 on both TRPV5 and TRPV6. WW1 and WW2 domains of Nedd4-2 may serve as a molecular switch to limit the ubiquitination of TRPV6/5 by the HECT domain (Zhang et al. 2010). Although Nedd4 and Nedd4-2 mediate TRPV5 degradation in vitro, it is unclear to what extent they do so in vivo. In addition, the degradation of TRPV5 is reduced by knocking down of Ubiquitin recoginition 4 (UBR4), another E3 ubiquitin ligase (Radhakrishnan et al. 2013). The physiological significance of these TRPV5 degradation pathways warrants further studies.

6.2.7 Ca^{2+}-Sensing Receptor

The mRNA and protein of Ca^{2+}-sensing receptor (CaR) are expressed in the DCT/CNT (Riccardi et al. 1996, 1998; Hoenderop et al. 1999). Activation of CaR increases TRPV5-mediated currents and elevates $[Ca^{2+}]_i$ in cells co-expressing TRPV5 and CaR (Topala et al. 2009). Phorbol-12-myristate-13-acetate (PMA)-insensitive PKC isoforms are likely involved in the signal pathway by which CaR stimulates TRPV5 (Topala et al. 2009). The stimulatory effect was abolished by mutation of two putative PKC phosphorylation sites, S299 and S654 in TRPV5, or by a dominant-negative CaR (R185Q) (Topala et al. 2009).

6.3 Regulation by Proteins in the Tubular Fluid

6.3.1 Plasmin

Plasminogen, which is elevated and can be filtered into the urine in nephrotic syndrome, is converted into active plasmin in the tubular fluid. When incubated with plasmin, either from commercial source or purified from nephrotic urine, TRPV5-mediated Ca^{2+} uptake in HEK-293 cells was inhibited (Tudpor et al. 2012). As a serine protease, plasmin does not cleave TRPV5 or alter TRPV5 surface abundance; instead, it affects TRPV5 by binding to protease-activated receptor-1 (PAR-1) (Tudpor et al. 2012). This activates the PAR-1/PLC/PKC pathway, which likely leads to phosphorylation of S144 within a CaM-binding site in the N terminus of TRPV5. The phosphorylation of S144 results in an alteration of CaM binding to TRPV5 and, in turn, a reduction in pore size and

open probability (Tudpor et al. 2012). Suppression of TRPV5 by plasmin likely takes effect in nephrotic patients.

6.3.2 Uromodulin

Uromodulin (also known as Tamm–Horsfall glycoprotein/THP) is a urinary glycoprotein secreted by the thick ascending loop of Henle (Bachmann et al. 1990). Co-expression with uromodulin increases TRPV5 current density and surface abundance in HEK293 cells (Wolf et al. 2013). Acting from the tubular luminal side, uromodulin decreases caveolin-mediated endocytosis of TRPV5, and the level of TRPV5 is lower in uromodulin KO mice (Wolf et al. 2013). This result suggests that uromodulin may act as a physiological regulator of TRPV5 to prevent kidney stone formation.

7 Regulation by Hormones

Trpv5 expression is regulated under physiological conditions. *Trpv5* expression in the kidney is upregulated under dietary Ca^{2+} restriction in mice (Song et al. 2003; Van Cromphaut et al. 2001); this regulation appears to be VDR dependent (Van Cromphaut et al. 2001). TRPV6 and TRPV5 in rat small intestine were decreased by immobilization and induced by endurance swimming, and a $1,25(OH)_2D_3$-dependent pathway is likely involved in these changes (Sato et al. 2006; Teeraporn-puntakit et al. 2009). TRPV5 was upregulated by high-salt intake and downregulated by low-salt intake or dehydration in rats; this was likely caused by the increase or decrease in the delivery of Ca^{2+} to the distal tubule in the kidney (Lee et al. 2012). Aging is associated with alterations in Ca^{2+} homeostasis, such as decreased Ca^{2+} absorption and increased urinary Ca^{2+} excretion. Duodenal TRPV5/6 mRNA level in adult (12-month old) rats was less than half of that in young (2-month old) rats (Brown et al. 2005). *Trpv5* KO mice develop age-related hyperparathyroidism and osteoporotic characteristics earlier than wild-type mice, possibly due to the age-related vitamin D resistance and less robust compensatory expression of intestinal TRPV6 in older mice (van Abel et al. 2006). Thus, as *Trpv5* KO mice age, vitamin D resistance prevents the animals to compensate the renal loss of Ca^{2+} through intestinal absorption, leading to a negative Ca^{2+} balance.

Hormonal regulation is at least in part behind the adaptation of TRPV5 expression in response to physiological conditions. Indeed, a number of hormones, including $1,25(OH)_2D_3$, PTH, sex hormones, and vasopressin, have been found to regulate TRPV5 (Table 2). The effects of these hormones on TRPV5 are summarized briefly below.

7.1 Vitamin D

In 1991, Bindels and colleagues reported that $1,25(OH)_2D_3$ increases transcellular Ca^{2+} absorption in primary culture of rabbit kidney CCD (Bindels et al. 1991)

Table 2 Hormones that regulate TRPV5

Hormones	Effects	References
1,25 $(OH)_2D_3$	Increases *Trpv5* transcription	Hoenderop et al. (2001a, 2002), Song et al. (2003)
PTH	Increases TRPV5 expression and increases TRPV5 activity via PKA and/or PKC	van Abel et al. (2005), Cha et al. (2008b), de Groot et al. (2009)
Estrogen	Acutely increases TRPV5 activity and chronically increases TRPV5 expression	Oz et al. (2007), van Abel et al. (2002), Irnaten et al. (2009)
Testosterone	Decreases TRPV5 expression	Hsu et al. (2010)
Vasopressin	Increase transepithelial Ca^{2+} transport by the activation of cAMP/PKA pathway	Diepens et al. (2004), Hofmeister et al. (2009), van Baal et al. (1996)

where TRPV5 was later cloned (Hoenderop et al. 1999). After the identification, TRPV5 mRNA was shown to be upregulated by $1,25(OH)_2D_3$ in the kidney of vitamin D-depleted rats (Hoenderop et al. 2001a). Moreover, a single injection of $1,25(OH)_2D_3$ in vitamin D-deficient mice induced ~three- to fourfold increase in TRPV5 mRNA in the kidney that peaked at 12 h after injection following the peak of duodenal TRPV6 (6 h after injection) (Song et al. 2003). This suggests that the increase in TRPV5 in the kidney might be a secondary event due to the increased Ca^{2+} absorption. In mice deficient in 25-hydroxyvitamin D_3-1α-hydroxylase with undetectable level of $1,25(OH)_2D_3$, the reduction of renal TRPV5 and hypocalcemia were normalized by high Ca^{2+} diet, suggesting that TRPV5 is upregulated by increased Ca^{2+} load to the distal tubule as a result of $1,25(OH)_2D_3$-induced Ca^{2+} absorption (Hoenderop et al. 2002). Although potential vitamin D response elements (VDREs) have been identified in the promoter region of *Trpv5* gene (Muller et al. 2000; Weber et al. 2001), their function has not been examined as those bona fide VDREs in *Trpv6* gene promoter (Meyer et al. 2006). At least in mice duodenum, FGF-23 could reduce circulating $1,25(OH)_2D_3$ and diminish 1,25 $(OH)_2D_3$-induced surge of TRPV6 and TRPV5 (Khuituan et al. 2012).

7.2 Parathyroid Hormone

PTH regulates both the expression and activity of TRPV5. In parathyroidectomized rats and calcimimetic compound NPS R-467-infused mice that had a lower PTH level, renal TRPV5, calbindin-D_{28k}, and NCX1 levels were decreased; and they were restored by PTH supplementation (van Abel et al. 2005). The decrease in other Ca^{2+} transport proteins by PTH is likely secondary to the reduction of TRPV5-mediated Ca^{2+} influx (van Abel et al. 2005).

PTH is also capable of acutely increasing Ca^{2+} transport in the distal tubule (Bacskai and Friedman 1990). Both PKA and PKC are involved in this regulation (Friedman et al. 1996). Indeed, activation of TRPV5 by PTH via both PKA and PKC has been reported (de Groot et al. 2009; Cha et al. 2008b). PTH activates the cAMP–PKA signaling cascade and increases TRPV5 open probability via phosphorylation of threonine 709 (T709) in TRPV5 (de Groot et al. 2009). This

regulation requires a strong buffering of intracellular Ca^{2+}. However, the PKA phosphorylation site T709 is not conserved in human TRPV5. In addition to PKA, the increase of TRPV5 activity by heterogeneously expressed PTH receptor was prevented by PKC inhibitor (Cha et al. 2008b). Mutation of PKC phosphorylation sites S299/S654 in TRPV5 abolished the regulation. Caveolae-mediated endocytosis of TRPV5 appears to be inhibited by PTH via a PKC-dependent pathway (Cha et al. 2008b).

Besides vitamin D and PTH, calcitonin is a hormone that regulates Ca^{2+} homeostasis. However, calcitonin affects renal Ca^{2+} reabsorption mainly through the thick ascending limb (Elalouf et al. 1984; Di Stefano et al. 1990). TRPV5, which is mostly expressed in the DCT and CNT, is not involved in the effects of calcitonin on urinary excretion of Ca^{2+}, Na^{+}, and K^{+} as no difference was observed between wild-type and *Trpv5* KO mice in this regard (Hsu et al. 2009).

7.3 Estrogen and Testosterone

As the most potent estrogen, 17β-estradiol increases TRPV5 mRNA and protein expression in the kidney (van Abel et al. 2002, 2003). Estrogen deficiency in aromatase-deficient mice results in decreased mRNA levels of renal TRPV5 and other Ca^{2+} transporters and Ca^{2+} wasting (Oz et al. 2007). In addition, 17β-estradiol (20–50 nM) rapidly increases TRPV5 current and $[Ca^{2+}]_i$ in rat CCD cells (Irnaten et al. 2009). Thus, estrogen acutely elevates TRPV5 activity and chronically increases its expression.

In contrast to the positive effects of estrogen, male hormone testosterone appears to have a negative effect on TRPV5. Male mice have higher urinary Ca^{2+} excretion than female mice (Hsu et al. 2010). Androgen deficiency increases renal TRPV5 mRNA and protein and decreases urinary Ca^{2+} excretion; these were normalized by testosterone treatment (Hsu et al. 2010). In addition, the negative effect of dihydrotestosterone on transcellular Ca^{2+} transport was demonstrated in primary rabbit CNT/CCD cells (Hsu et al. 2010).

7.4 Vasopressin

Arginine vasopressin stimulates transepithelial Ca^{2+} transport in primary cultures of rabbit CCD cells (van Baal et al. 1996). Vasopressin induces an increase in Ca^{2+} uptake and $[Ca^{2+}]_i$ in mpkDCT cells and freshly isolated late DCT and CNT cells, respectively, indicating that TRPV5 is a target of vasopressin (Diepens et al. 2004; Hofmeister et al. 2009).

8 Physiological Functions in Native Cells, Organs, Organ Systems

8.1 Ca^{2+} Reabsorption in the Kidney

TRPV5 is mainly distributed in the apical membrane of tubular cells in the DCT and CNT where it mediates the final reabsorption of Ca^{2+} via a transcellular pathway. TRPV5, calbindin-D_{28k}, NCX1, and PMCA are the major components of the pathway. Lines of evidence suggest that the expression of other Ca^{2+} transporters depends on TRPV5-mediated Ca^{2+} influx (van Abel et al. 2005; Hoenderop et al. 2003a). In this sense, TRPV5 is the rate-limiting component in the transcellular pathway of Ca^{2+} reabsorption.

TRPV5 is likely constitutively active in the apical membrane of tubular cells. At macroscopic level, TRPV5 acts as a facilitative transporter exhibiting saturable kinetics with apparent K_m values at sub-millimolar range, which are well suited for the luminal Ca^{2+} level in the distal tubule (Hoenderop et al. 1999; Peng et al. 1999, 2000a, b). The responsiveness of TRPV5 to vitamin D, PTH, Ca^{2+} load, pH, and tubular factors such as klotho, uromodulin, and plasmin makes TRPV5 well suited for the fine-tuning of Ca^{2+} reabsorption in the kidney.

8.2 Bone Resorption by Osteoclasts

TRPV5 also expresses in the ruffled border membrane of mouse osteoclasts (van der Eerden et al. 2005). Osteoclast numbers and area are increased, and paradoxically urinary bone resorption marker deoxypyridinoline was reduced in mice lacking TRPV5 (Hoenderop et al. 2003a). In vitro bone marrow culture system indicates that bone resorption by osteoclasts was impaired in *Trpv5* KO mice (van der Eerden et al. 2005). These results suggest a malfunction of osteoclast to some degree in the absence of TRPV5. However, bone resorption inhibitor alendronate normalizes the reduced bone thickness in *Trpv5* KO mice, even though it specifically increases bone TRPV5 expression (Nijenhuis et al. 2008). Furthermore, vitamin D analog ZK191784 partially restores the decreased bone matrix mineralization in *Trpv5* KO mice, suggesting the bone phenotype of *Trpv5* KO mice is secondary to the elevated $1,25(OH)_2D_3$ (van der Eerden et al. 2013). Thus, TRPV5 plays a role in osteoclast function, but it is not absolutely required for bone reabsorption. The role of TRPV5 in osteoclast function is not well understood, but TRPV5 is involved in receptor activator of NF-κB ligand (RANKL)-induced rise in $[Ca^{2+}]_i$ in human osteoclasts (Chamoux et al. 2010). This process is likely a part of the negative feedback loop to terminate RANKL-induced bone resorption (Chamoux et al. 2010).

8.3 Function in the Inner Ear

The low Ca^{2+} concentration of mammalian endolymph in the inner ear is required for normal hearing and balance. Marcus and colleagues showed that TRPV5 and TRPV6 may play roles in the function of inner ear (Yamauchi et al. 2005, 2010; Wangemann et al. 2007; Nakaya et al. 2007). TRPV5 was detected in native semicircular canal duct (SCCD) epithelial cells, cochlear lateral wall, and stria vascularis of adult rats along with other Ca^{2+} transport proteins (Yamauchi et al. 2010). TRPV5 protein was localized close to the apical membrane of strial marginal cells and in outer and inner sulcus cells of the cochlea (Yamauchi et al. 2010). TRPV5 transcript was responsive to $1,25(OH)_2D_3$ (Yamauchi et al. 2005); however, the protein level was not upregulated (Yamauchi et al. 2010). The levels of TRPV5 were decreased in the inner ear of older mice (Takumida et al. 2009). Mutations in pendrin (SLC26A4, an anion exchanger) cause the most common form of syndromic deafness. Reduced pH and utricular endolymphatic potential and increased Ca^{2+} concentration were found in pendrin KO mice (Wangemann et al. 2007; Nakaya et al. 2007). The reduced pH likely blocks the activity of TRPV5 and TRPV6, whose Ca^{2+} transport activity is reduced at low pH, similar to what was observed in primary SCCD cells (Nakaya et al. 2007). The elevation of endolymphatic Ca^{2+} level in pendrin KO mice may inhibit sensory transduction necessary for hearing and promote the degeneration of the sensory hair cells, which is necessary for the development of normal hearing (Wangemann et al. 2007).

8.4 Intestinal and Placental Ca^{2+} Transport

The levels of TRPV5 mRNA in intestine and placenta are much lower than TRPV6 (Peng et al. 2001). TRPV5 is regulated in the same direction with TRPV6 in the intestine in most cases, such as immobilization and exercise-induced alteration of gene expression (Teerapornpuntakit et al. 2009; Sato et al. 2006). In the placenta, Ca^{2+} transport in human syncytiotrophoblasts is insensitive to voltage and L-type Ca^{2+} channel modulators but is sensitive to TRPV5/6 blocker Mg^{2+} and ruthenium red (Moreau et al. 2002a). In cultured human trophoblasts isolated from term placenta, TRPV5 and TRPV6 expression correlated with the Ca^{2+} uptake potential along the differentiation of the trophoblasts (Moreau et al. 2002b). In contrast to the demonstrated role of TRPV6 in placental Ca^{2+} transport (Suzuki et al. 2008), the role of TRPV5 is unclear. TRPV5 likely plays a minor role in intestinal and placental Ca^{2+} transport due to its low levels of expression in these organs.

Table 3 Phenotype of *Trpv5* KO mice

Parameter	Alteration	Mechanism/explanation	References
Serum1,25 $(OH)_2D_3$	Elevated (2.9-fold)	Compensatory response	Hoenderop et al. (2003a)
Serum PTH	Elevated in older mice	Compensatory response	van Abel et al. (2006)
Ca^{2+} absorption	Increased (~30 % more). No increase in the absence of 25(OH) D_3 1α-hydroxylase	Due to the elevated 1,25$(OH)_2D_3$ and in turn increased intestinal TRPV6 and calbindin-D_{9k}	Hoenderop et al. (2003a), Renkema et al. (2005)
Ca^{2+} excretion	Increased (~sixfold)	TRPV5 plays a role in Ca^{2+} reabsorption	Hoenderop et al. (2003a)
Renal function	Increased urine volume and reduced urine pH	Activation of CaR in the collecting duct and subsequent upregulation of H^+–ATPase and reduction of aquaporin 2	Hoenderop et al. (2003a), Renkema et al. (2009b)
Bone	Reduced bone thickness	Prolonged elevation of 1,25 $(OH)_2D_3$	Hoenderop et al. (2003a), Nijenhuis et al. (2008), van der Eerden et al. (2013)

9 Lesson from *TRPV5* Knockout Mice

The most distinctive features of *Trpv5* KO mice include 2.9-fold elevation in serum 1,25$(OH)_2D_3$, sixfold increase in Ca^{2+} excretion, moderate reduced bone thickness in the femoral head, and normal level of plasma Ca^{2+} (Hoenderop et al. 2003a). In addition, polyuria and urine acidification were observed in *Trpv5* KO mice (Hoenderop et al. 2003a). These alterations in renal function are caused by the activation of CaR by the increased tubular Ca^{2+} in the collecting duct and subsequently upregulated proton secretion by the H^+–ATPase and reduced water reabsorption due to the downregulation of aquaporin 2 (Renkema et al. 2009b). These physiological adaptations reduce the risk of Ca^{2+} precipitations and stone formation. Intestinal Ca^{2+} absorption is increased in *Trpv5* KO mice as duodenal TRPV6 and calbindin-D_{9k} mRNAs are increased due to the elevated 1,25$(OH)_2D_3$ level (Hoenderop et al. 2003a). However, the serum Ca^{2+} levels are largely normal, even though the PTH level is elevated in older *Trpv5* KO mice (van Abel et al. 2006). The major phenotypes of *Trpv5* KO mice are listed in Table 3.

Studies with *Trpv5* KO mice reveal some important points in Ca^{2+} transport physiology: (1) the transcellular Ca^{2+} transport in the DCT and CNT, as represented by TRPV5, plays an important role in Ca^{2+} reabsorption. This is supported by the fact that removal of TRPV5 results in significant hypercalciuria. (2) TRPV5 is not indispensable to maintain a Ca^{2+} balance as long as vitamin D and related systems work normally. The body reset Ca^{2+} homeostasis by elevating the level of 1,25 $(OH)_2D_3$, which increases intestinal Ca^{2+} absorption to compensate the renal loss of

Ca^{2+} (Renkema et al. 2005). A new Ca^{2+} balance is achieved at higher intestinal absorption and higher urinary Ca^{2+} excretion in the absence of TRPV5. (3) The TRPV5 KO model shows an example that polyuria and reduced urinary pH are natural responses to increased Ca^{2+} excretion in order to prevent kidney stone formation. In addition, *Trpv5* KO mouse is a useful model to clarify some basic physiological mechanisms. For example, with this model it has been shown that calcitonin or thiazide-induced hypocalciuria occurs independent of TRPV5; thus, the responsible mechanisms likely reside in more proximal segments of the nephron (Hsu et al. 2009; Nijenhuis et al. 2005). All the studies indicate that TRPV5 does not alter the bulk Ca^{2+} reabsorption; rather, it provides a key mechanism for the fine-tuning of Ca^{2+} reabsorption.

10 Role in Hereditary and Acquired Diseases

As we learned from *Trpv5* KO mice, the lack of TRPV5 could be compensated through an elevation of $1,25(OH)_2D_3$, which regulates other proteins (e.g., TRPV6) in maintaining Ca^{2+} homeostasis. Thus, humans with defect in TRPV5 may have altered Ca^{2+} homeostasis, but could be clinically asymptomatic except for hypercalciuria. However, no mutation in the exons of *Trpv5* gene was found to be associated with autosomal dominant hypercalciuria (Muller et al. 2002). In another study involving 20 renal hypercalciuria patients, nonsynonymous variation of TRPV5 (A8V, R154H, and A561T) and synonymous variations were identified; however, these variants apparently do not alter the property of TRPV5 (Renkema et al. 2009a). Recently, S682P mutation of TRPV5 causes autosomal dominant hypercalciuria in mouse model (Loh et al. 2013). Interestingly, no significant functional differences were found between S682P and wild-type TRPV5 when expressed in *Xenopus* oocytes or HEK293 cells; the only difference was that S682P produced a lower baseline $[Ca^{2+}]_i$ than wild-type TRPV5 in HEK293 cells (Loh et al. 2013). However, mice with homozygous $TRPV5^{S682P}$ did show reduced TRPV5 immunostaining in the kidney and phenotype resembling that of *Trpv5* KO mice (Loh et al. 2013). This study suggests that mutations in TRPV5 could result in hypercalciuria; however, the functional changes of the TRPV5 mutations may not be detected in vitro. Thus, it remains to be further examined that mutations in *Trpv5* gene cause hypercalciuric disorders in humans.

In addition to a possible role of TRPV5 in hypercalciuria, TRPV5 expression and potentially activity are altered in response to a number of diseases and therapeutic conditions (Table 4). Mechanisms for the change in TRPV5 expression are often not fully understood. TRPV5 expression could be regulated by hormones, chemicals, and likely intracellular or extracellular Ca^{2+}. A change in *Trpv5* expression likely leads to alteration in urinary Ca^{2+} excretion; on the other hand, the alteration of filtered Ca^{2+} load or reabsorption of Ca^{2+} elsewhere in the nephron leads to increased or decreased delivery of Ca^{2+} to the DCT and CNT and in turn causes an elevation or reduction in *Trpv5* expression. If specific modulators for TRPV5 and TRPV6 could be developed, they could be useful to correct

Table 4 Alteration of *Trpv5* expression under disease and therapeutic conditions

Disease/therapeutic conditions	Model	Alterations	References
Arterial calcifications	TIF1alpha-deficient kidneys	mRNA ↑	Ignat et al. (2008)
Familial hypomagnesemia with hypercalciuria and nephrocalcinosis	*Cldn16*$^{-/-}$ mice	mRNA ↑	Will et al. (2010)
Streptozotocin-induced diabetes mellitus	Rats	mRNA/ protein ↑	Lee et al. (2006)
Idiopathic hypercalciuria	Genetic hypercalciuric stone-forming rats	mRNA/ protein ↓	Wang et al. (2008)
Preeclampsia	Primary culture of syncytiotrophoblasts from patients	mRNA/ protein ↓	Hache et al. (2011)
Gitelman syndrome	*Slc12a3* S707X knockin mice	mRNA/ protein↑	Yang et al. (2010)
Hydrochlorothiazide (high dose)	Rats	mRNA/ protein↓	Nijenhuis et al. (2003)
Hydrochlorothiazide (medium dose)	Rats	Protein↑	Jang et al. (2009)
Chlorothiazide(acute, low dose)	Mice	mRNA↑	Lee et al. (2004)
Furosemide	Mice	mRNA↑	Lee et al. (2007)
Gentamicin	Mice	mRNA↑	Lee et al. (2007)
Dexamethasone	Rats/mice	mRNA/ protein↑	Nijenhuis et al. (2004), Kim et al. (2009a, b)
Tacrolimus (FK506)	Rats	mRNA/ protein↓	Nijenhuis et al. (2004)
Chronic exposure to uranium	Rats	mRNA↓	Wade-Gueye et al. (2012)

Expression data were from mouse or rat kidney except for preeclampsia (human syncytiotrophoblasts) and chronic to uranium (cortical and trabecular bone)

abnormality in Ca^{2+} homeostasis under disease or therapeutic conditions. For example, under disease conditions caused by vitamin D signaling defect, such as in vitamin D-dependent rickets, type 2a (Online Mendelian Inheritance in Man #277440), intestinal and renal TRPV5 and TRPV6 expressions are likely disrupted as demonstrated in the *Vdr* KO mice (Van Cromphaut et al. 2001). Enhancing TRPV5- and TRPV6-mediated Ca^{2+} absorption would be helpful to achieve a positive Ca^{2+} under this condition. Similarly, enhancers for TRPV5 and TRPV6 are desirable to prevent osteoporosis in women after the menopause due to reduced estrogen level (Oz et al. 2007; van Abel et al. 2002; Irnaten et al. 2009). On the other hand, TRPV6 inhibitors would be needed in preventing absorptive hypercalciuria and kidney stone disease, and TRPV5 stimulator would be useful in preventing kidney stone formation in patients with renal Ca^{2+} leak. Stimulators of TRPV5 would be also helpful in tacrolimus-induced hypercalciuria (Nijenhuis et al. 2004) or other conditions associated with reduced expression of TRPV5 in Table 4.

11 Single Nucleotide Polymorphisms of TRPV5 in African Populations

TRPV5 and TRPV6 have been shown to have high frequency of SNPs in African populations. By analyzing SNP data from 24 African Americans and 23 European Americans in genes sequenced in SeattleSNPs, a 115-kb region in chromosome 7q34-35 of 4 contiguous genes, including *EPHB6*, *TRPV6*, *TRPV5*, and *KEL4*, was identified with features of a recent demographic selection (Stajich and Hahn 2005; Akey et al. 2004). *TRPV6* SNPs defined by three nonsynonymous SNPs (C157R, M378V, and M681T) exhibit most striking footprint of positive selection (Akey et al. 2006; Hughes et al. 2008). In addition, four nonsynonymous SNPs in TRPV5 were identified by SeattleSNPs: three of them (A8V, A563T, and L712F) were only present in African Americans, not in European Americans; R154H is common in both populations. The nonsynonymous SNP variations in TRPV6 ancestral haplotype are conserved in other species; in contrast, the variations in TRPV5 are newly derived as they are not commonly present in other species surveyed including chimpanzee, dog, rat, and mouse, with the exceptions of 563T in dog and 8V in rat (Na et al. 2009). In addition, the nonsynonymous SNPs of TRPV5 are not associated with each other as are those in TRPV6. By expression in *Xenopus* oocytes, we found that two of the SNPs, A563T and L712F, significantly increased TRPV5-mediated Ca^{2+} uptake by approx. 50 % and 25 %, respectively (Na et al. 2009). For A563T variant, the increased Ca^{2+} uptake activity was not associated with increased protein abundance in the plasma membrane; rather it was associated with increased apparent K_m for Ca^{2+} and increased sensitivity to extracellular Mg^{2+}, suggesting increased permeation of Ca^{2+} in the cation translocation pathway of the channel (Na et al. 2009). The A563 residue in the last transmembrane domain is 20 residues away from D542 residue in the Ca^{2+} filter in the pore. It is likely in the cation translocation path of the channel (Fig. 2).

African Americans exhibit lower urinary Ca^{2+} excretion than Caucasians (Braun et al. 2007; Pratt et al. 1996; Taylor and Curhan 2007), and the risk of kidney stone in African Americans is lower than that in Caucasians (Sarmina et al. 1987; Stamatelou et al. 2003). In addition, African Americans have higher bone mass (Bell et al. 1991) and lower incidence of osteoporosis-related fractures than whites (Bohannon 1999). Because of the high allele frequencies of TRPV5 and TRPV6 SNPs in African populations, these SNPs may contribute to the Ca^{2+} conservation mechanisms in African populations. Further population studies are necessary to clarify the relationship of SNPs in TRPV5 and TRPV6 and Ca^{2+} homeostasis in African populations.

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TRPV6 Channels

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Contents

Abstract

TRPV6 (former synonyms ECAC2, CaT1, CaT-like) displays several specific features which makes it unique among the members of the mammalian *Trp* gene family (1) TRPV6 (and its closest relative, TRPV5) are the only highly Ca^{2+}-selective channels of the entire TRP superfamily (Peng et al. 1999; Wissenbach et al. 2001; Voets et al. 2004). (2) Translation of *Trpv6* initiates at a non-AUG codon, at ACG, located upstream of the annotated AUG, which is not used for initiation (Fecher-Trost et al. 2013). The ACG codon is nevertheless decoded by

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methionine. Not only a very rare event in eukaryotic biology, the full-length TRPV6 protein existing *in vivo* comprises an amino terminus extended by 40 amino acid residues compared to the annotated truncated TRPV6 protein which has been used in most studies on TRPV6 channel activity so far. (In the following numbering occurs according to this full-length protein, with the numbers of the so far annotated truncated protein in brackets). (3) Only in humans a coupled polymorphism of *Trpv6* exists causing three amino acid exchanges and resulting in an ancestral *Trpv6* haplotype and a so-called derived *Trpv6* haplotype (Wissenbach et al. 2001). The ancestral allele encodes the amino acid residues $C_{197(157)}$, $M_{418(378)}$ and $M_{721(681)}$ and the derived alleles $R_{197(157)}$, $V_{418(378)}$ and $T_{721(681)}$. The ancestral haplotype is found in all species, the derived *Trpv6* haplotype has only been identified in humans, and its frequency increases with the distance to the African continent. Apparently the *Trpv6* gene has been a strong target for selection in humans, and its derived variant is one of the few examples showing consistently differences to the orthologues genes of other primates (Akey et al. 2004, 2006; Stajich and Hahn 2005; Hughes et al. 2008). (4) The *Trpv6* gene expression is significantly upregulated in several human malignancies including the most common cancers, prostate and breast cancer (Wissenbach et al. 2001; Zhuang et al. 2002; Fixemer et al. 2003; Bolanz et al. 2008). (5) Male mice lacking functional TRPV6 channels are hypo-/infertile making TRPV6 one of the very few channels essential for male fertility (Weissgerber et al. 2011, 2012).

Keywords
Calcium selective channel • Non AUG translation start • Prostate cancer • Breast cancer • Male fertility • Polymorphism • Epithelial calcium transport

1 Gene

The *Trpv6* gene is located on chromosome 7q33-q34 (human), chromosome 6 (mouse) and chromosome 4 (rat) in close proximity to its closest relative, *Trpv5* (7q35 in human). The deduced protein sequences comprise ~75 % identical amino acids (Peng et al. 1999, 2000; Hoenderop et al. 1999; Muller et al. 2000; Wissenbach et al. 2001; Hirnet et al. 2003). *Trpv5* and *Trpv6* arose by gene duplication from an ancestral gene, and the pufferfish *Takifugu rubripes*, for example, has only one gene which is slightly more similar to *Trpv6* than to *Trpv5* (Qiu and Hogstrand 2004; Peng 2011). The *TRPV5* gene is mainly expressed in the kidney of mammals, whereas *Trpv6* has a broader expression pattern. Therefore, it was speculated that gene duplication reflects the complex renal situation of land-living animals (Peng 2011). The *Trpv5-6*-like genes can be identified in primitive eukaryotic organisms like the choanoflagellate *Monosiga brevicollis* (King et al. 2008). In general *Trpv5-6*-like genes are not present in prokaryotic organisms,

protocysts, fungi and plants. However, the green algae *Chlamydomonas reinhardtii* and *Volvox carteri* exhibit *Trpv5-6*-like genes, and this may reflect horizontal gene transfer at a comparatively late time point during the evolution of these algae (Merchant et al. 2007). The chromosomal organization of *Trpv6* is conserved among several species. In the mouse genome *Trpv6* spans 15 exons and extends over a region of ~15.7 kb. Depending on the species, the deduced amino acid sequence is in the range of 703–767 amino acids.

1.1 Splice Variants and Polymorphisms

To date there are no splice variants known for human and mouse *Trpv6*. But in humans two alleles of the *Trpv6* gene were identified (Wissenbach et al. 2001): one ancestral variant (*Trpv6a*, red in Fig. 1a, b) and a so-called derived variant (*Trpv6b*, yellow in Fig. 1a, b). The cDNA sequences of the two alleles differ in five bases resulting in three amino acid substitutions with $R_{197(157)}$, $V_{418(378)}$ and $T_{721(681)}$ in the derived variant and $C_{197(157)}$, $M_{418(378)}$ and $M_{721(681)}$ in the ancestral variant. Additional polymorphisms found within the intronic regions of the *Trpv6* gene seem to be coupled to those polymorphisms in the coding region (Kessler et al. 2009). The frequencies of the two *Trpv6* alleles are highly variable between different ethnic groups. The percentage of the ancestral *Trpv6a* allele decreases with increasing distance to the African continent (Akey et al. 2006). In a few South African populations, the *Trpv6a* allele frequency is higher than 50 % of the tested alleles and decreases to less than 5 % in East Asian populations. From these data, it was assumed that a so far unknown selection pressure leads to the higher allele frequency of the *Trpv6b* variant in non-African humans (Akey et al. 2004; Stajich and Hahn 2005; Soejima et al. 2009). The physiological consequence of the polymorphisms is not clear yet. After heterologous expression of the *Trpv6a* and *Trpv6b* cDNAs, a non-significant faster Ca^{2+}-dependent inactivation of TRPV6b channels was noted (Hughes et al. 2008), but in general both channel variants revealed very similar biophysical properties (ion selectivity, reversal potential, Ca^{2+}-dependent inactivation, Mg^{2+} block). After *Trpv6* cRNA injection into *Xenopus laevis* oocytes, current amplitudes (measured as amplitudes of endogenous Ca^{2+}-activated Cl^- currents) (Sudo et al. 2010) or $^{45}Ca^{2+}$ uptake (Suzuki et al. 2008b) were larger for TRPV6a than for TRPV6b. Unfortunately, the latter publication lacks adequate controls. Interestingly, Akey et al. (2004) speculated that strong selection on the *Trpv6* locus is correlated to milk consumption and lactase persistence.

1.2 Initiation of Translation from a Non-AUG Codon

Recently our group showed (Fecher-Trost et al. 2013) that the full-length endogenous human TRPV6 protein is 40 amino acid residues longer at the N terminus as previously thought (Fig. 1c). Translation starts at an ACG codon upstream of the

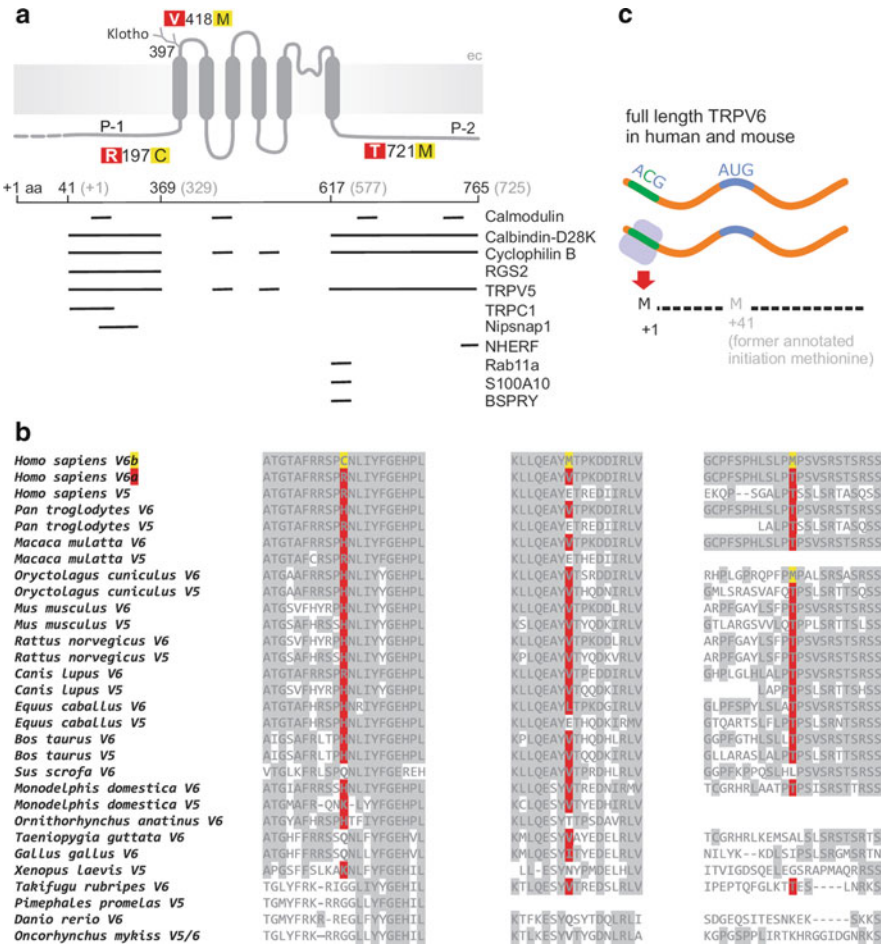

Fig. 1 (**a**) Structure of the TRPV6 channel non-synonymous polymorphisms are indicated: ancestral TRPV6a (*red*) and the derived variant TRPV6b (*yellow*). P-1, phosphorylation sites for *src* kinase (P-1, tyrosine$_{161}$), for PTP1B phosphatase (P-1) and for protein kinase C (P-2, threonine$_{742}$). Below numbering of the amino acids of the full-length TRPV6 (*black*) and the truncated annotated TRPV6 (*grey*). Protein interaction sites are indicated (see also Table 1 and text). (**b**) Multiple alignment of the non-synonymous polymorphism shown in (**a**), among several species (V6) aligned with the corresponding amino acid sequences of TRPV5 (V5). The derived TRPV6b variant (*yellow*) is only present in humans. The amino acid residues in TRPV5 proteins correspond to the amino acids present in TRPV6a. (**c**) Translation initiation of the full-length TRPV6 protein occurs at an ACG which is decoded by methionine and which is localized upstream of the annotated AUG codon

first AUG of the *Trpv6* mRNA. Although ACG codons are normally translated into threonine (T), the translational machinery incorporates methionine (M) instead. Translation of a non-AUG codon into methionine is a rare event, and to our knowledge, only the testis-specific PRPS3 protein (ribose-phosphate pyrophosphokinase 3, NM_175886.2) is also initiated from an ACG codon which

is apparently decoded by methionine (Taira et al. 1990). Alignments of the annotated 5′-untranslated *Trpv6* sequences indicate that in mammals but not in non-mammals, the TRPV6 protein appears to be translated from an ACG codon upstream of the annotated AUG. Accordingly, the murine TRPV6 protein also comprises a longer N terminus, and the initiation triplet is most likely the corresponding ACG codon (Fecher-Trost et al. 2013). Furthermore, analysis of 5′ regions of other *Trpv* genes indicates that translation initiation from an upstream non-AUG codon is an exclusive feature of *Trpv6* which is not met by any other *Trpv* including *Trpv5*.

2 Expression

The murine *Trpv6* is expressed in placenta, pancreas, prostate, epididymis and several parts of the small intestine including duodenum, oesophagus, stomach, colon, kidney and uterus as demonstrated by RT-PCR and Northern blots (Hirnet et al. 2003; Weissgerber et al. 2011, 2012; Lehen'kyi et al. 2012). Compared to mouse there are some differences of the *Trpv6* expression in humans. First, expression of *Trpv6* within small intestine (including duodenum) has not consistently been shown, and second, whereas *Trpv6* is highly expressed in the murine prostate, it is much less expressed in the human prostate; however, it is clearly overexpressed in prostate cancer as shown by Northern blots analysis and in situ hybridization (Wissenbach et al. 2001, 2004; Peng et al. 2001; Fixemer et al. 2003). *Trpv6* appears to be expressed in various cancer cell lines, but direct identification of the TRPV6 protein by mass spectrometry has only been shown in the human breast cancer cell line T47D (Fecher-Trost et al. 2013) and in the human lymph node prostate cancer cell line LNCaP (Fecher-Trost, Wissenbach, and Flockerzi, unpublished data).

In murine kidney only low levels of mRNA were detected by Northern blots and RT-PCR (Hirnet et al. 2003; Song et al. 2003; Peng 2011), but in immunostains the TRPV6 protein was identified in the apical domain of the distal convoluted tubules, in connecting tubules and cortical and medullary collecting ducts (Nijenhuis et al. 2003; Hoenderop et al. 2003a). In rat and in human kidney, *Trpv6* transcripts are not detectable by Northern blots (Peng et al. 1999; Wissenbach et al. 2001; Brown et al. 2005), but fragments of *Trpv6* transcripts could be weakly amplified in human kidney by RT-PCR (Peng et al. 2000; Hoenderop et al. 2001). Apparently, different age, variability of Ca^{2+} and vitamin D within the food and the hormonal state may confound detection of *Trpv6* expression in kidney (Lee et al. 2004; Hoenderop et al. 2005) and also in duodenum. In summary, data from Northern blots consistently confirm *Trpv6* expression in rat small intestine, in human and mouse placenta, pancreas and to a lower extent in the intestine, in human prostate cancer, in mouse placenta and in mouse epididymis. The *Trpv6* cDNAs were originally cloned from rat intestine (Peng et al. 1999) and human placenta (Wissenbach et al. 2001). Using antibodies which discriminate between corresponding tissues from wild-type and *Trpv6*-deficient mice, TRPV6 proteins have been identified in human placenta (Stumpf et al. 2008; Fecher-Trost

et al. 2013), mouse prostate and epididymis (Weissgerber et al. 2011, 2012); by mass spectrometry TRPV6 proteins were directly identified in placenta and the human cancer cell lines T47D (Fecher-Trost et al. 2013) and LNCaP cells.

2.1 Transcriptional Regulation of *Trpv6* by Vitamin D_3

Intestinal Ca^{2+} absorption (Bronner et al. 1986a, b) depends on two major pathways, a paracellular pathway and a transcellular pathway which can be stimulated by vitamin D_3 (Bronner and Pansu 1999; Bronner 2003; Kellett 2011; Lieben and Carmeliet 2012). The transcellular pathway includes a Ca^{2+} uptake channel at the luminal/apical membrane, soluble cytosolic Ca^{2+}-binding proteins and a Ca^{2+} extrusion mechanism at the basal membrane (Hoenderop et al. 2005; Suzuki et al. 2008c); a similar pathway appears to be relevant in placenta (Brunette 1988; Lafond et al. 1991; Belkacemi et al. 2002, 2003, 2004). Initial findings revealed that the amounts of *Trpv6* transcripts in the rat duodenum detected by Northern blots were not changed by prior treatment of the rats by 1,25-dihydroxyvitamin D_3 or by feeding them a Ca^{2+}-deficient diet (Peng et al. 1999), arguing that *Trpv6* expression is not regulated by vitamin D_3 or calcium deficiency in duodenum. In contrast, treatment of Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, by 1,25-dihydroxyvitamin D_3 upregulates *Trpv6* expression (Wood et al. 2001). In addition, *Trpv6* transcript levels were decreased in vitamin D receptor-deficient mice (Okano et al. 2004), whereas 1,25-dihydroxyvitamin D_3 supplementation of *25-hydroxyvitamin D_3-1α-hydroxylase*-deficient mice, which have undetectable levels of endogenous 1,25-dihydroxyvitamin D_3 and suffer from hypocalcaemia, resulted in increased expression of TRPV6, calbindin-D9K and PMCA1b and normalization of serum Ca^{2+} (van Abel et al. 2003). Binding sites for 1,25-dihydroxyvitamin D_3 and enhancer regions within the *Trpv6* promoter have been identified and characterized in detail (Pike et al. 2007), and it was concluded that transcellular Ca^{2+} uptake involves TRPV6 channels at the luminal intestinal side as primary Ca^{2+} uptake mechanism. Intracellular Ca^{2+} is then bound to calbindin 9K and transported across the cell to the basolateral side where it is extruded through the plasma membrane Ca^{2+}-ATPase (PMCA1b) and the Na^+/Ca^{2+} exchanger (van Abel et al. 2003). However, this model had to be reconsidered by the finding that duodenal Ca^{2+} uptake is normal in *Calbindin 9K*-deficient mice (Lee et al. 2007). In *Calbindin 9K*-/*Calbindin 28K*-double knockout mice, serum Ca^{2+} levels and bone length were decreased only under a low Ca^{2+} diet (Ko et al. 2009). In the *Trpv6*-deficient (Bianco et al. 2007; Kutuzova et al. 2008) and in the $Trpv6^{D/A/D/A}$ knock-in mouse lines (Weissgerber et al. 2011; Woudenberg-Vrenken et al. 2012), respectively, no functional TRPV6 channels are expressed; intestinal Ca^{2+} uptake was not affected in both lines (Kutuzova et al. 2008; Woudenberg-Vrenken et al. 2012) at a normal Ca^{2+} diet; at a low Ca^{2+} diet, intestinal Ca^{2+} uptake was slightly decreased in the $Trpv6^{D/A/D/A}$ knock-in mice. The finding that 1,25-dihydroxyvitamin D_3 administration increases intestinal Ca^{2+} uptake in *Trpv6*-deficient mice and in

Trpv6/Calbindin 9K-deficient double knockout mice put into question that TRPV6 and calbindin 9K are essential for vitamin D-induced active intestinal calcium transport (Benn et al. 2008). So in summary, it appears that *Trpv6* expression is regulated by vitamin D_3, but the TRPV6 channel is not the essential component of intestinal Ca^{2+} uptake; instead it might rather be involved in mechanisms preventing the loss of Ca^{2+} already present in the organism by reabsorbing Ca^{2+} (see also TRPV6 functions in mouse epididymis and prostate below).

2.2 Transcriptional Regulation of *Trpv6* by Steroid Hormones

Duodenal *Trpv6* (and *Trpv5*) expression was shown to be upregulated in ovariectomized rats after 17β-estradiol supplementation (van Abel et al. 2003), and in *oestrogen receptor*-deficient mice, duodenal *Trpv6* transcripts were reduced (Van Cromphaut et al. 2003). Decreased *Trpv6* transcript levels were also seen in an *aromatase*-deficient mouse line (Oz et al. 2007), a model of oestrogen deficiency. Ca^{2+} uptake of *Xenopus laevis* oocytes injected with the *Trpv6* cRNA (Bolanz et al. 2008) was reduced by the selective oestrogen receptor modulator tamoxifen. Similarly, basal cytosolic Ca^{2+} levels which were higher in the human breast cancer cell line MCF-7 which has been transfected with a *Trpv6* cDNA in comparison with non-transfected cells were also reduced in the presence of tamoxifen (Bolanz et al. 2009). Interestingly, this effect could be also demonstrated in the oestrogen receptor-negative MDA-MB-231 cells (Bolanz et al. 2009). Apparently, *Trpv6* expression may be regulated independently by oestrogen receptors and by tamoxifen. In the human breast cancer cell line T47D, the only breast cancer cell line in which the TRPV6 protein has been directly identified so far (Fecher-Trost et al. 2013), *Trpv6* expression appears to be increased in the presence of estradiol, progesterone and 1,25-dihydroxyvitamin D_3 and to be reduced in the presence of tamoxifen (Bolanz et al. 2008).

Duodenal Ca^{2+} uptake in mice was decreased after prednisolone treatment (Huybers et al. 2007) which resulted in reduced *Calbindin 9K* and *Trpv6* transcript expression in duodenum. Accordingly, it was concluded that reduced bone mineral density as unwanted effect from glucocorticoid treatment could result from downregulation of *Trpv6*. However, 5 days of dexamethasone administration affected bone metabolism, but expression levels of *Trpv6*, *calbindin 9K* and *PMCA1b* were not significantly different from tissues of untreated animals (Van Cromphaut et al. 2007). In contrast, dexamethasone-dependent downregulation of *Trpv6* was reported when five times higher dexamethasone doses had been applied (Kim et al. 2009a, b). Little or no expression of *Trpv6* was found in a variety of human and murine osteoblastic cells using qPCR (Little et al. 2011), indicating that TRPV6 plays no pivotal role in bone mineralization. These data are fully consistent with the data obtained from the $Trpv6^{D/A/D/A}$ mouse line (Weissgerber et al. 2011), which lacks functional TRPV6 channels, and did not show alterations of bone metabolism and bone matrix mineralization (van der Eerden et al. 2012).

In LNCaP, expression of *Trpv6* was shown to be upregulated in the presence of an androgen receptor antagonist whereas dihydrotestosterone reduced expression (Peng et al. 2001), but treatment of these cells did not affect Ca^{2+} entry (Bodding et al. 2003). Additional conditions have been reported to enhance *Trpv6* transcript expression including hypoxic conditions in human placenta (Yang et al. 2013) and developmental upregulation in intestine of neonatal mice at weaning (Song et al. 2003) which appeared to depend on vitamin D_3 and during pregnancy (Lee and Jeung 2007) but still occurs in *vitamin D receptor*-deficient mice (Van Cromphaut et al. 2003).

2.3 Transcriptional Regulation of *Trpv6* by Other Hormones

Intestinal Ca^{2+} absorption has been shown to be upregulated during pregnancy and lactation independent of vitamin D_3 (Boass et al. 1981; Brommage et al. 1990; Halloran and DeLuca 1980) or of the vitamin D receptor (Van Cromphaut et al. 2003). In accordance with these observations, prolactin had been shown to enhance intestinal Ca^{2+} absorption in vitamin D_3-deficient rats, to directly stimulate the transcellular Ca^{2+} transport in duodenal preparations (Charoenphandhu et al. 2001; Pahuja and DeLuca 1981) and to regulate vitamin D metabolism and induction of *Trpv6* mRNA expression (Ajibade et al. 2010).

During pregnancy, an active Ca^{2+} transport through the placenta (Sibley and Boyd 1988) was found to be regulated by the parathyroid hormone-related protein PTHrP (Tobias and Cooper 2004). In *PTHrP*-deficient mice, significant differences in placental Ca^{2+} transport compared to wild-type mice were observed, but the transcript levels of *Trpv6* and plasma membrane Ca^{2+} ATPase (*Pmca*) 1 and 4 were unaltered (Bond et al. 2008; Karaplis et al. 1994) arguing against transcriptional regulation of *Trpv6* and *Pmca* by PTHrP.

3 The Channel Protein Including Structural Aspects

The endogenous human full-length TRPV6 protein consists of 765 amino acids (accession number KF534785; Fecher-Trost et al. 2013) compared to the 725-aa annotated TRPV6 protein, which is truncated. The additional 40-aa sequence shows no similarity to any known protein sequence; nine out of the 40 amino acid residues are proline residues, and they might well constitute motifs involved in protein–protein interaction mediated by SH3 domains. The N terminus contains a series of 6 repeats with similarity to domains found in ankyrin repeat proteins (Wissenbach et al. 2001). Erler et al. (2004) identified ankyrin repeat 3 and 5 as critical components involved in the assembly of functional channel complexes. The crystal structure of the six ankyrin repeat domains (aa 124–345 or aa 84–305 in the truncated version) revealed conserved helical-turn-helix conformations, very similar to those present in TRPV1 and 2, but with a variable long-loop region between ankyrin repeat 3 and 4 (Phelps et al. 2008). Various short intracellular C-terminal

fragments of TRPV6 (and TRPV5) were used for circular dichroism (CD) and NMR spectroscopy in the presence and absence of calmodulin, and the results obtained suggest an association of calmodulin and the TRPV6 C terminus (Kovalevskaya et al. 2011, 2012).

TRPV6 proteins most likely co-assemble to homotetrameric channels. Heterotetrameric TRPV6/TRPV5 channels have been reported after co-expression of the respective cRNAs in oocytes (Hoenderop et al. 2003b), but the in vivo expression pattern of both genes does hardly overlap. An exception might be the kidney, where both genes are expressed, but *Trpv5* expression is much more abundant than *Trpv6* (van Abel et al. 2005). TRPV6 proteins are glycosylated at Asn 397 (357) which is located within the extracellular S1–S2 linker (Hirnet et al. 2003; Chang et al. 2005). The beta-glucuronidase klotho stimulates TRPV6 (and TRPV5) channel activity by sugar hydrolysis at the Asn 397 glycosylation site which is conserved in the TRPV5 protein (Chang et al. 2005). The higher expression of klotho within kidney tissue might be an indicator that in vivo the target for klotho-dependent regulation is TRPV5 (Kuro-o et al. 1997).

4 TRPV6-Interacting Proteins

Studies were performed using yeast two-hybrid screens, pull-down assays and antibody-based affinity purifications from primary tissues and HEK cells expressing the TRPV6 cDNA to identify proteins which are transiently or stably associated with TRPV6. A few proteins have been identified including calmodulin, klotho, S100A10-annexin 2, the PDZ domain-containing protein Na^+/H^+ exchanger regulatory factor 4 (NHERF4) and Rab11a which regulate the trafficking, plasma membrane anchoring and activity of the TRPV6 channel. The TRPV6-binding sites for these proteins have been mapped within extracellular and intracellular linkers of transmembrane regions and within the cytosolic C- and N-termini of TRPV6 (Fig. 1a, Table 1).

Calmodulin is known to regulate TRPV6 channel activity, and strong evidence exists for a functional high-affinity calmodulin association at the C terminus of human/mouse TRPV6 between aa 735 and 756 (Niemeyer et al. 2001; Hirnet et al. 2003; Derler at al. 2006; Cao et al 2013). The calcium-dependent calmodulin binding to the C terminus facilitates channel inactivation and is counteracted by protein kinase C-mediated phosphorylation within the calmodulin-binding site at threonine residue 742 (Niemeyer et al. 2001). Stumpf and co-workers used the high-affinity binding of TRPV6 for calmodulin to copurify TRPV6 and interacting proteins from human placenta with a calmodulin column (Stumpf et al. 2008).

The C-terminal high-affinity calmodulin-binding site is also sensitive for phosphatidylinositol 4,5-bisphosphate (PIP2) binding (Zakharian et al. 2011; Cao et al 2013), which interferes with the binding of calmodulin and inversely regulates TRPV6 activity, but presumably not through a direct competition mechanism. The latter study shows a complex interplay between calmodulin and PIP2 and

Table 1 TRPV6-interacting proteins

Protein	Putative function	References
Calmodulin	Channel inactivation/modulation	Niemeyer et al. (2001), Hirnet et al. (2003), Lambers et al. (2004), Derler et al. (2006), Stumpf et al. (2008), Kovalevskaya et al. (2012)
	Competitive PIP2/calmodulin regulation	Cao et al. (2013), Zakharian et al. (2011)
Klotho	Activation through increase of plasma membrane level	Chang et al. (2005)
Calbindin D28K	Binding	Lambers et al. (2006)
BSPRY	Binding	van de Graaf et al. (2006b, c)
PKC	Competitive regulation of calmodulin-dependent inactivation	Niemeyer et al. (2001)
PTBIP, SRC	Channel inhibition	Sternfeld et al. (2007)
Cyclophilin B	Channel activation	Stumpf et al. (2008)
NHERF	Protein transport to plasma membrane, activation	Kim et al. (2007)
Rab11a	Protein transport to plasma membrane, channel activation	van de Graaf et al. (2006a)
Nipsnap1	Channel inhibition	Schoeber et al. (2008)
RGS2	Channel inhibition	Schoeber et al. (2006)
S100A10	Increase in plasma membrane level, activation	Borthwick et al. (2008), van de Graaf et al. (2003)
TRPC1	Suppression of plasma membrane targeting, inhibition of TRPV6 activity	Schindl et al. (2012), Courjaret et al. (2013)
TRPV5	Heterooligomerization, binding	Hellwig et al. (2005), Hoenderop et al. (2003a), Semenova et al. (2009)

demonstrates that calcium, calmodulin and the depletion of PIP2 contribute to the inactivation of TRPV6 channels.

Three additional calmodulin-binding sites have been reported (Lambers et al. 2004), N- and C-terminal and within the intracellular S2–S3 linker (see Fig. 1), but their functions have not been confirmed (Derler at al. 2006; Cao et al 2013). In addition, the presumed N-terminal calmodulin-binding site forms a part of the ankyrin repeat core domain and is therefore unlikely to serve as calmodulin interaction site (Phelps et al. 2008). In line with this, Derler and co-workers show that the N terminus is not involved in channel inactivation by calmodulin (Derler at al. 2006).

Hydrolysis of the extracellular N-linked sugar residues at asparagine 397 by the glucuronidase klotho (see above) entraps the channel proteins in the plasma membrane (Chang et al. 2005) and, as a consequence, results in a higher channel activity. Klotho is the first mammalian proteo-hormone, which binds to TRPV6 from the extracellular side. Replacing the asparagine residue by a glutamine and thereby

creating a glycosylation-deficient TRPV6 mutant abolished klotho-dependent channel activation. The stimulatory effect of klotho is restricted to TRPV5 and TRPV6 and not detectable for TRPV4 and TRPM6, which are also expressed in the kidney (Lu et al. 2008).

In summary, some TRPV6-interacting proteins, like calmodulin or klotho, have been investigated by several studies, and it has been shown consistently that they modulate/regulate channel activity, whereas most of the other candidate proteins were only reported with little or without biochemical or functional characterization (see also http://trpchannel.org/summaries/TRPV6).

5 Biophysical Properties of TRPV6 Channels

TRPV6 (and TRPV5) represent Ca^{2+}-selective ion channels (Peng et al. 1999; Nilius et al. 2000; Vennekens et al. 2000; Wissenbach et al. 2001; Hirnet et al. 2003). Under physiological conditions, TRPV6 conducts only Ca^{2+} ions, but in the absence of divalent cations, the channel conducts monovalents such as Na^{+} (Vennekens et al. 2000; Nilius et al. 2001; Wissenbach et al. 2001; Voets et al. 2003). Interestingly, Na^{+} currents do not inactivate, whereas Ca^{2+} currents quickly inactivate indicating a Ca^{2+}-dependent inactivation mechanism (Hoenderop et al. 2005). Another mechanism of inactivation includes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Thyagarajan et al. 2008). Al-Ansary and co-workers mapped an ATP binding site in the N-terminal region of the TRPV6 protein which indicates that the channel is regulated by the cytosolic ATP concentration (Al-Ansary et al. 2010). The channel permeability was estimated to be about 100 times more selective for Ca^{2+} than for Na^{+}, and the single aspartate residue D581 in the murine TRPV6 (D541 in the truncated protein) and D582 in the human TRPV6 (D542 in the truncated protein) were identified to be part of the selectivity filter (Nilius et al. 2001, 2003; Voets et al. 2003, 2004; Vennekens et al. 2008). In the absence of Ca^{2+}, TRPV6 also conducts Mg^{2+} and the initially large currents quickly inactivate (Voets et al. 2001, 2003). Depending on the membrane potential, Mg^{2+} ions block and unblock the channel pore (Voets et al. 2003) and influence the inward rectification behaviour of TRPV6. Some inward rectification also appears in the absence of Mg^{2+} and seems to be an intrinsic property of TRPV6 (Hoenderop et al. 2005). TRPV6 and TRPV5 conduct Ba^{2+}, but the permeability and inactivation properties of Ba^{2+} currents are different between TRPV6 and TRPV5 although the pore sequences are identical (Nilius et al. 2002; Hoenderop et al. 2005). Also Cd^{2+} and Zn^{2+} can permeate and contribute to the toxicity of these heavy metal ions (Kovacs et al. 2013). Interestingly, Ba^{2+}-dependent current properties seem to be determined by residues in the transmembrane regions 2 and 3 indicating that structures quite distant to the pore region may influence pore properties as well as the inactivation behaviour of the channel.

The single channel conductance of TRPV6 was estimated to be 40–70 pS, and by cysteine scanning the pore width was calculated to be 5.4 Å (Hoenderop et al. 2001, 2005; Voets et al. 2004). Apparently, most of the electrophysiological data

described were obtained from HEK293 cells overexpressing the truncated Trpv6 cDNA. The comparison of the biophysical properties of channels obtained after expressing the full-length cDNA or the truncated cDNA yields very similar results so far (Fecher-Trost et al. 2013). However, the full-length TRPV6 is more efficiently translocated to the plasma membrane, and five times more of the truncated TRPV6 protein is required to produce similar current amplitudes (Fecher-Trost et al. 2013).

Another yet unsolved problem is that in acutely isolated primary cells which do endogenously express Trpv6 transcripts like pancreatic acinar cells, TRPV6-like currents could not be recorded although the protocols applied yield impressive currents in HEK293 cells expressing the *Trpv6* cDNA. Instead, in primary cells and tissues, $^{45}Ca^{2+}$ uptake measurements are the method of choice to monitor TRPV6 activity. Using this method TRPV6 activity was identified in epididymal epithelia (Weissgerber et al. 2011, 2012).

6 Pharmacology of TRPV6 Channels

Xestospongin C, a natural isolate from a sponge (Vassilev et al. 2001), inhibits TRPV6 in the lower μM range. Ruthenium red also blocks TRPV6 but works more potently on TRPV5 (Hoenderop et al. 2001). Some antifungal drugs such as econazole and miconazole inhibit TRPV5 and TRPV6 (Hoenderop et al. 2001). A newly synthesized derivative of a substance called TH-1177 inhibits TRPV6 currents with an IC50 for TRPV6 of 0.44 μM (Haverstick et al. 2000; Landowski et al. 2011). This compound is five times more effective on TRPV6 than on TRPV5. 2-APB (2-aminoethoxydiphenyl borate), a rather non-selective TRP channel blocker/activator, was shown to block TRPV6 but surprisingly not TRPV5 (Kovacs et al. 2012). Recently a peptide, soricidin, derived from short-tailed shrew (Bowen et al. 2013) was shown to exhibit an analgesic effect and to suppress growth of some tumour cells. It was shown that the C-terminal part of the peptide is sufficient for growth inhibition, and the authors demonstrate that one target of this peptide is TRPV6: TRPV6 currents in HEK293 cells are partially blocked with an IC50 in the low nM range. Apparently, soricidin represents the most potent TRPV6 channel blocker [see also Owsianik et al. (2006) and Vennekens et al. (2008)].

7 Physiological Functions of TRPV6

Since the identification and the cloning of the cDNAs and the initial expression studies, both TRPV6 and TRPV5 have been implicated in epithelial Ca^{2+} uptake. In agreement with this function, the *Trpv6* gene was shown to be expressed in placenta, pancreas, salivary gland and several parts of the small intestine including duodenum by Northern blots and in situ hybridization (Peng et al. 1999; Wissenbach et al. 2001; Hirnet et al. 2003). Using antibodies for TRPV6, immunostaining was demonstrated in enterocytes of the intestinal villi in the

murine duodenum with predominant expression at the apical cell membrane at the tips of the villi (Little et al. 2011) as well as in exocrine organs including pancreas, prostate and mammary gland (Zhuang et al. 2002). In addition, the TRPV6 protein was detected within the mouse kidney at the apical domain of the late distal convoluted tubule, the connecting tubule and the cortical and medullary collecting ducts (Nijenhuis et al. 2003). Several groups demonstrated the expression of TRPV6 in the proximal part of duodenum (Zhuang et al. 2002; van de Graaf et al. 2003; Walters et al. 2006; Huybers et al. 2007) in line with a role for TRPV6 in intestinal Ca^{2+} absorption, but others did not detect *Trpv6* expression in duodenum and kidney (Wissenbach et al. 2001).

Appropriate controls for antibody specificity including immunostains from knockout mice strongly support results in general, but have not been described in most studies referred to above. One of the few exceptions is the demonstration that TRPV6 proteins are expressed in the apical membrane of the murine epididymal epithelium and prostatic epithelium (Weissgerber et al. 2011, 2012). It should be mentioned here that expression of *Trpv6* transcripts in human prostate is low or even not detectable depending on the method (Northern blot, in situ hybridization or PCR) but is upregulated in human prostate cancer (see below).

7.1 TRPV6 Channels in Bone

Laser scanning microscopy revealed that TRPV6 proteins—as well as TRPV5—are expressed at the apical domain of murine osteoclasts cultured on cortical bone slices predominantly at the bone-facing site (van der Eerden et al. 2005). However, TRPV6 activity did not compensate the reduced bone resorption occurring in mice lacking *Trpv5* (Hoenderop et al. 2003c). Additional immunohistochemical analysis revealed only weak staining for TRPV6 in osteoblasts and no staining in cortical or trabecular osteocytes nor in the growth plate (Little et al. 2011), whereas *Trpv6* mRNA was detectable in the murine bone (Nijenhuis et al. 2003) with considerable expression in the bone marrow.

7.2 TRPV6 Channels in Placenta

The *Trpv6* mRNA was detected in syncytiotrophoblasts (Wissenbach et al. 2001) and uterus (Moreau et al. 2002) suggesting a role for basal Ca^{2+} influx in placental cells and from there to the foetus. Whereas the placental Ca^{2+} transport and the involved proteins are not completely understood, so far TRPV6 protein was decreased in placenta from women suffering from pre-eclampsia compared to healthy placental tissue (Hache et al. 2011). Pre-eclampsia (PE) is a multisystemic disorder that represents a major factor for maternal and perinatal mortality, and it affects 7–10 % of pregnancies worldwide (Sibai 2005; Walker 2000). In mice, *Trpv6* is highly expressed in the extraplacental yolk sac (the foetal side of the placenta) and only weakly expressed on the maternal side of the placenta during the last

trimester of gestation, but *Trpv6* transcript expression was strongly upregulated from embryonic day 15 to day 18 (Suzuki et al. 2008a) indicating involvement in the increase of maternal-foetal Ca^{2+} transport which supports foetal bone mineralization.

8 Lessons from Knockouts

At present, three independent mouse lines exist with targeted germ line mutations within the *Trpv6* gene (Bianco et al. 2007; Weissgerber et al. 2011). One *Trpv6*-deficient line was generated using a classical knockout strategy replacing exons 9–15 and exons 15–18 of the adjacent *Ephb6* gene by a neomycin resistance cassette (Bianco et al. 2007). In an independent approach, a Cre-loxP strategy was used to delete exons 13–15 of *Trpv6* encoding part of transmembrane domain 5, the pore, the transmembrane domain 6, the cytosolic C terminus and exons 17–18 of the *Ephb6* gene (Weissgerber et al. 2012). For homologous recombination, genomic sequences of appropriate length are required within the gene-targeting vector, and this is the reason that deletion of the exons 13–15 of the *Trpv6* gene also affected parts of the closely adjacent *Ephb6* gene. In a third mouse line, a single aspartate (D) residue at position 581 (541 in the truncated protein) within the pore region was replaced by an alanine (A) residue ($Trpv6^{D/A/D/A}$). This residue is a critical constituent of the TRPV6 selectivity filter, and replacement by an alanine (D/A) completely abolished the Ca^{2+} permeability (Nilius et al. 2001; Voets et al. 2004; Weissgerber et al. 2012).

Bianco et al. (2007) reported that their $Trpv6^{-/-}$ mice were viable but show decreased intestinal Ca^{2+} absorption, lower femoral bone mineral density, lower body weight, growth retardation and reduced fertility of homozygous males and females, and 80 % of all mice developed alopecia and dermatitis (Table 2). Serum Ca^{2+} levels were normal, but under Ca^{2+}-restricted conditions, the $Trpv6^{-/-}$ mice developed hypocalcaemia. Furthermore, the total Ca^{2+} levels in the serum and amniotic fluid of $Trpv6^{-/-}$ foetus were significantly lower than those in wild-type foeti (Bianco et al. 2007; Suzuki et al. 2008a). Analysis of active intestinal Ca^{2+} transport revealed no difference between $Trpv6^{-/-}$ and wild-type mice on a standard diet (Benn et al. 2008).

The analysis of the $Trpv6^{-/-}$ and the $Trpv6^{D/A/D/A}$ mice from Weissgerber et al. (2011, 2012) demonstrated that homozygous males but not females from both mouse lines showed severely impaired fertility despite normal copulation behaviour. Furthermore, the spermatozoa showed markedly reduced motility, fertilization capacity and viability. Northern blot and immunohistochemical analysis demonstrated that *Trpv6* was expressed in the apical membrane of the epididymal epithelium but not in spermatozoa or the germinal epithelium. The Ca^{2+} concentration within the fluid of the cauda epididymis was significantly increased in both mouse lines $Trpv6^{D/A/D/A}$ and $Trpv6^{-/-}$ compared to wild-type controls and led to reduced sperm viability. These results indicate that appropriate Ca^{2+} concentration is essential for the development of viable and fertilization-competent spermatozoa. In addition, deletion of the *Trpv6* gene (Weissgerber et al. 2012) did not further

Table 2 Comparison of *TRPV6*$^{D/A/D/A}$ and *Trpv6*-deficient mouse lines

Analysed parameter	*Trpv6*$^{D/A/D/A}$ (Weissgerber et al. 2011)	*Trpv6*$^{-/-}$ (Weissgerber et al. 2012)	*Trpv6*$^{-/-}$ (Bianco et al. 2007)
Femoral bone mineral density	No difference	Not analysed	Decreased
Body weight	No difference	No difference	Decreased in homozygous males and females
Growth retardation	Not detectable	Not detectable	Decreased in homozygous males and females
Fertility defect	Homozygous males	Homozygous males	Homozygous males and females
Sperm motility	Impaired	Impaired	Not analysed
Sperm viability	Impaired	Impaired	Not analysed
Ca^{2+} homeostasis			
On normal calcium diet:			
–Intestinal Ca^{2+} absorption	No change	Not analysed	Decreased
–Serum PTH and 1,25-dihydroxyvitamin D	Not analysed	Not analysed	Elevated
–Urine osmolality	Not analysed	Not analysed	Altered
–Ca^{2+} excretion	No change	No change	Increased
–Polyuria	No change	No change	Increased
–Serum Ca^{2+} level	No change	No change	No change
On Ca^{2+} restricted diet:			
–Serum Ca^{2+} level	Unaltered	Not analysed	Decreased (hypocalcaemia)
–Intestinal Ca^{2+} uptake	Decreased	Not analysed	Decreased
Alopecia	Not detectable	Not detectable	Yes
Dermatitis	Not detectable	Not detectable	Yes

aggravate the phenotype observed in *TRPV6*$^{D/A/D/A}$ mice (Weissgerber et al. 2011), arguing against residual channel activity of the mutated TRPV6$^{D/A}$ protein. TRPV6 proteins were also identified in the apical epithelial membranes of prostatic epithelium, and *Trpv6* deletion results in Ca^{2+} precipitates within the enlarged prostatic ducts. Van der Eerden et al. (2011) showed that *Trpv6*$^{D/A/D/A}$ mice do not have altered bone mass, and other morphological parameters such as trabecular and cortical bone microarchitecture were similar compared to wild-type mice. However, bone size was affected as shown by reductions in femoral length as well as femoral head, cortical bone and endocortical bone volumes. Intestinal Ca^{2+} uptake under a Ca^{2+}-restricted diet was significantly impaired in *Trpv6*$^{D/A/D/A}$ mice compared to wild-type mice (Woudenberg-Vrenken et al. 2012) demonstrating a specific role of TRPV6 in transepithelial Ca^{2+} absorption under conditions of limited Ca^{2+} supplies.

Differences in Ca^{2+} absorption, weight gain, hair coat or fertility of homozygous females (Bianco et al. 2007) were not observed in the *Trpv6*$^{-/-}$ or *Trpv6*$^{D/A/D/A}$ mice generated by Weissgerber et al. (2011, 2012; Table 2). The reason for the discrepancies between the *Trpv6*-deficient mouse lines is not known, but different exons were deleted in either strategy and differences in the genetic background between the mouse lines cannot be excluded. In addition, the neo-cassette introduced for gene targeting has been removed from the mouse lines of Weissgerber et al. (2011, 2012) before phenotyping. If this foreign DNA remains in the genomic locus, it may influence the expression of neighbouring genes. Because it contains cryptic splice sites, its persisting presence can also fortuitously create a hypomorphic allele. Finally, the number of animals used to characterize a phenotype is crucial; for example, weight gain was studied with four animals (Bianco et al. 2007) and 19–31 animals (Weissgerber et al. 2011).

9 Roles in Hereditary and Acquired Disease

Changes of *Trpv6* transcript expression have been shown in various transgenic mouse models of human diseases (Table 3) but whether these changes occur in humans and whether there is a link between the changes of transcript expression and the pathophysiology of these diseases have only preliminarily been investigated. For example, Lowe disease patients carry mutations within the *phosphatidylinositol bisphosphate (PIP2) 5-phosphatase* gene resulting in increased levels of the substrate phosphatidylinositol 4,5-bisphosphate (PIP2), and this lipid accumulates in the renal proximal tubule cells of these patients (Devuyst and Thakker 2010). PIP2 positively regulates the activity of TRPV6 (Thyagarajan et al. 2008; Zakharian et al. 2011; Wu et al. 2012; Cao et al 2013) and might therefore contribute to the intestinal hyperabsorption of Ca^{2+} in these patients.

From the very beginning, it became apparent that *Trpv6* transcripts are overexpressed in cancerous tissue and in cell lines derived thereof (Table 4, and expression profiles HG-U95A and GDS1746 available at http://www.ncbi.nlm.nih.gov/geoprofiles). In prostate cancer, *Trpv6* transcripts are overexpressed and the expression pattern correlates with the aggressiveness of the disease (Wissenbach et al. 2001, 2004; Peng et al. 2001; Fixemer et al. 2003). Likewise, in the human lymph node prostate cancer cell line LNCaP, upregulation of *Trpv6* transcripts by vitamin D treatment enhanced proliferation rate and resistance to apoptosis (Lehen'kyi et al. 2007, 2011, 2012). The proliferation rate of HEK293 cells expressing the *Trpv6* cDNA was also enhanced (Schwarz et al. 2006). Nude mice in which HEK293 cells stably expressing *Trpv6* were injected subcutaneously as xenografts developed tumours (Wissenbach 2013), whereas control mice injected with non-transfected HEK293 cells or with HEK293 cells stably expressing *Trpc4* did not. As described above, soricidin (Bowen et al. 2013), a peptide isolated from the venom of the short-tailed shrew, inhibits TRPV6 currents. Tumours derived from injection of the ovarian cancer cell line SKOV-3 and the cell line DU145

Table 3 *Trpv6* expression in animal models of human disease

Animal model: disease	Link to *Trpv6*	References
Slc26a4 knockout mouse: animal model for Pendred syndrome	Expression of *Trpv6* (and *Trpv5*) in the cochlea (immunohistochemistry)	Wangemann et al. (2007)
$TNF_{\Delta ARE}$ mouse: animal model of Crohn's-like disease	Transcript ↓(qPCR, duodenum)	Huybers et al. (2008)
(*Clcn5* knockout mouse: animal model of Dent disease)	(Mutations of *OCRL* cause Lowe syndrome and Dent disease) Co-expression of *Trpv6* (immunohistochemistry) and the oculocerebrorenal syndrome of Lowe (*OCRL*) gene product, the phosphatidyl inositol 4,5-bisphosphate 5-phosphatase in rat Intestinum. In *Xenopus laevis* oocytes endogenous OCRL suppressed TRPV6-mediated Ca^{2+} uptake	Wu et al. (2012)
$Slc12A3_{truncated}$ knock-in mouse: animal model of Gitelman syndrome	*Trpv6* transcript ↑(qPCR kidney)	Yang et al. (2010)
Hypercalciuric stone-forming (GHS) rat: animal model for kidney stones	*Trpv6* transcript ↑(qPCR, kidney)	Frick et al. (2013)

Table 4 *Trpv6* expression in malignant human tissues and cancer cell lines

Malignancy	Method	References
Prostate cancer	RT-PCR, Northern, ISH	Fixemer et al. (2003), Peng et al. (2001), Wissenbach et al. (2001, 2004)
LNCaP	Northern, Western	Bodding et al. (2003), Peng et al. (2001)
DU-145	RT-PCR	Lehen'kyi et al. (2011), Peng et al. (2001)
PC3	RT-PCR	Peng et al. (2001)
Breast cancer	RT-PCR, immunostaining[a]	Bolanz et al. (2008), Zhuang et al. (2002)
MCF7	RT-PCR, Western blot[a]	Bolanz et al. (2009)
T47D	MS/MS, RT-PCR	Bolanz et al. (2008), Fecher-Trost et al. (2013)
Colon cancer	Immunostaining[a]	Zhuang et al. (2002)
SW480	Northern	Peng et al. (2000)
LS-180	RT-PCR	Zheng et al. (2012)
Caco	RT-PCR, Western[a]	Fleet et al. (2002), Taparia et al. (2006)
Ovarian cancer	Immunostaining[a]	Zhuang et al. (2002)
SKOV-3	Peptide staining of xenograft	Bowen et al. (2013)
Thyroid cancer	Immunostaining[a]	Zhuang et al. (2002)
Endometrial cancer	ISH	Wissenbach and Niemeyer (2007)
Leukaemia	–	–
K-562	*Northern*	*Peng et al. (2000), Semenova et al. (2009)*

[a]No data available on antibody specificity

derived from brain metastases of prostate cancer could be stained with soricidin-derived peptides in vivo (Bowen et al. 2013) apparently through labelling TRPV6. In addition to prostate cancer, *Trpv6* transcripts are overexpressed in breast cancer and breast cancer-derived cell lines (Zhuang et al. 2002) as well as in oestrogen receptor-negative breast cancer tumours (Peters et al. 2012). The authors also showed that overexpression of *Trpv6* most likely results from genomic amplification of the *Trpv6* gene. Gene amplification of *Trpv6* was also found in prostate tumours (Kessler and Wissenbach, unpublished). In summary, the data indicate that overexpression of *Trpv6* is involved in carcinogenesis of several human cancers. They demonstrate the importance of TRPV6 which not only represent a marker for prostate cancer progression but may serve as a target for therapeutic strategies in the malignancies listed above.

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Part III

The TRPM Subfamily

TRPM1

Shoichi Irie and Takahisa Furukawa

Contents

Abstract

The transient receptor potential (TRP) channels play a wide variety of essential roles in the sensory systems of various species, both invertebrates and vertebrates. The TRP channel was first identified as a molecule required for proper light response in *Drosophila melanogaster*. We and another group recently revealed that TRPM1, the founding member of the melanoma-related transient receptor potential (TRPM) subfamily, is required for the photoresponse in mouse retinal ON-bipolar cells. We further demonstrated that Trpm1 is a component of the transduction cation channel negatively regulated by the metabotropic glutamate receptor 6 (mGulR6) cascade in ON-bipolar cells through a reconstitution experiment using CHO cells expressing Trpm1, mGluR6, and Goα. Furthermore, human *TRPM1* mutations are associated with congenital stationary night blindness (CSNB), whose patients lack rod function

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and suffer from night blindness starting in early childhood. In addition to the function of transduction cation channel, TRPM1 is one of the retinal autoantigens in some paraneoplastic retinopathy (PR) associated with retinal ON-bipolar cell dysfunction. In this chapter, we describe physiological functions of the TRPM1 channel and its underlying biochemical mechanisms in retinal ON-bipolar cells in association with CSNB and PR.

Keywords
TRP channel • Retina • Cation channel • mGluR6 • ON-bipolar cell • Congenital stationary night blindness • Autoantigen • Paraneoplastic retinopathy

1 Gene, Promoter, Splicing, etc.

The melanoma-related transient receptor potential (TRPM) family genes are well conserved through evolution in both invertebrates and vertebrates and encode nonselective cation channels involved in many biological functions. There are eight TRPM members in human and mouse, four members in *Caenorhabditis elegans*, and a single member in *Drosophila melanogaster*. *TRPM1* is expressed in skin melanocytes and eye, but its expression level is reduced in the highly metastatic cell line, suggesting a possible role for *TRPM1* in body color formation and melanoma metastasis (Duncan et al. 1998; Bellone et al. 2008). The *TRPM1* transcript is alternatively spliced to produce a *TRPM1-S* in humans (GenBank accession number HM135791 for human *TRPM1-L*, #AF071787 for human *TRPM1-S*) (Duncan et al. 1998; Hunter et al. 1998; Fang and Setaluri 2000; Xu et al. 2001). Although mouse *Trpm1-S* was previously identified as *melastatin* (GenBank accession number #AF047714 for mouse *Trpm1-S*), mouse *Trpm1-L* had not been identified until recently (Duncan et al. 1998). A mouse *Trpm1-L* cDNA corresponding to the human *TRPM1* long form was identified as being a highly expressed gene in the retina (GenBank accession number #AY180104) (Koike et al. 2010b). Human *TRPM1* maps to the q13.3 region of chromosome 15; mouse *Trpm1* maps to chromosome 7 (Hunter et al. 1998).

The regulation of *Trpm1* expression has been demonstrated. Basic helix-loop-helix transcription factor b4 (Bhlhb4) and microphthalmia-associated transcription factor (MITF) are reported as candidate transcription factors to regulate *Trpm1* expression. In the *bHLHb4* mutant retina, the expression of *Trpm1* mRNA was slightly reduced (Kim et al. 2008). In mice and humans, the promoter region of *TRPM1* contains four consensus binding sites for a melanocyte transcription factor, MITF (Hunter et al. 1998; Zhiqi et al. 2004). We have previously shown that Otx2, the *Otx*-like homeobox gene, is required not only for photoreceptor cell fate determination but for the maturation of bipolar cells (Koike et al. 2007). The 5 kb upper proximal promoter region of *TRPM1* contains five consensus binding sites for

Otx2 (data not shown). To investigate if *TRPM1* is a downstream target of Otx2, *in situ* hybridization is conducted in retinas from rod bipolar-specific *Otx2* conditional knockout mice (Otx2 flox/flox; L7-Cre). Intriguingly, the expressions of both *Trpm1-L* and *-S* were almost disappeared. These data suggest that Otx2 regulates the expression of *Trpm1* predominantly in the retina.

2 Expression

TRPM1-S, initially named melastatin, was the first mammalian TRPM subfamily cloned (Duncan et al. 1998). *TRPM1-S* expression was correlated with pigmentation in melanoma cell lines, while reversely correlated with the metastatic potential of melanoma cells as a tumor suppressor (Duncan et al. 1998; Fang and Setaluri 2000). Northern blot analysis revealed the presence of mouse *Trpm1-L* and *-S* transcripts in the retina; however, only the latter was detected in the skin (Koike et al. 2010b). *In situ* hybridization detected the presence of substantial *Trpm1-L* transcripts, specifically in the inner nuclear layer (INL), at postnatal day 8 (P8) through P9 corresponding to the localization of Chx10, a pan-bipolar marker (Koike et al. 2010b). A faint *Trpm1-S* signal was also detected in INL. The expression of Trpm1-S was reported in the retinal pigment epithelial (RPE) during embryonic stages; however, Trpm1-L was not detected in the RPE (Koike et al. 2010b). An antibody against Trpm1-L was raised and the localization of Trpm1-L was examined. Around the stage that retinal cell differentiation complete, Trpm1-L proteins were observed diffusely in bipolar cell bodies, and during retinal maturation, the proteins localized at the tips of Goα-expressing and mGluR6-expressing dendrites in the outer plexiform layer (Koike et al. 2010b).

3 The Channel Protein Including Structural Aspects

TRP channel proteins share a common structure of six transmembrane domains and a reentrant P-loop, which is involved in forming the pore of the channel, as it does in voltage-gated ion channels (Morgans et al. 2010). In addition to these features, a highly conserved 23–25 amino acid residue TRP domain containing an "EWKFAR" TRP-box1 and a proline-rich region TRP-box2 is located near the sixth transmembrane domain in the cytoplasmic region of TRPC, TRPM, and TRPN channels (Venkatachalam and Montell 2007; Morgans et al. 2010). A large body of research has shown that an abundance of TRP channels have been discovered and implicated in sensing activities, including taste, olfaction, hearing, and touch, in addition to thermosensation and osmosensation. In addition, some TRP channels function as effector channels in neurons.

TRPM channels have intracellular N- and C-terminals, six transmembrane segments, and a hydrophobic pore loop between transmembrane segments five

and six. This family possesses a TRPM family homology region of about 700 amino acid residues along with a coiled-coil (CC) domain within the N-terminal region and a TRP-box and CC domain within the C-terminal region (Perraud et al. 2003). The CC domain within the C-terminal region is required for assembly, tetrameric formation, and proper trafficking of channel proteins (Erler et al. 2006; Tsuruda et al. 2006; Fujiwara and Minor 2008). The mouse *Trpm1-L* encodes a predicted 1,622 amino acid protein, containing six transmembrane domains, a pore region, and a TRP domain, as do other TRP family members, whereas TRPM1-S is an N-terminal truncated form lacking the six transmembrane domains and the other domains as well (Koike et al. 2010b).

4 Interacting Proteins

It was reported that TRPM1-S interacts directly with TRPM1-L to suppress TRPM1-L channel activity by inhibiting its translocation to the plasma membrane *in vitro* (Xu et al. 2001). The leucine-rich proteoglycan protein, nyctalopin, is a membrane-anchored protein that is expressed at the dendritic tips of ON-bipolar cells in the retina, where it closely colocalizes with mGluR6, forming prominent synaptic puncta (Morgans et al. 2006; Gregg et al. 2007). It was demonstrated that *in vitro* nyctalopin can form protein–protein interactions with two principle components of the ON-bipolar cell signal transduction pathway: the effector channel Trpm1 and the receptor mGluR6 (Cao et al. 2011). The Trpm3 channel, a member of the TRPM family, interacts biochemically with the Trpm1 channel to form functional heteromultimeric channels in co-transfected cultured cells (Lambert et al. 2011).

5 A Biophysical Description of the Channel Function, Permeation, and Gating

Functional separation of neuronal signaling into the ON and OFF pathways that contribute to visual contrast recognition is a fundamental feature of the vertebrate vision. After absorption of light, photoreceptor cells—rods and cones—transmit signals to depolarizing ON-bipolar cells and hyperpolarizing OFF-bipolar cells by glutamate release, which is reduced by light-evoked hyperpolarization (Dowling 1978; DeVries and Baylor 1993). In mammalian retinas, rod photoreceptor cells form synapses with ON-type rod bipolar cells, and cone photoreceptor cells connect with cone bipolar cells, which are subdivided into ON and OFF types. The expression pattern of different glutamate receptors (GluRs) at the postsynaptic region generates the functional diversity of bipolar cells (Nakanishi et al. 1998). ON-bipolar cells express a unique metabotropic GluR, mGluR6, on its dendrites. The ON-bipolar dendrite forms an invaginating single synapse with both rod and

cone photoreceptor terminals. On the other hand, OFF-bipolar cells express ionotropic GluRs (AMPA/Kainate receptors), glutamate-gated cation channels, on its dendrites. The OFF-bipolar dendrite makes multiple flat synapses with cone photoreceptor terminals (Nomura et al. 1994; Morigiwa and Vardi 1999; Haverkamp et al. 2001). In the dark, the neurotransmitter glutamate is released at a high rate from rod terminals into the synaptic cleft and depolarizes OFF-bipolar cells through activation of ionotropic glutamate receptor, whereas glutamate hyperpolarizes ON-bipolar cells through mGluR6 activation leading to the closure of cation channels and a decrease in cationic conductance (Shiells et al. 1981; Slaughter and Miller 1981; de la Villa et al. 1995; Masu et al. 1995; Euler et al. 1996). The transduction cation channel of retinal ON-bipolar cells was hypothesized to be a cyclic guanosine monophosphate (cGMP)-gated cation channel that is closed by increasing the rate of cGMP hydrolysis through phosphodiesterases (PDEs) by a G-protein-mediated process, based on analogous light transduction machinery in photoreceptor cells (Nawy and Jahr 1990; Arshavsky et al. 2002). Several previous studies reported that the response of the ON pathway for phototransduction is mediated by turning off the G-protein signaling that is activated in the dark when glutamate binds to mGluR6 (Nomura et al. 1994; Vardi and Morigiwa 1997; Vardi et al. 2000). In ON-bipolar cells, it has been reported that mGluR6 couples to a heterotrimeric G-protein complex Go (Weng et al. 1997; Vardi 1998; Nawy 1999; Dhingra et al. 2000, 2002). Goα is required for phototransduction, which closes a downstream cation channel upon the activation of ON-bipolar cells (Nawy and Jahr 1990; Shiells and Falk 1990; 1992a, b, c; de la Villa et al. 1995; Euler et al. 1996; Weng et al. 1997; Nawy 1999; Dhingra et al. 2000, 2002). From these results, it appears that Goα is required for the closure of the cation channel, but there is no evidence that PDEs are involved in the regulation in terms of function and distribution for the cation channel (Nawy 1999). Furthermore, it is reported that PDEs are not essential elements in the signal transduction of ON-bipolar cells (Nawy 1999). Although a regulator of G-protein signaling (RGS), Ret-RGS1, which interacts with Goα in transfected cells and the retina, was identified (Dhingra et al. 2004), a transduction cation channel in the downstream of the mGluR6 pathway in retinal bipolar cells was not identified until recently. Koike et al. generated *Trpm1* null mutant (*Trpm1*$^{-/-}$) mice by targeted gene disruption and reported that the *Trpm1*$^{-/-}$ mice showed a complete lack of an electroretinogram (ERG) b-wave, whereas the a-wave was normal (Koike et al. 2007). Another group had been focusing on the Trpv1 channel as a candidate for the ON-bipolar transduction channel (Shen et al. 2009). Based on the report by Koike et al., Shen et al. examined and reported that ERG analysis in the *Trpm1*$^{-/-}$ mice showed a complete lack of a b-wave, whereas the b-wave of *Trpv*$^{-/-}$ mice was normal (Shen et al. 2009). After decades of searching for the ON-bipolar transduction channel, TRPM1 was finally identified as the responsible channel which was negatively regulated in the downstream of the mGluR6 transduction pathway (Koike et al. 2010b) (Fig. 1).

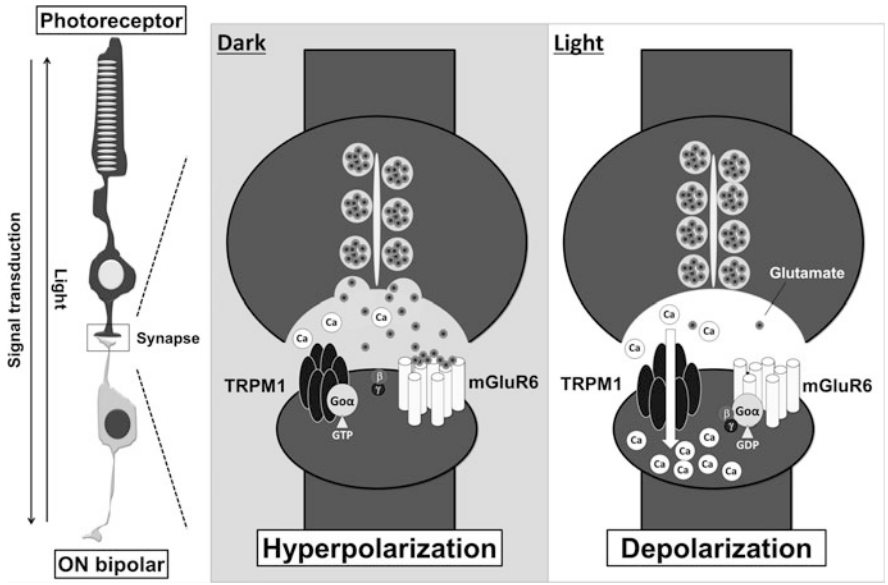

Fig. 1 Schematic representation of the mechanism for an ON-bipolar cell response to the light. Schematic diagram of a photoreceptor and an ON-bipolar cell which form a ribbon synapse between them (*left*). Upon light stimulation, the conversion of photons to neural signals in the photoreceptor cell decreases the concentration of glutamate released around the synaptic ribbon area of a photoreceptor cell as shown in the *middle diagram*. The decrease in the synaptic glutamate concentration inactivates an mGluR6 channel, leading to an open state of TRPM1 which is a constitutive active nonselective cation channel. This led to depolarization of the ON-bipolar cells (*middle*). In contrast, in the dark, a decrease in the rate of photon absorption by photoreceptor cell increases the concentration of glutamate. An increase of glutamate activates an mGluR6 channel, results in activation of Goα, and finally inactivates the TRPM1 channel with a decrease in cationic conductance (*right*)

6 Physiological Functions in Native Cells, Organs, and Organ Systems

To verify whether or not TRPM1-L satisfies the properties of a nonselective cation channel regulated by the mGluR6 cascade, measurements were made of ionic currents under a whole-cell voltage clamp in a reconstitution system that mimicked the postsynaptic membrane of retinal ON-bipolar cells by transfection of CHO cells with *Trpm1-L*, *mGluR6*, and *Goα* (Koike et al. 2010b). In CHO cells expressing mGluR6, Goα, and Trpm1-L, constitutively active inward currents were observed, whereas in Goα-transfected CHO cells stably expressing mGluR6 but not Trpm1-L, a whole-cell current was negligible (Koike et al. 2010b). Furthermore, constitutively active currents were detected in Trpm1-L expressing cells even after replacing extracellular cations with Na^+, K^+, Ca^{2+}, or Mg^{2+}, supporting the idea

that Trpm1-L is a nonselective active cation channel (Koike et al. 2010b). Furthermore, in CHO cells expressing mGluR6, Goα, and Trpm1-L, the administration of glutamate to the bath solution suppressed constitutively active cationic currents, while subsequent washout of glutamate restored the suppressed currents to levels comparable to those prior to glutamate administration (Koike et al. 2010b). The effect of Go activation on the Trpm1-L current was further investigated by measuring whole-cell current in Trpm1-L expressing CHO cells transfected with either wild type of *Goα* or a constitutively active mutant of *Goα* (*Goα-Q205L*) (Koike et al. 2010b). The current density obtained in *Trpm1-L*-transfected cells was significantly larger than that in *Trpm1-L* and *Goα*-co-transfected cells, while suppression of the current density in *Trpm1-L* and *Goα-Q205L*-co-transfected cells was observed at a level comparable to that in *Trpm1-L*- and *Goα*-co-transfected cells intracellularly applied GMP-PNP, an unhydrolyzable analog of GTP (Koike et al. 2010b). To demonstrate more directly that Trpm1-L is regulated by Go protein, the effect of application of the purified Go protein from the intracellular side on Trpm1-L activity was examined in the excised inside-out patch recordings (Koike et al. 2010b). Application of the purified Goα protein gradually, but strongly, suppressed open probability of Trpm1-L, whereas administration of heat-denatured Goα protein failed to suppress it with GMP-PNP. Application of another G-protein subunit, Gβγ, did not suppress the open probability of Trpm1-L (Koike et al. 2010a). Patch-clamp analysis on the *Trpm1-L*-transfected CHO cells effectively demonstrated that Trpm1-L is a nonselective cation channel and that Trpm1-L activity is negatively regulated by Goα protein downstream of the mGluR6 signaling cascade (Koike et al. 2010b).

There are several modulatory mechanisms for Trpm1 function. A G-protein β subunit, $G\beta 3_3$, is expressed in ON-bipolar cells and plays an essential role for proper localization of mGluR6 and TRPM1 on rod bipolar dendrites (Dhingra et al. 2012). Activation of PKCα reduces the inhibition of Trpm1 function by Mg^{2+} (Rampino and Nawy 2011). In the retina, Zn^{2+} is co-released with glutamate from the terminals of rod photoreceptors. An experiment with chimeric channels between Trpm1 and Trpm3 revealed that the Trpm1 channel was inhibited by Zn^{2+} (Redenti and Chappell 2005; Redenti et al. 2007; Lambert et al. 2011).

TRPM1 may also be involved in melanocytogenesis and pigmentation processes. Reduced expression of TRPM1 in human melanocytes and mouse melanoma cell lines correlates with decreased melanin content, suggesting that TRPM1-mediated Ca^{2+} homeostasis plays a role in the regulation of melanogenesis (Devi et al. 2009; Oancea et al. 2009). Interestingly, another group reported that human melanocytes express mGluR6, but not Goα, and that TRPM1 activity in human melanocytes is positively regulated by the mGluR6 signaling pathway (Devi et al. 2013). It should be noted that the coat color of *Trpm1* null mutant mice was unaffected compared with that of the wild-type mice (Koike et al. 2010b).

7 Lessons from Knockouts

Koike et al. generated *Trpm1* null mutant ($Trpm1^{-/-}$) mice by targeted gene disruption (Koike et al. 2007, 2010b). It should be noted that *Trpm1* is a host gene for *miR-211*; however, the *miR-211* gene is retained in the $Trpm1^{-/-}$ allele (Boyle et al. 2011). They revealed that neither their rod bipolar cells nor their cone ON-bipolar cells showed the photoresponses by using whole-cell patch-clamp analysis, suggesting that there are no functional transduction cation channels in ON-bipolar cells of $Trpm1^{-/-}$ mice (Koike et al. 2010b). On the other hand, light stimulation on both wild-type (WT) and $Trpm1^{-/-}$ mice cone OFF-bipolar cells evoked photoresponses, and there were no significant differences in either the amplitude of the light responses or the time for half-maximal amplitude after the light was turned off (Koike et al. 2010b). Examination of the optokinetic responses and electroretinograms (ERGs) of 2-month-old WT and $Trpm1^{-/-}$ mice revealed optokinetic deficiencies similar to those of mice lacking mGluR6 and WT mice injected intravitreally with the mGluR6 agonist, L-2 amino-4-phosphonobutyric acid (Slaughter and Miller 1985; Euler et al. 1996; Iwakabe et al. 1997; McGillem and Dacheux 2001; Rohrer et al. 2004; Koike et al. 2010b). The ERGs evoked by light stimuli in WT mice show normal a-waves and b-waves, originating mainly from photoreceptor cells and bipolar cells, respectively. In the dark-adapted state, the ERG b-wave in $Trpm1^{-/-}$ mice was absent and their ERG waveforms were very similar to those of $mGluR6^{-/-}$ mice, whereas the a-wave amplitudes of $Trpm1^{-/-}$ mice were equal to those from WT mice (Masu et al. 1995; Koike et al. 2007, 2010b). In the light-adapted state, the ERG b-wave was severely attenuated or absent leaving only the ERG a-wave in the $Trpm1^{-/-}$ mice (Koike et al. 2007). These ERG results suggested that the function of both rod and cone bipolar cells was severely impaired in $Trpm1^{-/-}$ mice and Trpm1 plays a crucial role in the synaptic transmission from photoreceptor cells to ON-bipolar cells.

8 Role in Hereditary and Acquired Diseases (We Therefore Do Not Include a Chapter on TRPs and Disease)

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous group of retinal disorders associated with lifelong deficient vision in the dark, while their day vision is normal. Two types of CSNB can be distinguished on the basis of the standard flash ERG: the complete form (cCSNB) and the incomplete form (icCSNB). The complete form, also known as type1 CSNB or CSNB1, is characterized by a complete loss of the b-wave in response to a dim flash and an electronegative maximum response with a normal a-wave under dark adaptation (Audo et al. 2008). The photopic single flash and 30 Hz flicker ERG has a normal a-wave amplitude with a broadened trough and a sharply rising peak with no photopic oscillatory potentials and a reduced b/a ratio (Audo et al. 2008). The incomplete form (icCSNB), also known as type 2 CSNB or CSNB2, is characterized by a reduction of b-wave but a normal a-wave in the maximal

response, and that photopic responses are severely reduced and delayed in response to both a single flash and 30 Hz flicker compared with cCSNB (Miyake et al. 1986; Audo et al. 2008). The primary cause of complete CSNB is postsynaptic defects in ON-bipolar cell signaling with the OFF-bipolar cell pathway preservation. X-linked cCSNB has been associated with mutations in genes expressed and localized in ON-bipolar cells including *nyctalopin* (*NYX*) on chromosome X for X-linked CSNB1A (Bech-Hansen et al. 1998; Pusch et al. 2000). *mGluR6* (*GRM6*) for CSNB1B (Dryja et al. 2005; Zeitz et al. 2005), *TRPM1* for CSNB1C (Audo et al. 2009; Li et al. 2009; van Genderen et al. 2009; Nakamura et al. 2010), and *G-protein-coupled receptor 179* (*GPR179*) for CSNB1E (Audo et al. 2012; Peachey et al. 2012b) are autosomal recessive form of cCSNB. Whole-exome sequencing on cCSNB patients' genome led to the identification of heterozygous mutations in exon 4 of *LRIT3* as a cause of autosomal recessive cCSNB. Immunostaining of the human retina using an anti-LRIT3 antibody exhibited a characteristic synaptic punctate labeling at the dendritic tips of depolarizing bipolar cells (Zeitz et al. 2013). Understanding of the exact functional role of LRIT3 in ON-bipolar cells awaits future analysis. Incomplete CSNB has been implicated in mutations in several genes coding for proteins important for continuous glutamate release at the photoreceptor synapse, including *calcium channel voltage-dependent alpha-1F subunit* (*CACNA1F*) on chromosome X for X-linked icCSNB. *Calcium-binding protein 4* (*CABP4*) and *calcium channel voltage-dependent alpha-2/delta subunit 4* (*CACNA2D4*) (Bech-Hansen et al. 1998; Strom et al. 1998; Zeitz et al. 2006) are involved in autosomal recessive form of icCSNB.

A single incomplete dominant gene, leopard complex (*LP*), is essential for determining the Appaloosa coat-spotting pattern in horses. Homozygosity for *LP* (*LP*/*LP*) directly causes CSNB in Appaloosa horses, characterized by a congenital and nonprogressive scotopic visual deficit (Bellone et al. 2008). Furthermore, Bellone et al. mapped a 6-cM *LP* candidate region on ECA1 where the *TRPM1* gene is located. They found that in the retina of CSNB (*LP*/*LP*) horses, *TRPM1* expression was downregulated whereas *TRPM1* was marginally downregulated in horses heterozygous for Appaloosa spotting (*LP*/*lp*), suggesting that *TRPM1* is the ethology of CSNB; however, no *TRPM1* mutation in *LP*/*LP* horses was reported (Bellone et al. 2008). In an analysis of a large South Asian family with CSNB, Li et al. identified a large region of homozygosity on chromosome 15q which contains *TRPM1* gene (Li et al. 2009). Screening identified a single homozygous mutation in the affected mother (IVS16 + 2T > C) (Li et al. 2009). In a Caucasian non-consanguineous family, the affected proband was found to have two likely disease-causing missense mutations in the *TRPM1* gene (G138fs and Y1035X) (Li et al. 2009). These mutations cause a premature termination codon which would cause the mRNA to succumb to nonsense-mediated decay (Li et al. 2009). The proband of non-consanguineous Caucasian family was found to harbor heterozygote for two missense mutations in the *TRPM1* gene (R74C and I1002F) (Li et al. 2009). Furthermore, two groups have identified other homozygous mutations, including a predicted premature stop codon, silent mutations, and missense mutations (2567G > A, 1-27C > T, 40C > T, and 1418G > C), and a

homozygous mutation for a 36,445 bp deletion of exons two to seven which produce a null allele for all isoforms of *TRPM1* (Y72-K365del) (Audo et al. 2009; van Genderen et al. 2009). In addition to homozygous mutations, current studies in *TRPM1* identified compound heterozygous mutations which comprise nonsense mutations, a deletion leading to a predicted premature stop codon, splice-site mutations, silent mutations, and missense mutations (Audo et al. 2009; Li et al. 2009; van Genderen et al. 2009; Nakamura et al. 2010). Nakamura et al. also identified five different novel mutations in the human *TRPM1* gene, IVS2-3C > G, IVS8 + 3_6felAAGT, R624C (c.1870C > T), S882X (c.2645C > A), and F1075S (c.3224T > C)) in three unrelated patients who were compound heterozygous (Nakamura et al. 2010). Biochemical and cell biological analyses revealed that the two intron mutations (IVS2-3C > G and IVS8 + 3_6delAAGT) can cause splicing abnormalities leading to defects in protein production, and the two missense mutations (R624C and F1075S) resulted in the failure of the transportation of the missense mutant channels to the dendritic tips of the ON-bipolar cells (Nakamura et al. 2010). A recent study reported a mouse mutant of *TRPM1*, *tvrm27*, which was identified through an ERG screen of chemically mutagenized mice (Won et al. 2011; Peachey et al. 2012a). The *tvrm27* mutant allele is caused from a point mutation (A1068T) in the *TRPM1* gene that results in a missense mutation in the pore domain of the TRPM1 protein (Peachey et al. 2012a). $TRPM1^{tvrm27/tvrm27}$ retinal histology is normal, whereas mice heterozygous for the $TRPM^{/tvrm27}$ allele showed decreased ERG b-wave (Peachey et al. 2012a).

Paraneoplastic retinopathy (PR), including melanoma-associated retinopathy (MAR) and cancer-associated retinopathy (CAR), is a progressive retinal disorder caused by antibodies generated against neoplasms not associated with the eye (Thirkill et al. 1989; Chan 2003; Heckenlively and Ferreyra 2008; Adamus 2009). Patients with PR can suffer from night blindness, photopsia, ring scotoma, and attenuated retinal arteriole and show abnormal ERGs. Recent studies reported that TRPM1 is an autoantigen targeted by autoantibodies in some patients with MAR or CAR (Dhingra et al. 2011; Kondo et al. 2011). The ERG findings in a patient with lung CAR showed a selective ON-bipolar dysfunction with normal OFF response, indicating that the defect is in the signal transmission between photoreceptor cells and ON-bipolar cells (Kondo et al. 2011). Western blot analysis using sera from CAR and MAR patients exhibited a significant immunoreactive band against *TRPM1*-transfected cell lysates (Kondo et al. 2011). Furthermore, immunohistochemical analysis using the serum from the CAR patient exhibited a significant immunolabeling on the bipolar side of the INL in the monkey retina where TRPM1 proteins localize (Kondo et al. 2011). TRPM1 is also identified as a target of an anti-bipolar cell antibody produced in a patient with paraneoplastic vitelliform retinopathy, which is a MAR-like retinopathy (Wang et al. 2012). A recent study reported that TRPM1 autoantibodies from MAR patient sera bind to an epitope in the intracellular N-terminal domain of the TRPM1 channel and that intravitreal injection of autoantibodies reduces the ERG b-wave in mouse eyes, although no specific autoantibody immunoreactivity was demonstrated using Western blot analysis (Xiong et al. 2013). These observations, the expression of TRPM1

in melanocytes and its downregulation in melanoma cells, suggest that TRPM1 is one of the retinal autoantigens in some CAR or MAR associated with retinal ON-bipolar cell dysfunction (Duncan et al. 1998).

It was reported that a novel homozygous 15q13.3 microdeletion is associated with a complex neurodevelopmental disorder characterized by severe visual impairment, hypotonia, profound intellectual disability, and refractory epilepsy (Lepichon et al. 2010). The deleted region of 1.5 to 2.0 Mb encompasses at least seven genes, *ARHGAP11B*, *MTMR15*, *MTMR10*, *TRPM1*, *KLF13*, *OTUD7A* and *CHRNA7* (Lepichon et al. 2010).

There is evidently a high level of allelic heterogeneity in *TRPM1*, without any clear pattern with regard to location of mutations. These mutations affect residues throughout TRPM1, the intracellular N terminus, the transmembrane domains, and C terminus. There is no noticeable genotype–phenotype relationship. However, TRPM1 channel is essential for the depolarizing ON-bipolar cells in humans as well as in mice.

After decades of sizeable efforts on the ON-bipolar transduction channel, TRPM1 was finally identified as a nonselective and constitutively active cation channel negatively regulated by mGluR6 in retinal ON-bipolar cells, based on *Trpm1* mutant mouse phenotypes and a reconstitution system with TRPM1, mGluR6, and Goα, using culture cells (Koike et al. 2010b). Molecular genetic analysis on CSNB patients supports the notion that TRPM1 plays an essential role in mediating the photoresponse in retinal ON-bipolar cells. It should be noted that recent studies reported that other components or modulators, including GPR179 and LRIT3, function within the ON-bipolar cell pathway. Extensive future studies on both mouse retinas and human CSNB patients will be very useful to gain a deeper understanding on the detailed molecular mechanisms that are the foundation of ON-bipolar signal transduction mechanisms.

TRPM1 is also proposed to play roles in other processes, including melanocytogenesis, pigmentation, and melanoma metastasis. However, melanin production in the *Trpm1* null mutant mice seems unaffected (Koike et al. 2010b). Therefore, further investigation of Trpm1 is still needed to fully understand Trpm1 biological functions and its underlying biochemical mechanisms.

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TRPM2

Malika Faouzi and Reinhold Penner

Contents

Abstract

TRPM2 is the second member of the transient receptor potential melastatin-related (TRPM) family of cation channels. The protein is widely expressed including in the brain, immune system, endocrine cells, and endothelia. It embodies both ion channel functionality and enzymatic ADP-ribose (ADPr) hydrolase activity. TRPM2 is a Ca^{2+}-permeable nonselective cation channel embedded in the plasma membrane and/or lysosomal compartments that is primarily activated in a synergistic fashion by intracellular ADP-ribose (ADPr) and Ca^{2+}. It is also activated by reactive oxygen and nitrogen species (ROS/NOS) and enhanced by additional factors, such as cyclic ADPr and NAADP, while inhibited by permeating protons (acidic pH) and adenosine monophosphate (AMP). Activation of TRPM2 leads to increases in intracellular Ca^{2+} levels, which can serve signaling roles in inflammatory and secretory cells

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through release of vesicular mediators (e.g., cytokines, neurotransmitters, insulin) and in extreme cases can induce apoptotic and necrotic cell death under oxidative stress.

Keywords
Calcium • Non-selective cation • Transient receptor potential channel • Reactive oxygen species • ADP ribose • Nucleoside diphosphate hydrolase • Inflammation • Apoptosis • Cancer • Diabetes

1 Gene

TRPM2 was first isolated from human brain in 1998 and given the designation TRPC7 (transient receptor potential-related channel 7) (Nagamine et al. 1998). The protein was later categorized more appropriately as a member of the long TRPC subfamily nomenclature and referred to as LTRPC2 (Harteneck et al. 2000). In 2002, a unified nomenclature assigned it to the melastatin subfamily of TRP channels as TRPM2 (Montell et al. 2002).

The gene coding human TRPM2 is located between two markers D21S400 and D21S171 on human chromosome 21q22.3 and consists of 32 exons spanning 90 kb and mapping a 1503 amino acid long protein (Nagamine et al. 1998). An additional exon has been reported (Uemura et al. 2005), indicating two transcription start sites in the human TRPM2 gene that yield two forms of TRPM2: a 6.5 kb transcript encoding the 1503 amino acid full-length long form TRPM2 (TRPM2-L) that is widely expressed and starts from a noncoding exon associated with a CpG island, and a shorter 5.5 kb transcript that starts from intron 4 and encodes a 1289 amino acid striatum short form TRPM2 (TRPM2-SSF) that lacks N-terminal 214 amino acid residues of the long form. In addition, various TRPM2 splice variants have been identified: TRPM2-ΔN, TRPM2-ΔC, TRPM2-ΔNΔC, and TRPM2-S (see Sect. 3 for specifics). A recent study found that 17β-estradiol (E2) treatment induces an increase in TRPM2 transcripts in human endometrial cells and identified a functional estrogen response element (ERE) in the 3′-untranslated region (UTR) of the TRPM2 gene (Hiroi et al. 2013a).

The mouse Trpm2 gene contains 34 exons and spans about 61 kb. In contrast to the human gene, it has only one transcription start site and no second promoter to produce a shorter mRNA. The mouse gene also does not exhibit any predicted CpG islands (Uemura et al. 2005).

2 Expression

TRPM2 is widely expressed in the central nervous system (CNS), including hippocampus, thalamus, striatum, and cerebral cortex, as well as in microglia (Nagamine et al. 1998; Kraft et al. 2004; Fonfria et al. 2005, 2006a, b; Lipski et al. 2006; Olah

et al. 2009; Roedding et al. 2013). However, its presence at mRNA and/or protein levels is not ubiquitous throughout all CNS regions and within all neuronal subtypes. Indeed, TRPM2 could not be detected within either cultured astrocytes or granule cells of the cerebellum (Kraft et al. 2004). Additionally, while hippocampal CA1 pyramidal neurons possess functional TRPM2 channels (Olah et al. 2009; Belrose et al. 2012), hippocampal CA1 stratum radiatum interneurons show no functional evidence of TRPM2 expression (Olah et al. 2009).

TRPM2 is also detected in other tissues such as the bone marrow, spleen, heart, liver, lung, placenta endometrium, and gastrointestinal tract and in different cell types like pancreatic β-cells (Fonfria et al. 2006b; Togashi et al. 2006; Ishii et al. 2006a, b; Lange et al. 2009; Uchida and Tominaga 2011; Uchida et al. 2011; Hiroi et al. 2013a), salivary gland (Liu et al. 2013), endothelial cells (Hecquet et al. 2008, 2010; Hecquet and Malik 2009; Sun et al. 2012), heart and vasculature (Yang et al. 2006; Takahashi et al. 2012; Miller et al. 2013), and immune cells (neutrophils, megakaryocytes, monocytes, macrophages, B lymphoblast cells, T lymphocytes, and mast cells) (Heiner et al. 2003a, b; Carter et al. 2006; Yamamoto et al. 2008; Lange et al. 2008; Wenning et al. 2011; Roedding et al. 2012; Kashio et al. 2012; Magnone et al. 2012; Oda et al. 2013; Hiroi et al. 2013b; Knowles et al. 2013).

Although originally described as a plasma membrane channel, TRPM2 has been found to also function as a lysosomal Ca^{2+} release channel in pancreatic β-cells and dendritic cells (Lange et al. 2009; Sumoza-Toledo et al. 2011). It shares this cellular localization in the endosomal pathway with the mucolipin channels TRPML1–3 (Piper and Luzio 2004; Dong et al. 2010; Cheng et al. 2010) and the two-pore channels TPC1–3 (Calcraft et al. 2009; Brailoiu et al. 2009; Galione et al. 2009; Zong et al. 2009; Ruas et al. 2010; Pitt et al. 2010). Intracellular localization, albeit not in lysosomes, has also been reported for other TRP channels, including TRPV1 (Morenilla-Palao et al. 2004), TRPC5 (Bezzerides et al. 2004), TRPC3 (Singh et al. 2004), TRPM8 (Thebault et al. 2005), and TRPM7 (Oancea et al. 2006). The factors that would determine the cellular localization of TRPM2 and various other TRP channels remain to be defined, as well as whether the cellular localization serves a particular cellular function.

3 The Channel Protein Including Structural Aspects

The full-length TRPM2 consists of an intracellular N terminus of ~700 amino acids, the TRPM homology region, followed by a region of approximately 300 amino acids (residues 762–1048) containing six putative transmembrane domains (S1–S6), a pore-forming loop domain located between S5 and S6, an approximately 100 amino acid region of high coiled-coil character (CCR), a short 30 amino acid linker region, and a unique intracellular C-terminal adenosine diphosphate ribose (ADPr) pyrophosphatase domain (residues 1236–1503, Nudix-like or NUDT9 homology domain) (Fig. 1) (Perraud et al. 2001, 2003a; Sano et al. 2001; Fleig and Penner 2004a, b).

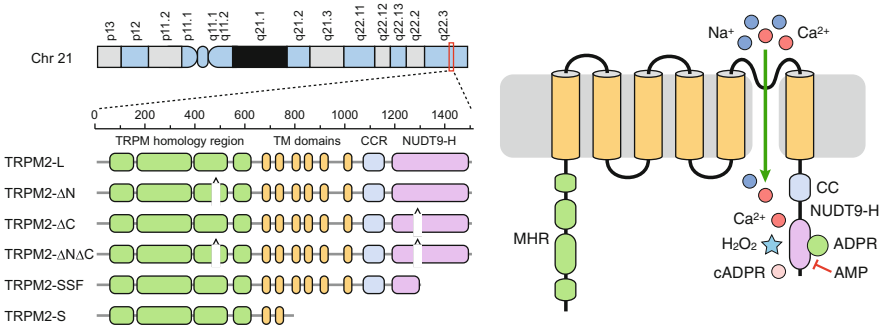

Fig. 1 Schematic of TRPM2 gene and encoded protein isoforms (*left*) and membrane topology (*right*). The human chromosome 21 schematic (*top left*) shows the location of TRPM2 gene in the sub-band 3 of band 2 of the second region of the long arm q of the chromosome 21 (21q22.3). The gene encodes a full-length TRPM2 form (TRPM2-L) and various splice variants (*bottom left*). The full-length protein is composed of 1503 amino acids (1507 in mouse and rat). Segments in the N terminus denote the four domains of the TRPM homology region (MHR), followed by six transmembrane segments (TM: S1–S6) with the putative pore-forming region (S5–S6). The C-terminal region contains a coiled-coil region (CCR) and a NUDT9-homology region (NUDT9-H). The caret (^) denotes the deletions within the N- and C-terminal domains of TRPM2 variants. The membrane topology of TRPM2 (*right*) shows that both N- and C-termini are in the cytosol. ADP-ribose (ADPr) binds to the NUDT9-H region to induce channel gating and enable calcium (Ca^{2+}) and sodium (Na^{+}) influx. The NUDT9-H enzymatic activity hydrolyses ADPr to ribose 5-phosphate and adenosine monophosphate (AMP). AMP, in turn, acts as a negative regulator of TRPM2. TRPM2 gating by ADPr is facilitated by hydrogen (H_2O_2), cyclic ADPr (cADPr), and Ca^{2+}

The TRPM2 N terminus has four homologous domains and a calmodulin (CaM)-binding IQ-like motif located at 406–416AA, which plays a role in modulating channel activation (Perraud et al. 2001; Sano et al. 2001; McHugh et al. 2003; Fleig and Penner 2004a, b; Tong et al. 2006). It was also reported that deletion of a stretch of 20 amino acid residues (Δ537–556) in the N terminus, corresponding to the TRPM2-ΔN splice variant in neutrophils, abolishes any channel function (Wehage et al. 2002). This dysfunction is believed to be related to undetermined motifs within the ΔN-stretch, but not the IQ-like motif, and two SH3-binding (PxxP) motifs found in this region (Kühn et al. 2009). Unlike their close relatives in the TRPC and TRPV subfamilies, TRPM channels contain a pair of cysteine residues in the pore region (positions 996 and 1008 for TRPM2), whose substitutions with either alanine or serine did not affect protein expression/trafficking or localization, but generated TRPM2 channels that were functionally unresponsive to ADPr (Mei et al. 2006a). Furthermore, a substitution mutation of I1045K on the distal part of the S6 domain was crucial for the selectivity of TRPM2, transforming TRPM2 from a cation to an anion channel (Kühn et al. 2007).

The CCR region is hypothesized to be involved in several important functions, including protein trafficking, channel tetrameric assembly, and gating (Perraud et al. 2003a; Jiang 2007). CCR deletion or site-directed mutagenesis did not affect protein expression, but resulted in severe disruption of the TRPM2 subunit

assemblies and substantial loss of ADPr-evoked channel currents (Mei et al. 2006b). This was due to reduced trafficking of TRPM2 subunits and proper localization at the membrane level.

The NUDT9-H region that gives TRPM2 the chanzyme designation is named after the mitochondrial ADP-ribose pyrophosphatase NUDT9 with whom it shares 39 % homology (Shen et al. 2003). The NUDT9-H region contains a Nudix box sequence motif G*X*(5)E*X*(7)REU*X*EE*X*U (*X* represents any amino acid residue, and U represents a large hydrophobic residue) that is characteristic of a family of diverse pyrophosphatases that accept nucleoside diphosphate substrates like ADPr (Kühn and Lückhoff 2004; Mildvan et al. 2005). Biochemical analyses have indicated that NUDT9 consists of two domains, a C-terminal CORE domain containing the structures required for ADPrase activity and an N-terminal CAP domain which enhances the CORE domain's affinity for ADPr (Perraud et al. 2003a). Deletion of the NUDT9-H domain strongly decreases TRPM2 plasma membrane expression, indicating its vital role for normal channel assembly and surface trafficking (Perraud et al. 2005). This role has been specifically linked to the NUDT9-H CORE domain, since the TRPM2-ΔC channels that lack amino acids in the NUDT9-H CAP region are properly expressed at the cell surface (Perraud et al. 2003a). Additionally, the NUDT9-H region is directly involved in TRPM2 channel gating by virtue of binding ADPr at multiple sites (Perraud et al. 2001, 2003a; Kühn and Lückhoff 2004). It appears that the binding of ADPr rather than enzymatic activity of TRPM2's NUDT9-H domain is critical for channel gating, as mutations that eliminate ADPrase activity retain channel gating capacity (Perraud et al. 2003b, 2005). Three-dimensional reconstruction of purified tetrameric TRPM2 using transmission electron microscopy has yielded first insights into the structure of the channel protein at 2.8 nm resolution, revealing a swollen, bell-shaped structure of 18 nm in width and 25 nm in height (Maruyama et al. 2007).

In addition to the full-length TRPM2 (TRPM2-L), physiological TRPM2 splice variants missing one or both exons 11 and 27 (Fig. 1) have been identified in human hematopoietic cells (HL-60 monocytes and neutrophil granulocytes): TRPM2-ΔN is characterized by a deletion in the N terminus (residues K538-Q557), TRPM2-ΔC lacks residues in the C terminus (T1292–L1325), and TRPM2-ΔNΔC carries both deletions (Wehage et al. 2002). An additional short variant, TRPM2-S, contains only the N terminus and the first two transmembrane segments and is generated by an additional stop codon (TAG) at the splice junction between exons 16 and 17 (Fig. 1). This variant has been found in the bone marrow, brain and pulmonary arteries, and aorta (Zhang et al. 2003; Yang et al. 2006; Vázquez and Valverde 2006; Hecquet and Malik 2009) and may act as a dominant negative inhibitor of TRPM2 activity (Zhang et al. 2003). The TRPM2-ΔC proteins encoded by exon 27 deletion transcripts carry a deletion within the NUDT9-H. While TRPM2-ΔN fails to respond to either ADPr or H_2O_2, it has been reported that the TRPM2-ΔC variant responds to H_2O_2 but not to ADPr, indicating a possible direct activation of TRPM2 by H_2O_2 (Wehage et al. 2002).

4 Interacting Proteins

Only a few studies have investigated TRPM2 channel interaction with other proteins (So et al.). It has been reported that the TRPM2-S isoform acts as a suppressor of H_2O_2-induced calcium influx through the full-length TRPM2 (TRPM2-L) channels when heterologously expressed in HEK-293T cells. This effect involves a direct interaction between the two isoforms and not a modification in subcellular localization of TRPM2-L (Zhang et al. 2003). A study examining the TRPM2 protein partners that regulate cell survival has found that the protein tyrosine phosphatase-L1 (PTPL1) interacts with TRPM2 channels to decrease their tyrosine phosphorylation and activity and thereby reduce H_2O_2- and TNF-α-induced cell death in HEK-293 cells (Zhang et al. 2007). This interaction was examined and confirmed endogenously in the human monocytic U937-ecoR cells, supporting the relevance of TRPM2 in the cell-death resistance phenotype within the PTPL1-overexpressing tumors. Furthermore, immunoprecipitation analysis has demonstrated physical interaction of the N- and C-terminal cytoplasmic tails of TRPM2 with the EF-hand domain-containing protein 1 (EFHC1), whose mutation causes juvenile myoclonic epilepsy (JME) via mechanisms including neuronal apoptosis (Katano et al. 2012). This study also reported that this interaction significantly potentiated cell death mediated by H_2O_2, ADPr-induced Ca^{2+} responses, and cationic currents via recombinant TRPM2 in HEK-293 cells.

An important functional interaction is provided by the calcium sensor calmodulin (CaM). Its involvement in TRPM2 modulation appears to be responsible, at least in part, for the Ca^{2+}-dependent activation of TRPM2 (Tong et al. 2006). Thus, overexpression of a dominant negative mutant of CaM was able to compete with endogenous CaM and inhibit TRPM2-mediated increases in $[Ca^{2+}]_i$ and immunoprecipitation confirmed a direct interaction between CaM and TRPM2. A strong CaM binding region was identified in the TRPM2 N terminus (amino acids 1–730) and weak binding region in the C terminus (amino acids 1060–1503). CaM is believed to bind to an IQ-like consensus binding motif on the TRPM2 N terminus (amino acids 406–416) since a substitution mutant of this motif (TRPM2-IQMUT1) reduced the CaM-TRPM2 binding (Tong et al. 2006). The IQ-like motif was shown to be the mechanism mediating Ca^{2+}-activated TRPM2 currents (Du et al. 2009a). Additionally, intracellular perfusion of cells with CaM in the patch pipette significantly increased ADPr-activated TRPM2 currents, whereas exposure to 2 μM calmidazolium, a known CaM antagonist, prevented ADPr-mediated TRPM2 currents (Starkus et al. 2007).

One study has found that the ΔC splice variant of TRPM2 co-immunoprecipitates with CD38 in HeLa cells and the authors proposed that this close interaction may form the basis for hypertonicity-induced gating of this splice variant (Numata et al. 2012).

Finally, proteome-wide site-specific quantifications of endogenous putative ubiquitylation sites indicate posttranslational modifications of TRPM2 (Wagner et al. 2011; Kim et al. 2011), although their physiological context and functional consequences remain to be explored.

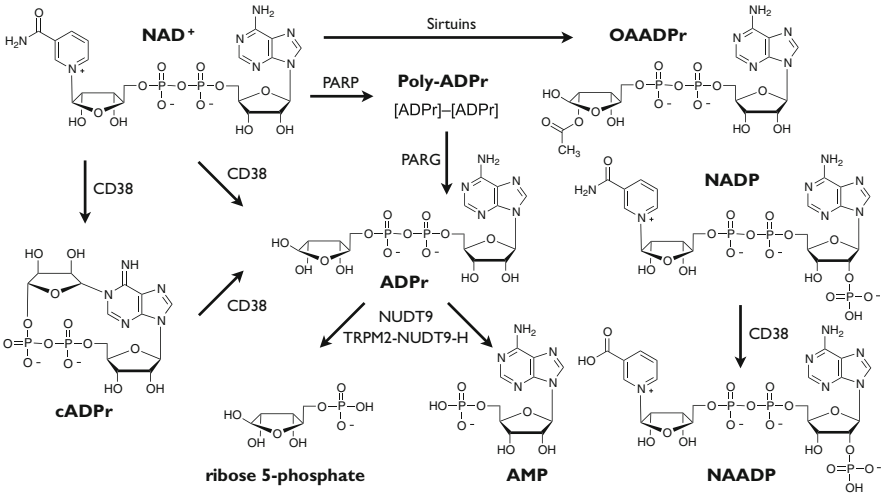

Fig. 2 Schematic of metabolic pathways of pyridine nucleotides acting on TRPM2. The primary activator of TRPM2 is ADP-ribose (ADPr), which can be produced from several sources through various enzymatic reactions. Nicotinamide adenine dinucleotide (NAD^+) can be directly converted to ADPr by CD38 NADase activity or indirectly through the intermediate cyclic ADPr (cADPr)—a facilitator of ADPr-mediated activation of TRPM2—that is produced by CD38's ADP-ribosyl cyclase activity and can further be converted to ADPr via cADPr hydrolase activity of CD38. NAD^+ is also the substrate of poly(ADPr) polymerase (PARP), which creates ADPr polymers that can be hydrolyzed to free ADPr by the poly(ADPr) glycohydrolase (PARG) and sirtuins, which generate the TRPM2 agonist 2′-*O*-acetyl-ADPr (OAADPr). ADPr itself is the substrate of the ADPr pyrophosphatase NUDT9 as well TRPM2's endogenous NUDT9 homology domain in the N terminus, yielding the inactive metabolite ribose 6-phosphate and the TRPM2 inhibitor adenosine monophosphate (AMP). Finally, it is thought that NAADP, another facilitator of ADPr-mediated TRPM2 gating, can be formed from NADP by a base-exchange reaction via CD38

5 A Biophysical Description of the Channel Function, Permeation, and Gating

TRPM2 is a homo-tetrameric nonselective cation permeable channel that exhibits a perfectly linear I/V curve (Perraud et al. 2003a; Csanády and Törocsik 2009). The channel activates in response to low micromolar levels of cytosolic ADPr with half-maximal effective concentrations (EC_{50}) of 1–90 μM (Perraud et al. 2001; Sano et al. 2001; Inamura et al. 2003; Beck et al. 2006; Gasser et al. 2006; Starkus et al. 2007; Lange et al. 2008). The variability in EC_{50} values may arise from the modulatory mechanisms expressed in a given cell type. At the cellular level, free ADPr is mainly produced by the hydrolysis of NAD^+ and/or cADPr by glycohydrolases, including the ectoenzymes CD38 and CD157, as well as the mitochondrial NADase (Lund et al. 1995, 1998; Lund 2006; Malavasi et al. 2006). A further source of ADPr is provided by the combined action of poly

(ADPr) polymerases (PARP) and poly(ADPr) glycohydrolases (PARG), which indirectly generate ADPr via formation and hydrolysis of poly-ADPr when hyperactivated in response to DNA damage (Esposito and Cuzzocrea 2009; Caiafa et al. 2009; Fauzee et al. 2010). Figure 2 illustrates the various adenine nucleotides and their metabolic pathways.

The ability of other adenine nucleotide second messengers, metabolically related to ADPr, to activate TRPM2 channels has been described. These include cyclic ADPr (cADPr; EC_{50} ~0.7 mM) (Kolisek et al. 2005; Lange et al. 2008) and nicotinic acid adenine dinucleotide phosphate (NAADP; EC_{50} ~0.73 mM) (Beck et al. 2006; Lange et al. 2008). Even though activation of TRPM2 by high concentrations of nicotinamide adenine dinucleotide (NAD^+; EC_{50} ~1–1.8 mM) has been observed (Sano et al. 2001; Hara et al. 2002; Naziroğlu and Lückhoff 2008), its status as a direct agonist for TRPM2 remains uncertain, since at least in some studies, contaminations with ADPr or metabolism of NAD^+ may account for the observed TRPM2 activation (Beck et al. 2006; Grubisha et al. 2006). The relatively high concentrations of cADPr and NAADP required to activate TRPM2 directly are above physiological levels; however, these adenine nucleotide second messengers can synergize with ADPr and increase TRPM2 sensitivity at much lower doses. In fact, it has been reported that 10 μM of cADPr may facilitate TRPM2 function such that nanomolar (possibly ambient) cytosolic levels of ADPr can activate the channel (Kolisek et al. 2005). Whether these nucleotides bind directly to the Nudix domain, or to different cooperative sites, or are converted to ADPr is not clearly understood.

A significant enzymatic source of ADPr is CD38, a multifunctional ectoenzyme that is widely expressed in hematopoietic and non-hematopoietic cells. It uses NAD^+ as a substrate to catalyze the production of ADPr, cADPr, and NAADP (Lund et al. 1995, 1998). In neutrophils both CD38 and TRPM2 channels are present in the plasma membrane, possibly establishing a signaling pathway that involves CD38, ADPr production, and TRPM2 activation. Indeed, CD38 knockout (KO) neutrophils stimulated with the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) show a reduced Ca^{2+} response when compared to wild-type cells (Partida-Sánchez et al. 2003). Similarly, fMLP-treated TRPM2 KO neutrophils have defects in Ca^{2+} influx (Yamamoto et al. 2008). Additionally, the fMLP-induced Ca^{2+} entry in neutrophils is inhibited with the ADPr and cADPr antagonists 8Br-ADPr and 8Br-cADPr, respectively (Partida-Sánchez et al. 2004, 2007). Although ADPr is the main product of CD38 and evidence points to TRPM2 as a mediator of Ca^{2+} entry, there are still open questions such as to whether and how the extracellular ADPr generated by CD38 crosses the plasma membrane and acts on the cytosolic Nudix domain of TRPM2 channels (Franco et al. 1998; Bruzzone et al. 2001). A further metabolite coupling to TRPM2 is the sirtuin-generated acetyl-ADP-ribose product 2′-*O*-acetyl-ADP-ribose (OAADPr), which also induces TRPM2 currents by direct binding to the Nudix domain with an EC_{50} of ~100 μM (Grubisha et al. 2006; Tong and Denu 2010). OAADPr is produced by a histone/protein deacetylase reaction mediated by a family of silent information regulator 2 (Sir2 or sirtuin)-related NAD-dependent protein deacetylases. Indeed,

the mammalian sirtuins SIRT2 and SIRT3 have been suggested to generate the OAADPr that leads to TRPM2-dependent cell death induced by puromycin, while specific RNAi knockdown in TRPM2-expressing cells protects these cells from cell death (Grubisha et al. 2006).

The gating and full activation of TRPM2 channels by ADPr is highly sensitive to Ca^{2+}, as either absence of external Ca^{2+} or strong buffering of internal Ca^{2+} to low levels (<30 nM) substantially inhibit gating of TRPM2 channels by ADPr (McHugh et al. 2003; Starkus et al. 2007; Csanády and Törocsik 2009). This Ca^{2+} effect is not mimicked by other divalent cations such as Mg^{2+}, Ba^{2+}, or Zn^{2+} (Starkus et al. 2007). Moreover, 200 μM external Ca^{2+} is sufficient and as efficient as 1 mM Ca^{2+} in promoting TRPM2 activation (Starkus et al. 2007). It has also been suggested that Ca^{2+} may gate the channel directly in a dose-dependent manner with an EC_{50} of 17 μM (Du et al. 2009a), possibly as a result of conformational changes due to Ca^{2+}-dependent binding of CaM with the TRPM2 IQ-like motif or other intracellular sites (Du et al. 2009a). However, other groups have not observed Ca^{2+}-induced activation in the absence of ADPr (McHugh et al. 2003; Starkus et al. 2007; Csanády and Törocsik 2009), and it is therefore possible that TRPM2 activation is secondary to ADPr production or ADPr release from mitochondria caused by high Ca^{2+} concentrations. Similarly to the facilitating role of intracellular Ca^{2+}, it has been suggested that intracellular chloride ions may also provide a facilitating effect on ADPr- and H_2O_2-induced activation of TRPM2, promoting ADPr/Ca^{2+}-induced TRPM2 gating with an EC_{50} of ~18 mM (Hong et al. 2010). This effect has been attributed to a critical lysine residue K1110 that is located between TRPM2's transmembrane domains and the coiled-coil region and whose mutation inhibited channel activation by both ADPr and H_2O_2 (Kim et al. 2013).

TRPM2 channels can also be activated by micromolar levels of H_2O_2 and agents that produce reactive oxygen/nitrogen species, providing a direct link to inflammation, oxidative stress, and cell death (Hara et al. 2002; Kolisek et al. 2005; Ishii et al. 2006b; Yamamoto et al. 2008; Takahashi et al. 2011; Haraguchi et al. 2012). Whether or not H_2O_2 can gate TRPM2 directly and independently of ADPr remains unclear. Wehage et al. found that TRPM2-ΔC channels expressed in HEK293 cells, which fail to respond to ADPr, could still be activated by H_2O_2, suggesting distinct and independent gating mechanisms of ADPr and H_2O_2 (Wehage et al. 2002). However, a later study in Chinese Hamster Ovary cells could not confirm direct H_2O_2 activation (Kühn and Lückhoff 2004). Kolisek et al. reported that H_2O_2 by itself, like cADPr, was not effective in activating TRPM2, but strongly facilitated ADPr-mediated gating. Hence, an alternative explanation for the capacity of H_2O_2 to induce TRPM2 activation may relate to its ability to both mobilize ADPr from mitochondria (Perraud et al. 2005) and, at the same time, synergize with ADPr in gating the channel (Kolisek et al. 2005). The notion that release of ADPr from mitochondria could be a critical mechanism leading to TRPM2 gating (Ayub and Hallett 2004) was confirmed by experiments showing that H_2O_2-induced TRMP2 currents were suppressed when reducing the ADPr concentration within the mitochondria (Perraud et al. 2005).

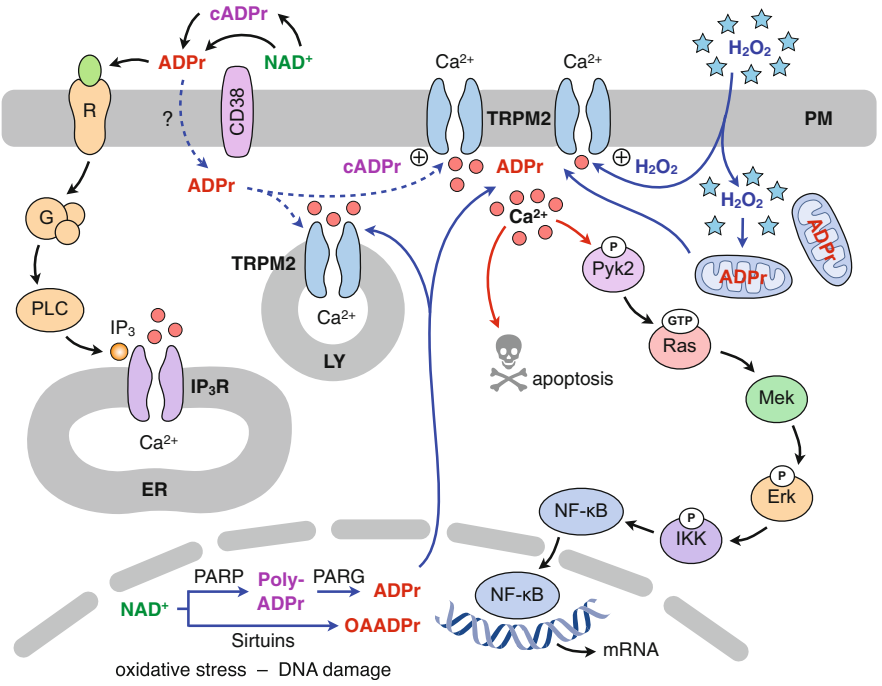

Fig. 3 Upstream and downstream signaling mechanisms for TRPM2 activation. External NAD^+ and reactive oxygen species (ROS), including H_2O_2, accumulate during inflammation and tissue damage. NAD^+ may be converted to ADPr and cADPr by the ectoenzyme CD38. Extracellular ADPr may then bind to G-protein-coupled purinergic receptors and increase $[Ca^{2+}]_i$ through Ca^{2+} release from stores via G-proteins and the phospholipase C (PLC) pathway with subsequent IP_3 production. ADPr may also translocate across the plasma membrane (PM) to gate TRPM2. H_2O_2 can also cross the plasma membrane and mobilize ADPr from mitochondria and both H_2O_2 and cADPr can synergize with ADPr to activate TRPM2. Additionally, ADPr is also generated from NAD^+ via poly-ADPr during ROS-induced DNA damage through activation of the PARP/PARG pathway. NAD^+ can also be used to generate *O*-acetyl-ADPr, another agonist of TRPM2, through nuclear and cytosolic sirtuins. Free cytosolic ADPr or OAADPr can act on the NUDT9-H of both lysosomal and plasma membrane TRPM2 channels, enabling Ca^{2+} influx across the plasma membrane and/or release of lysosomal Ca^{2+}, raising the Ca^{2+} concentration in the cytosol. Intracellular Ca^{2+} increases will activate different physiological processes including gene expression through Ca^{2+}-dependent signaling pathways such as MAP Kinase and NF-κB. Ca^{2+} overload may also trigger programmed cell death (apoptosis) and possibly necrosis

In addition to mitochondrial sources, ADPr may also be generated in the nucleus through the activation of the PARP/PARG pathway following oxidative stress and DNA damage (Fonfria et al. 2004). Poly(ADP-ribosyl)ation is regulated by the synthesizing enzyme poly(ADP-ribose) polymerase-1 (PARP-1) and the degrading enzyme poly(ADP-ribose) glycohydrolase (PARG) (Esposito and Cuzzocrea 2009; Caiafa et al. 2009; Fauzee et al. 2010), resulting in the production of free ADPr that can then activate TRPM2. The involvement of this mechanism has been demonstrated pharmacologically through the use of PARP inhibitors, which

effectively suppress H_2O_2-mediated and PARP-dependent Ca^{2+} increases through TRPM2 channels (Fonfria et al. 2004). Similarly, genetic PARP ablation in DT40 cells, which express TRPM2, results in loss of oxidative stress-induced Ca^{2+} responses normally seen in wild-type DT40 (Buelow et al. 2008). PARP-1 knockout mice have also implicated this enzyme, in combination with androgen receptor signaling, to be responsible for male-specific TRPM2 channel activation and neuronal injury (Shimizu et al. 2013). Figure 3 illustrates some of the most important signaling pathways for TRPM2 activation.

In pancreatic beta cells, the gating of TRPM2 appears to be influenced by temperature. It has been reported for rat insulinoma RIN-5F cells that temperatures higher than 35 °C can directly activate TRPM2 channels and potentiate ADPr- and cADPr-induced activation of TRPM2 (Togashi et al. 2006). A similar temperature-dependent potentiation of cADPr-induced Ca^{2+} signals via TRPM2 has been observed in NG108-15 neuronal cells (Amina et al. 2010). The underlying mechanisms and the possible physiological consequences of these effects remain to be identified.

A somewhat unusual activation mechanism has been proposed for the ΔC splice variant of TRPM2, which is insensitive to adenine nucleotides. Yet in HeLa cells, this variant has been suggested to function as a poorly Ca^{2+}-permeable cation channel that is activated by hypertonicity via nucleotide transport activity of CD38 (Numata et al. 2012).

In addition to the facilitating modulators of TRPM2 discussed above, the channel can also be inhibited. The first such negative regulator described was adenosine monophosphate (AMP) (Kolisek et al. 2005; Beck et al. 2006; Lange et al. 2009; Tóth and Csanády 2010), which represents a breakdown product of TRPM2's endogenous enzymatic domain, hydrolyzing the physiological agonist ADPr into AMP and ribose 5-phosphate (Perraud et al. 2001, 2003b). AMP can also be elevated as a result of ischemia and may attempt to limit Ca^{2+} entry through TRPM2. It remains to be determined whether the inhibitory effect of AMP is direct or indirectly mediated by AMP-dependent signals such as AMP kinase.

In addition to AMP, TRPM2 channels are negatively regulated by protons and cellular acidification (Du et al. 2009b; Starkus et al. 2010; Yang et al. 2010). Thus, TRPM2 currents are completely suppressed when cells are externally or internally exposed to pH of 5–6 (Du et al. 2009b; Starkus et al. 2010), although conflicting interpretations with respect to proton permeation through TRPM2 channels and the site of inhibitory action of protons have been presented. One study proposed that protons inhibit at the extracellular side (Du et al. 2009b), whereas two other laboratories suggest that the mechanism is linked to protons competing with Na^+ and Ca^{2+} ions for channel permeation, and channel closure results from a competitive antagonism of protons at an intracellular Ca^{2+}-binding site (Starkus et al. 2010; Csanády 2010).

Additional inhibition of TRPM2 currents has been observed with various divalent heavy metal cations, including Cu^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+}, Se^{2+} (Zeng et al. 2012), and Zn^{2+} (Yang et al. 2011). Of these ions, Cu^{2+}, Hg^{2+}, and Zn^{2+} are the most potent

and have been shown to act as extracellular pore-blocking antagonists (Yang et al. 2011; Zeng et al. 2012).

6 Physiological Functions in Native Cells, Organs, and Organ Systems

The TRPM2 expression profile throughout the body (Fonfria et al. 2006b) and the channel's role in Ca^{2+} mobilization from both extracellular and intracellular compartments makes it a strong candidate to mediate significant calcium-dependent physiological processes. The abundant presence of TRPM2 in the CNS has been investigated and related to some physiological functions, including TRPM2's contribution to synaptic transmission in hippocampal CA3-CA1 synapses and its activation following Ca^{2+} increases mediated by voltage-dependent Ca^{2+} channels and glutamate receptors (Olah et al. 2009; Xie et al. 2011). Additional roles for TRPM2 in the CNS are related to its presence in microglia, the host macrophages of the brain, where TRPM2 appears to be responsible for physiological microglia activation through ROS- and LPS-mediated signaling (Kraft et al. 2004; Fonfria et al. 2006a; Wehrhahn et al. 2010). However, the majority of studies place TRPM2 into the context of pathophysiological events of stroke/ischemia and neurodegeneration (Xie et al. 2011), where TRPM2's roles include numerous mechanisms that result in the promotion of cytokine release, the exacerbation of inflammation, and the initiation of neuronal death.

In addition to the neuronal and microglial populations of the CNS, TRPM2 is also localized in various cell types of the peripheral immune system, including neutrophils (Heiner et al. 2003a, b, 2006; Partida-Sanchez et al. 2007; Lange et al. 2008; Hiroi et al. 2013b), monocytes (Perraud et al. 2001; Yamamoto et al. 2008; Wehrhahn et al. 2010), macrophages (Kashio et al. 2012; Zou et al. 2013), dendritic cells (Partida-Sanchez et al. 2007; Sumoza-Toledo et al. 2011), and lymphocytes (Beck et al. 2006; Buelow et al. 2008; Roedding et al. 2012). In most cells, TRPM2 has been investigated in the context of inflammation, mediating responses to oxidative stress and/or chemoattractants, acting as a plasma membrane-resident mediator of stimulus-induced Ca^{2+} influx. Thus, Ca^{2+} influx through TRPM2 induced by H_2O_2 and ROS in monocytes, macrophages, and lymphocytes can directly mediate cytokine release and contribute to recruitment and activation of inflammatory cells to the site of injury (Sano et al. 2001; Yamamoto et al. 2009; Sumoza-Toledo et al. 2011; Kashio et al. 2012; Magnone et al. 2012; Oda et al. 2013; Knowles et al. 2013). Additionally, TRPM2-deficient mice show decreased levels of cytokines IL-12 and IFNγ and are more susceptible to infection with Listeria monocytogenes (Knowles et al. 2011). Interestingly, dendritic cells express TRPM2 exclusively intracellularly, where it acts as a lysosomal Ca^{2+} release channel and plays a role in cell maturation via chemokine production and cell migration (Sumoza-Toledo et al. 2011).

Paradoxically, TRPM2 has also been shown to inhibit ROS production in phagocytic cells and prevent endotoxin-induced lung inflammation

(Di et al. 2012). This has been linked to the dampening of NADPH oxidase-mediated ROS production through depolarization of the plasma membrane. As a result, TRPM2-KO mice exposed to endotoxin show enhanced inflammatory responses and reduced survival compared to WT mice.

Outside of the immune context, TRPM2 has also been identified in endocrine cells such as pancreatic β-cells (Qian et al. 2002; Togashi et al. 2006; Ishii et al. 2006a, b; Lange et al. 2009; Bari et al. 2009), where its activity has been demonstrated to contribute to glucose-induced insulin release and alloxan- and H_2O_2-mediated apoptosis (Herson and Ashford 1997, 1999; Togashi et al. 2006; Uchida and Tominaga 2011; Uchida et al. 2011). Uchida and collaborators have shown that glucose tolerance was impaired and insulin secretion was decreased in TRPM2 knockout mice. They also found that basal blood glucose levels were higher in TRPM2-KO mice than in WT mice, while plasma insulin levels were similar. β-cells isolated from TRPM2-KO mice produced smaller Ca^{2+} signals in response to high concentrations of glucose and incretin hormone than WT cells, resulting in reduced insulin secretion from pancreatic islets of these mice. Insulin secretion via TRPM2 seems to not only depend on the control of intracellular Ca^{2+} concentrations, but also occurs through Ca^{2+} influx-independent mechanisms (Uchida and Tominaga 2011; Uchida et al. 2011). Additionally, TRPM2 deletion is thought to protect mice from developing diet-induced obesity and insulin resistance (Zhang et al. 2012).

TRPM2 downregulation has also been shown to protect vascular endothelial cells from both H_2O_2- and tumor necrosis factor (TNF)α-induced apoptotic cell death (Sun et al. 2012). TRPM2 channels may further be important for disrupting the bronchial epithelial tight junctions, since their activation by oxidative stress induced the attenuation of the junctions through phospholipase Cγ1 (PLCγ1) and the protein kinase Cα (PKCα) signaling cascade (Xu et al. 2013b).

A somewhat unusual role and activation mechanism has been proposed for the ΔC splice variant of TRPM2 found in HeLa cells. Here it has been suggested that the truncated TRPM2 channel is activated following exposure to hypertonic solutions.

7 Lessons from Knockouts

Different strategies have been applied to study TRPM2 pathophysiological functions, including gene knockout. Several studies carried out in mice have shown that TRPM2 channels play a crucial role in the inflammatory process. Indeed, It was found that antigen-stimulated degranulation was significantly reduced in mucosal-type bone marrow-derived mast cells (mBMMCs) isolated from TRPM2-KO mice (Oda et al. 2013). Moreover, macrophages and microglia derived from this model organism show reduced production of chemokine (C-X-C motif) ligand-2 (CXCL2) and nitric oxide synthase induction (Haraguchi et al. 2012). Additionally, TRPM2 ablation revealed a prominent role of TRPM2 in the dextran sulfate sodium (DSS)-induced chronic experimental colitis mouse

model (Yamamoto et al. 2008), in which monocytes, neutrophils, and macrophages are the primary mediators of inflammation. This study demonstrated that in monocytes from TRPM2-deficient mice, the H_2O_2-induced Ca^{2+} influx and the production of the macrophage inflammatory protein-2 (CXCL2) were impaired. The impaired chemokine production in cells lacking TRPM2 was linked to a defect in TRPM2-mediated Ca^{2+} influx that consequently resulted in defective activation of the Ca^{2+}-dependent kinase Pyk2 and downstream activation of the Erk/NF-κB pathway (Yamamoto et al. 2008, 2010). In the DSS-induced colitis inflammation model, CXCL2 expression, neutrophil infiltration, and ulceration were all attenuated by TRPM2 disruption, suggesting that TRPM2-mediated Ca^{2+} influx controls the ROS-induced signaling cascade responsible for chemokine production and the aggravation of inflammation (Yamamoto et al. 2008).

Given that ROS play an important role in airway disorders such as adult respiratory distress syndrome (ARDS), cystic fibrosis, idiopathic fibrosis, chronic obstructive pulmonary diseases (COPD), and asthma, it is surprising that TRPM2 channels appear to not be critical for at least two airway inflammation models. Two recent publications that took advantage of TRPM2-KO mice have found no obvious or significant role for TRPM2 channels in chronic obstructive pulmonary disease in mice exposed to ozone, LPS, or tobacco smoke (Hardaker et al. 2012) or in a mouse airway inflammation model of OVA-induced severe allergic asthma (Sumoza-Toledo et al. 2011).

Since TRPM2 is also expressed in pancreatic β-cells, its role in insulin release has been confirmed through the use of transgenic animals. TRPM2-KO mice show impaired glucose tolerance and reduced insulin secretion, suggesting that TRPM2 contributes to the Ca^{2+} signals and insulin secretion in pancreatic β-cells and might represent a new factor involved in diabetes (Uchida and Tominaga 2011; Uchida et al. 2011).

8 Role in Hereditary and Acquired Diseases

Based on their reported physiological functions, much attention has been dedicated to investigating the role of dysfunctional expression and/or activity of TRPM2 channels in various pathological contexts. Since TRPM2 is most abundantly expressed in the brain, it is not surprising that TRPM2 has also been associated with CNS pathologies, including ischemia and neurodegenerative diseases (Xie et al. 2010). TRPM2 activation following in vitro ischemia increases cell death of male hippocampal neurons (Verma et al. 2012), and in stroke models, TRPM2 inhibition or knockdown is neuroprotective against ischemia in vitro and in vivo (Jia et al. 2011). TRPM2 appears to also be involved in mediating neuronal death of striatal neurons, which are particularly vulnerable to hypoxia-/ischemia-induced damage, and free radicals are thought to be prime mediators of this neuronal destruction (Smith et al. 2003). Recent work suggests that the observed preferential susceptibility of male neurons to TRPM2-mediated cell death may additionally involve androgen signaling and activation of the PARP pathway (Shimizu

et al. 2013). Furthermore, TRPM2 may contribute to neuropathic pain by aggravating pro-nociceptive inflammatory responses and sensitizing the pain-signaling pathway (Haraguchi et al. 2012).

Patients with bipolar disorders type I present high basal $[Ca^{2+}]_i$, and the chromosome region 21q22.3 harbors genes that confer susceptibility to this pathology, including TRPM2 (Xu et al. 2006, 2009, 2013a; Roedding et al. 2012, 2013). Although TRPM2 variants with a single amino substitution (e.g., Asp543Glu) have been detected in patients with bipolar disorder, the relevance of these variants in the pathogenesis of the disease remains to be elucidated. Additionally, TRPM2 has been shown to contribute to the expression of juvenile myoclonic epilepsy (JME) phenotypes by mediating disruptive effects of JME mutations of EFHC1 protein on biological processes such as cell death (Katano et al. 2012). TRPM2 has also been linked to amyotrophic lateral sclerosis and parkinsonism–dementia (Hermosura and Garruto 2007). Here, a TRPM2 mutation (P1018L) results in channels that inactivate more rapidly than wild-type channels, resulting in reduced Ca^{2+} entry. Again, the cellular and functional context of TRPM2 in these pathologies remains to be demonstrated.

The presence of TRPM2 in pancreatic β-cells and its role in glucose-induced insulin secretion suggest a possible role of this channel in diabetes (Herson and Ashford 1997, 1999; Togashi et al. 2006; Uchida et al. 2011). Insulin release was shown to be impaired in the TRMP2-KO mice treated by glucose and incretin hormone (Uchida et al. 2011). In contrast, Romero and collaborators reported the absence of any correlation between type 2 diabetes mellitus and the genetic TRPM2 variants rs2838553, rs2838554, rs4818917, rs1619968, rs1785452, rs2238725, rs2010779, rs9979491, and rs1573477 (Romero et al. 2010). However, the variants rs2838553, rs2838554, and rs4818917 showed negative association with a homeostatic model assessment of β-cell function, which determines insulin resistance and β-cell function, hinting at the possibility that TRPM2 activity may indeed regulate β-cell function. Further studies examining other variants are necessary to establish a role of TRPM2 in diabetes.

The above-described role of TRPM2 in the immune system function makes it a good candidate in promoting inflammatory diseases. TRPM2 expressed in macrophages and microglia aggravates peripheral and spinal pro-nociceptive inflammatory responses and contributes to the pathogenesis of inflammatory and neuropathic pain (Haraguchi et al. 2012). TRPM2 may also be the target of NLRP3 inflammasome-associated inflammatory disorders, since TRPM2 was shown to be the key factor that links oxidative stress to the NLRP3 inflammasome activation (Zhong et al. 2013). In cardiac tissue, accumulation of neutrophils in the reperfused area mediated by TRPM2 activation is likely to play a crucial role in myocardial I/R injury (Hiroi et al. 2013b). While TRPM2 is clearly linked to several inflammatory pathology models, it has been shown inconsequential in others. Hardaker and collaborators have reported that TRPM2 has no role in inflammatory mouse models of COPD (Hardaker et al. 2012), and Sumoza-Toledo et al. showed that TRPM2 is not required for airway inflammation in OVA-induced airway inflammation (Sumoza-Toledo et al. 2013).

Finally, TRPM2 may also play a role in cancer, where cytokine secretion by cancer cells contributes to cancer-induced symptoms and angiogenesis. The sirtuin SIRT6 was shown to promote cytokine secretion and migration in pancreatic cancer cells by increasing intracellular levels of ADP-ribose and consequently TRPM2-mediated Ca^{2+} mobilization. This calcium entry activates the Ca^{2+}-dependent transcription factors (NFAT) and thereby the expression of proinflammatory, proangiogenic, and chemotactic cytokines (TNF and IL-8) (Bauer et al. 2012). In human lung cancer A549 cells, activation of TRPM2 channel, which mediates ATP release, plays significant roles in the cellular responses to DNA damage induced by γ-irradiation and UVB irradiation (Masumoto et al. 2013). Similarly, therapeutic irradiation treatment as used in head and neck cancer treatments leads to activation of TRPM2 via stimulation of PARP1 and contributes to irreversible loss of salivary gland function (Liu et al. 2013). Finally, TRPM2 isoforms have been shown to play a crucial and differential role in neuroblastoma. Indeed, overexpression of TRPM2-S isoform in the neuroblastoma SH-SY5Y cell line results in increased proliferation through phosphatidylinositol 3-kinase/Akt and ERK pathways while overexpression of TRPM2-L isoform in confers protection against oxidative stress-induced cell death through FOXO3a and SOD (Chen et al. 2013). A more direct role in cell proliferation has been established in prostate cancer, where selective knockdown of TRPM2 inhibited the growth of prostate cancer cells but not of noncancerous cells. Moreover, subcellular localization of this protein was also remarkably different between cancerous and noncancerous cells, with benign cells expressing TRPM2 homogenously near the plasma membrane and in the cytoplasm, whereas in cancerous cells, a significant amount of the TRPM2 protein was clustered in the nucleus (Zeng et al. 2010).

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TRPM3

Johannes Oberwinkler and Stephan E. Philipp

Contents

Abstract

Like most other members of the TRP family, the *Trpm3* gene encodes proteins that form cation-permeable ion channels on the plasma membrane. However, TRPM3 proteins have several unique features that set them apart from the other members of this diverse family. The *Trpm3* gene encodes for a surprisingly large number of isoforms generated mainly by alternative splicing. Only for two of the (at least) eight sites at which sequence diversity is generated the functional

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consequences have been elucidated, one leading to nonfunctional channels, the other one profoundly affecting the ionic selectivity. In the *Trpm3* gene an intronic microRNA (miR-204) is co-transcribed with *Trpm3*. By regulating the expression of a multitude of genes, miR-204 increases the functional complexity of the *Trpm3* locus. Over the past years, important progress has been made in discovering pharmacological tools to manipulate TRPM3 channel activity. These substances have facilitated the identification of endogenously expressed functional TRPM3 channels in nociceptive neurons, pancreatic beta cells, and vascular smooth muscle cells, among others. TRPM3 channels, which themselves are temperature sensitive, thus have been implicated in sensing noxious heat, in modulating insulin release, and in secretion of inflammatory cytokines. However, in many tissues where TRPM3 proteins are known to be expressed, no functional role has been identified for these channels so far. Because of the availability of adequate pharmacological and genetic tools, it is expected that future investigations on TRPM3 channels will unravel important new aspects and functions of these channels.

Keywords
TRPM3 variants • Alternative splicing • Channel pore • MicroRNA

1 Genomic Organization of the *Trpm3* Gene, Which Encodes Many Different Variants

The human gene is located on chromosome 9 (9q21.11–q21.12), a region that is possibly linked to Kabuki syndrome (Kuniba et al. 2009) and which lies in close proximity to regions linked to coronary artery disease (Wellcome Trust Case Control Consortium 2007). The *Trpm3* genes of rat and mouse are located on chromosomes 1q51 and 19 B (Abramowitz and Birnbaumer 2007), respectively. The organization of the genes is highly conserved in mammals indicating common functions of their gene products. In humans the *Trpm3* gene is the largest gene on chromosome 9 (Humphray et al. 2004), and similarly the mouse gene spans more than 870.77 kb (Fig. 1a; Oberwinkler et al. 2005). It contains 28 exons where exons 1 and 2 as well as exons 2 and 3 are separated by huge introns of 309 and 249 kb, respectively (Fig. 1a). The large separation of exons 1, 2, and 3 and the differences of the hitherto known 5′ untranslated regions (5′ UTR) of the transcripts suggest the existence of alternative promoters located upstream of these exons. Accordingly, three transcription start sites located upstream of exons 1, 2, and 3 have been proposed based on the RefSeq data presented in the UCSC genome browser (Shaham et al. 2013). Furthermore, binding sites of the transcription factor Pax6 have been identified downstream of exon 2 (Shaham et al. 2013). Exon 1 and exon 2 appear to be expressed in a mutually exclusive way, as no cDNA clones have been described having both of these exons. The different promoters may regulate

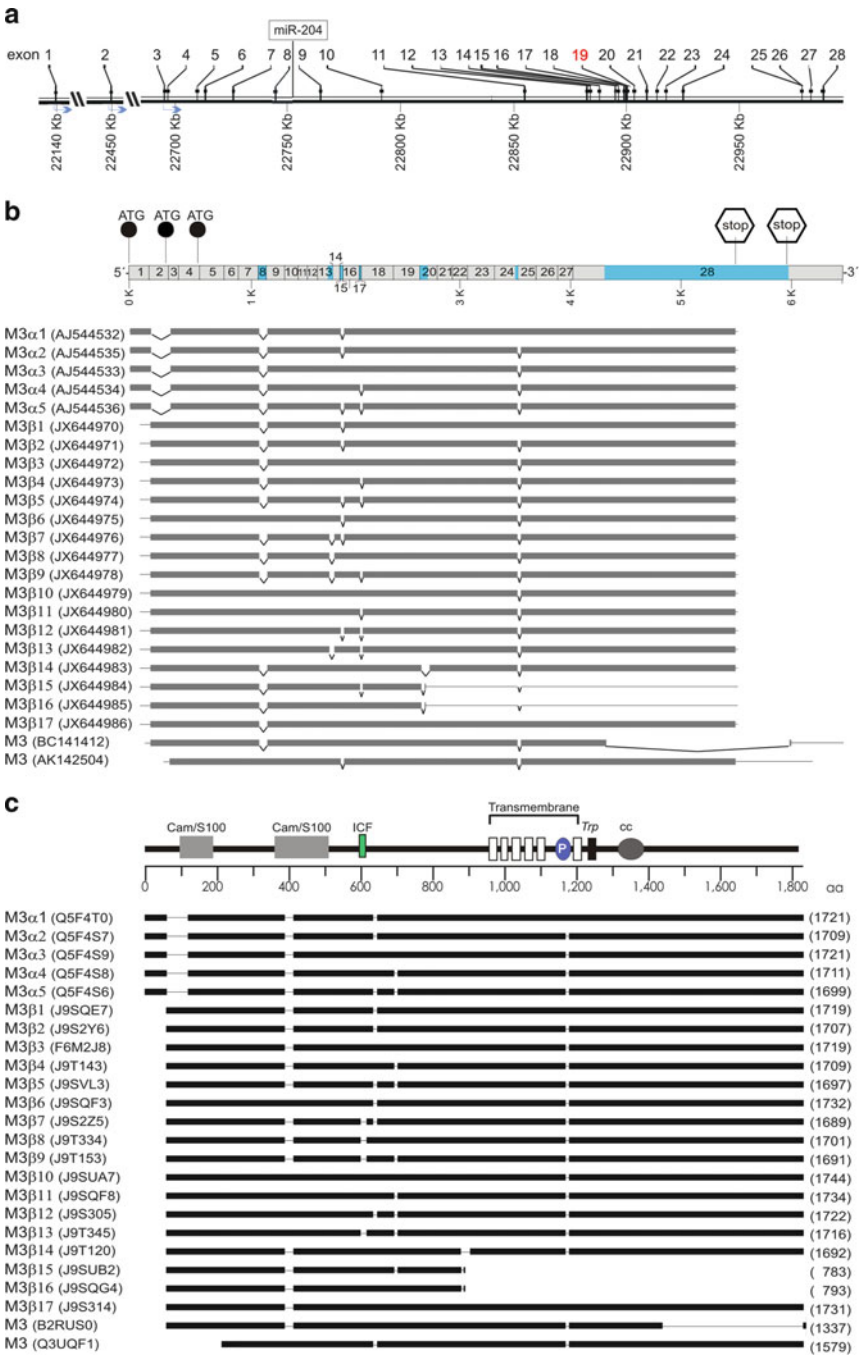

Fig. 1 The *Trpm3* gene, its transcripts and the encoded proteins. (**a**) Genomic organization of the mouse *Trpm3* gene on chromosome 19 B comprising 28 exons and spanning 870.77 kb. Predicted transcription start sites are indicated by *arrows*. A microRNA sequence (miR-204) is located in intron

expression of different isoforms called TRPM3α (α1–α5) starting with exon 1 and lacking exon 2 (Oberwinkler et al. 2005), isoforms called TRPM3β (β1–β 17) starting with exon 2 (Frühwald et al. 2012; Grimm et al. 2003), and finally isoforms starting with a ATG codon located at the very end of exon 4 which is the start codon for transcripts starting with exon 3 (Fig. 1b, c; Lee et al. 2003). However, to date neither 5′ UTRs of *Trpm3* transcripts nor promoter sequences of *Trpm3* genes have been analyzed systematically. Thus, the origin of the different TRPM3 amino termini remains to be demonstrated.

Within the TRP family, the *Trpm3* gene is unique because it encodes the largest number of isoforms (Fig. 1b, c). The majority of different TRPM3 isoforms originate from alternative splicing at internal splice sites, generating an enormous number of different TRPM3 proteins and thus setting TRPM3 apart from the other members of the TRP family. The splicing pattern appears to be evolutionary well conserved between mouse and human as revealed from a variety of database entries. From these entries, it is apparent that splicing of *Trpm3* transcripts follows a common pattern at least as exons 8, 13, 15, 17, 20, and 24 are concerned (Fig. 1b). Most of the splice events do not cause a switch of the reading frame. Consequently, the resulting variants differ only by the presence or absence of short stretches of 10–27 amino acid residues (Fig. 1c). However, splicing within exon 20 may also lead to a frame shift and to truncation of the proteins upstream of the transmembrane region as it is the case in TRPM3β15 and TRPM3β16. In the choroid plexus of the mouse brain, all these splice events occur with a frequency of at least 4.9 % (Frühwald et al. 2012). Furthermore, splicing within exon 28 replaces 389 amino acid residues by an alternative carboxy terminus of 7 residues in mouse (Strausberg et al. 2002) and in human (Grimm et al. 2003), keeping the functional activity of the truncated ion channel proteins unaffected (Grimm et al. 2003; Klose et al. 2011; Naylor et al. 2010).

Fig. 1 (continued) 8. Exon 19 which was subject of the targeting strategy to obtain a TRPM3 deficient mouse line (Hughes et al. 2012; Vriens et al. 2011) is highlighted in *red*. Please note that the numbering of exons and introns differs from other publications, which invariably omit one or several of the known exons. In our numbering scheme, which is identical to the one we previously published (Oberwinkler and Philipp 2007), all described 28 exons are considered, and we therefore believe that this numbering scheme should be adopted for enumerating the exons of the *Trpm3* gene. (**b**) Structure of *Trpm3* transcripts identified in mouse tissues. Their reading frames are flanked by stop codons establishing entire protein-coding sequences. The GenBank accession numbers of the cDNA clones are indicated in *brackets*. The coding parts of the transcripts are shown as *gray bar* and the noncoding parts as *thin line*. The *upper bar* indicates the relative size of the protein-coding exons 1–28 with spliced parts highlighted in *blue*. Start codons (ATG) present in exons 1, 2, and 4 and stop codons (stop) in exon 28 are indicated. (**c**) Schematic presentation of TRPM3 protein isoforms (*black bars*) scaled to their relative size with protein identifiers and numbers of amino acid residues (aa) indicated in *brackets*. Internal protein domains removed by alternative splicing are indicated as *thin lines*. The organization of domains of TRPM3 proteins is shown above, with calmodulin/S100 protein-interacting regions (CamBS/S100), ICF region, transmembrane region including the six transmembrane domains (*white rectangles*), the channel pore (P), the TRP motif (TRP), and a coiled-coil region (cc) as indicated

Further entries of TRPM3 variants displaying variations at other positions exist in the databases. However, at present it is not entirely clear whether such variants occur consistently within a tissue since these entries rely on single clones and/or fragmentary sequence information. In addition, since similar entries from other species are missing, the significance of these variants is unclear. Splicing of *Trpm3* transcripts may dramatically change the properties of the encoded channels. Splicing within the pore-forming exon 24 (Fig. 1c) modifies the ion selectivity of TRPM3 channels (Oberwinkler et al. 2005; see Sect. 5). On the other hand, removal of 54 nucleotides by splicing within exon 13 eliminates a domain of 18 amino acid residues, which is indispensable for TRPM3 channel function (Frühwald et al. 2012). In its absence, the protein no longer forms active channels but rather interferes with the channel function of other isoforms providing a mechanism to fine-tune TRPM3 channel activity (Frühwald et al. 2012). Splicing within exons 8, 15, and 17 does not change TRPM3-mediated Ca^{2+} signaling (Frühwald et al. 2012) in an apparent way, and the significance of these splice events is still to be discovered.

2 A MicroRNA Gene Is Located Within the *Trpm3* Locus

MicroRNAs (miRNAs) play important roles in posttranscriptional regulation of gene expression by targeting mRNAs for cleavage or translational repression. A microRNA called miR-204 has been identified in mouse eye (Lagos-Quintana et al. 2003), which originates from intron 8 of the *Trpm3* gene (Fig. 1a; Rodriguez et al. 2004). Interestingly, a highly similar microRNA called miR-211, which derives from the closely related *Trpm1* gene, is expressed in the eye, too [see Irie and Furukawa (2014)]. Both microRNAs belong to the same family and their sequence differs by only one or two nucleotides in mouse and human, respectively. In contrast, no other *Trp* gene contains microRNA sequence information. This unique feature adds additional functional properties to the *Trpm1* and *Trpm3* genes, which have to be considered when analyzing physiological functions of TRPM1 and TRPM3 channels by comparing TRPM3 mutant animals with their wild-type counterparts. Knocking out the TRPM1 or TRPM3 gene might also change the level of expression of miR-204 and miR-211, respectively. This should be tested experimentally, because it might substantially contribute to the phenotype of the TRPM-deficient mice.

miR-204 is transcribed in the same orientation as the *Trpm3* transcript (Karali et al. 2007), and it can be assumed that its pre-miRNA is excised from a common precursor RNA. Thus, it is very likely that *Trpm3* and miR-204 share the same promoter(s). Accordingly, the pattern of miR-204 expression broadly coincides with TRPM3. For example, in the eye miR-204 and TRPM3 could both be detected in the neuronal retina, ciliary body, and retinal pigment epithelium (Deo et al. 2006; Hackler et al. 2010; Karali et al. 2007; Wang et al. 2010; see Table 1) in which miR-204/211 may play a critical role in the maintenance of the epithelial barrier (Wang et al. 2010). Furthermore, co-expression of both *Trpm3* and miR-204 could

Table 1 TRPM3 expressing tissues and cell types and the methods of their detection

Tissue/cell type	RT-PCR	RT-qPCR	Microarray	Northern	ISH	Western	IHC/ICC	cDNA library	Transgene	Function
Nervous system										
Total brain	Fantozzi et al. (2003), Grimm et al. (2003), Hoffmann et al. (2010), Inoue et al. (2006), Jang et al. (2012), Lee et al. (2003), Wagner et al. (2008)	Fonfria et al. (2006), Lee et al. (2003)		Gilliam and Wensel (2011), Grimm et al. (2003), Lee et al. (2003), Oberwinkler et al. (2005)		Hoffmann et al. (2010); Wagner et al. (2008)				
Cerebrum		Kunert-Keil et al. (2006)					Hoffmann et al. (2010)			
Brain stem							Hoffmann et al. (2010)			
Locus coeruleus		Lee et al. (2003)								
Spinal cord		Lee et al. (2003)								
Hippocampus		Kunert-Keil et al. (2006)			Oberwinkler et al. (2005)		Hoffmann et al. (2010)			
Forebrain		Kunert-Keil et al. (2006)								
Corpus callosum							Hoffmann et al. (2010)			

Chor. plexus epithel.		Hasselblatt et al. (2009), Lee et al. (2003)	Hasselblatt et al. (2009)	Oberwinkler et al. (2005)	Deo et al. (2006), Karali et al. (2007), Kastenhuber et al. (2013), Oberwinkler et al. (2005)				
Tenia tecta					Oberwinkler et al. (2005)				
ILSN[a]					Oberwinkler et al. (2005)				
Hypothalamus		Lee et al. (2003)							
Cerebellum		Kunert-Keil et al. (2006), Lee et al. (2003)					Hoffmann et al. (2010), Zamudio-Bulcock et al. (2011)		
Purkinje cell							Zamudio-Bulcock et al. (2011)		Zamudio-Bulcock et al. (2011)
Oligodendrocyte	Hoffmann et al. (2010)						Hoffmann et al. (2010)		
Oli-neu/OLN-93cell						Hoffmann et al. (2010)			Hoffmann et al. (2010)
Basal ganglia/ substantia nigra		Kunert-Keil et al. (2006), Lee et al. (2003)							

(continued)

Table 1 (continued)

Tissue/cell type	RT-PCR	RT-qPCR	Microarray	Northern	ISH	Western	IHC/ICC	cDNA library	Transgene	Function
Dorsal root ganglion	Jang et al. (2012)	Jang et al. (2012), Staaf et al. (2010), Vandewauw et al. (2013), Vriens et al. (2011)			Vriens et al. (2011)	Vriens et al. (2011)				Straub et al. 2013a, b, Vriens et al. (2011)
Trigeminal ganglion	Nealen et al. (2003)	Jang et al. (2012), Vriens et al. (2011)			Vriens et al. (2011)	Vriens et al. (2011)				Vriens et al. (2011)
Nodose ganglion		Staaf et al. (2010)								
SH-SY5	Bollimuntha et al. (2005)									
Sensory system										
Eye (total)			Hughes et al. (2012)	Oberwinkler et al. (2005)	Karali et al. (2007)					
Retina	Gilliam and Wensel (2011)	Hackler et al. (2010), Krol et al. (2010)		Gilliam and Wensel (2011)	Gilliam and Wensel (2011), Karali et al. (2007), Kastenhuber et al. (2013)			Wistow et al. (2008)	Hughes et al. (2012)	
Iris								Wistow et al. (2002)		
Lens		Xie et al. (2013)			Karali et al. (2007), Kastenhuber et al. (2013), Shaham et al. (2013)					

Retinal pig. epithel.		Adijanto et al. (2012), Wang et al. (2010)	Gilliam and Wensel (2011), Karali et al. (2007), Shaham et al. (2013)		Schulz et al. (2004), Wistow et al. (2008)	
Müller cell	Hughes et al. (2012)					Hughes et al. (2012)
Ciliary body			Gilliam and Wensel (2011), Karali et al. (2007), Shaham et al. (2013)			Hughes et al. (2012)
Inner ear	Cuajungco et al. (2007), Gabashvili et al. (2007)	Asai et al. (2010)		Takumida et al. (2009)	Gabashvili et al. (2007)	
Cardiovascular system						
Heart	Jang et al. (2012)	Kuster et al. (2013)				
Pulmonary artery	Fantozzi et al. (2003), Yang et al. (2006)	Courboulin et al. (2011), Yang et al. (2006)				
Coronary artery	Inoue et al. (2006)					
Mesenteric artery	Inoue et al. (2006)					
Femoral artery				Naylor et al. (2010)		
Aorta	Inoue et al. (2006), Naylor et al. (2010), Yang et al. (2006)	Yang et al. (2006)				Naylor et al. (2010)

(continued)

Table 1 (continued)

Tissue/cell type	RT-PCR	RT-qPCR	Microarray	Northern	ISH	Western	IHC/ICC	cDNA library	Transgene	Function
Saphenous vein	Naylor et al. (2010)	Naylor et al. (2010)					Naylor et al. (2010)			Naylor et al. (2010)
Endocrine system										
Pituitary gland		Fonfria et al. (2006)								
Pancreas (total)	Grimm et al. (2003)									
Pancreatic islet cell	Müller et al. (2011), Wagner et al. (2008)					Wagner et al. (2008)				Lambert et al. (2011), Wagner et al. (2008, 2010)
INS1	Müller et al. (2011), Wagner et al. (2008)	Xu et al. (2013)		Wagner et al. (2008)		Klose et al. (2011), Mayer et al. (2011), Wagner et al. (2008)				Klose et al. (2011), Wagner et al. (2010), Wagner et al. (2008)
Urinary system										
Kidney (total)	Grimm et al. (2003), Jang et al. (2012)	Fonfria et al. (2006), Lee et al. (2003)		Grimm et al. (2003), Lee et al. (2003)		Grimm et al. (2003)				
Collect. tub. epithel.					Lee et al. (2003)					
MDCKII cell	Langford et al. (2012)									
ccRCC[b]		Mikhaylova et al. (2012)								

Reproductive system					
Ovary	Grimm et al. (2003)				
Testis	Jang et al. (2012)	Jang et al. (2012), Lee et al. (2003)	Lee et al. (2003)		
Sperm cell	Li et al. (2008)				
Prostate	Wang et al. (2007)	Wang et al. (2007)			
Other tissues/cells					
MG-63 cell	Abed et al. (2009)				
SaOS cell	Abed et al. (2009)				
U2 OS cell	Abed et al. (2009)				
Odontoblast	Son et al. (2009)				
Adipose tissue		Fonfria et al. (2006)			
Buccal mucosa		Lee et al. (2010)			
Synoviocyte	Ciurtin et al. (2010)			Ciurtin et al. (2010)	Ciurtin et al. (2010)
Glioma		Ying et al. (2013)			

[a]Intermediate lateral septal nuclei
[b]Clear cell renal cell carcinoma

be demonstrated in the choroid plexus (Deo et al. 2006), retina (Karali et al. 2007; Krol et al. 2010), lens (Shaham et al. 2013), and insulinoma cells (Xu et al. 2013). In the mouse retina the levels of miR-204 and miR-211 are upregulated by increasing levels of illumination similar to *Trpm1* and *Trpm3* mRNA (Krol et al. 2010).

Interestingly, miR-204 is also involved in beta-cell function and regulates insulin production (Xu et al. 2013). It has been shown that thioredoxin-interacting proteins (TXNIP) of beta cells substantially reduce phosphorylation of signal transducer and activator of transcription 3 (STAT3; Xu et al. 2013), which suppresses miR-204 expression (Courboulin et al. 2011). Increased amounts of miR-204 target and downregulate expression of MafA, a known transcription factor for insulin, which is then followed by a reduction of insulin (Xu et al. 2013). Thus, it is reasonable to assume that STAT3 is also involved in the regulation of *Trpm3* expression, and, accordingly, STAT3 binding to two putative binding sites located downstream of the human *Trpm3* gene could be demonstrated by chromatin immunoprecipitation (Courboulin et al. 2011).

miR-204 also induces downregulation of a number of other genes which have already been experimentally confirmed for some of them. At present, 33 validated target genes of miR-204 are available at TarBase 6.0 (Vergoulis et al. 2012) and even 78 are present in miRTarBase (Hsu et al. 2011). Among them are many genes that are involved in tumorigenesis (Chung et al. 2012; Findlay et al. 2008; Garzon et al. 2008; Lee et al. 2010; Master et al. 2010). Furthermore, miR-204 is essential for the development of the eye in the medaka fish (Conte et al. 2010; Shaham et al. 2013) and might be implicated in the etiology of pulmonary arterial hypertension (Courboulin et al. 2011). However, a direct interference of miR-204 with the expression of the *Trpm3* host gene or any other *Trp* gene is not documented.

3 Expression Pattern of TRPM3

Systematic expression analyses of human and mouse TRPM channels in a variety of tissues by RT-qPCR indicated the strongest expression of TRPM3 in the brain, pituitary gland, kidney, and adipose tissue but low abundance or absence of TRPM3 transcripts in other tissues (Fonfria et al. 2006; Kunert-Keil et al. 2006). In situ hybridization (ISH) of whole-mount zebra fish during embryonic and larval stages showed prominent expression of *Trpm3* in various brain areas (Kastenhuber et al. 2013). However, as shown in Table 1, further data indicate that TRPM3 is expressed in quite a number of tissues and cell types that serve many different functions. The best investigated tissues are the central nervous system and the eye. In these tissues, the *Trpm3* gene is expressed not only in neurons and epithelia but also in oligodendrocytes, where TRPM3 channels have been proposed to participate in differentiation and CNS myelination (Hoffmann et al. 2010). In mouse brain, transcripts could be detected in several regions (Table 1) with the most prominent expression in epithelial cells of the choroid plexus (Oberwinkler et al. 2005) where the function of TRPM3 channels is still elusive. For neuronal TRPM3 channels, their occurrence in dorsal root ganglia (DRG) and their function as sensor of

noxious heat are well established (Vriens et al. 2011). In adult lumbar DRG from mouse, TRPM3 expression is highest compared to other *Trpm* transcripts (Staaf et al. 2010), and RT-qPCR data indicate that TRPM3 is as strongly (or even stronger) expressed in DRGs as the other well-expressed TRP channels (e.g., TRPA1, TRPM8, and TRPV1; Vriens et al. 2011). A systematic and detailed RT-qPCR analysis of all *Trp* genes expressed in DRG of different sections of the vertebral column is available (Vandewauw et al. 2013).

A comprehensive overview of *Trpm3* cDNA clones identified in different tissues of the eye from different organisms is available at the NEIBank (http://neibank.nei.nih.gov) which is a database of assembled EST data from eye tissue libraries (Wistow et al. 2008). Furthermore, a comprehensive analysis of the expression of all TRP channels in mouse retina has been published recently (Gilliam and Wensel 2011). Using in situ hybridization, it has been shown that *Trpm3* is expressed in different regions of the neuronal retina, retinal pigment epithelium (RPE), and ciliary body, and, accordingly, a number of cDNA clones were isolated from these tissues. Expression of TRPM3 in Müller cells of the retina and in the ciliary body was confirmed using a lacZ reporter gene of TRPM3 lacZ knock-in mice (Hughes et al. 2012). However, expression of the lacZ reporter gene in RPE remains to be detected (Hughes et al. 2012).

In human kidney, a prominent TRPM3 expression has been shown with a variety of methods (Fonfria et al. 2006; Grimm et al. 2003; Lee et al. 2003), and in MDCK cells which derive from the dog kidney, even a change of the splicing frequency in exon 8 could be observed after stimulation with hepatocyte growth factor (Langford et al. 2012). However, in mouse kidney *Trpm3* expression was not detectable by Northern (Gilliam and Wensel 2011; Grimm et al. 2003; Oberwinkler et al. 2005) and Western analysis (Grimm et al. 2003). Using standard RT-PCR at least, a weak signal could be amplified from mouse kidney, but RT-qPCR could not substantiate this finding (Jang et al. 2012). Thus, it may be assumed that TRPM3 may fulfill additional functions in human but not in mouse kidney.

4 Structural Aspects of TRPM3 Proteins and TRPM3 Interacting Partners

Except TRPM3β15 and TRPM3β16, all TRPM3 isoforms show the typical features of a TRP protein with six putative membrane spanning domains, a conserved TRP motif, and a coiled-coil region in its C terminus (Fig. 1c; Grimm et al. 2003; Lee et al. 2003; Oberwinkler et al. 2005). Ankyrin repeats, present in TRPC and TRPV proteins and supposed to be involved in channel assembly of TRPV6 (Erler et al. 2004), are lacking in TRPM3 proteins, and assembly of TRPM3 channels is therefore independent of these structures. The importance of the coiled-coil region for assembly of TRPM subunits has been analyzed in detail for TRPM8 (Erler et al. 2006; Phelps and Gaudet 2007; Tsuruda et al. 2006) and TRPM2 (Mei et al. 2006). It has been demonstrated that the TRPM8 coiled coil is necessary for channel assembly and sufficient for tetramer formation (Tsuruda et al. 2006).

Because of the high sequence conservation of this motif throughout the TRPM subfamily, a similar function has been proposed for TRPM3, and accordingly, the isolated TRPM3 peptide showed a similar tendency to self-assemble as the coiled coil of TRPM8 (Tsuruda et al. 2006). To date further experimental evidence for such a central role of the TRPM3 coiled coil in channel assembly is missing, but a direct homomultimeric interaction of TRPM3 proteins has been demonstrated (Frühwald et al. 2012; Hoffmann et al. 2010; Lambert et al. 2011). Furthermore, a physical interaction of recombinant TRPM3 and TRPM1 proteins has been confirmed with independent methods (Lambert et al. 2011) and might have significance for visual transduction since both proteins are expressed in the retina.

Comparing different TRPM3 splice variants, we recently identified a region of 10 amino acid residues in the amino terminus of TRPM3 that is indispensable for channel function and therefore is called ICF region (Fig. 1c; Frühwald et al. 2012). Variants lacking this domain display neither Ca^{2+} entry nor ionic currents. They show reduced interaction with other TRPM3 isoforms, and their occurrence at the cell membrane is diminished (but not abolished). The ICF region is conserved throughout the TRPM family, and its presence in TRPM8 proteins is also necessary for function (Frühwald et al. 2012). Thus, a central role of the ICF domain for correct protein folding of TRPM proteins can be assumed.

A comparison of the TRPM3 amino acid sequence with sequences of calmodulin-binding proteins indicated the presence of four putative calmodulin binding sites within the amino terminus of TRPM3 (Oberwinkler and Philipp 2007), and recently the Ca^{2+}-dependent binding of calmodulin (CaM) to two of them has been confirmed experimentally (Holakovska et al. 2012). The N-terminal binding site might be unique for TRPM3β variants, which therefore may display a different kind of Ca^{2+}-dependent regulation. Interestingly, S100A1 binds to the very same regions implying dual regulation of TRPM3 channels by calmodulin/S100A1. Finally, PtdIns(4, 5)P_2 also interacts with a CaM/S100A1 binding site implying a central role of these regions for the regulation of TRPM3 ion channels (Holendova et al. 2012).

5 Biophysical and Pharmacological Description of TRPM3

The biophysical properties of TRPM3 channels have mainly been investigated in heterologous overexpression systems. Recently, however, studies were published investigating the biophysical properties of endogenously expressed TRPM3 channels, most notably in pancreatic β-cells (Wagner et al. 2008, 2010; Klose et al. 2011), in smooth muscle cells (Naylor et al. 2010), and in nociceptive DRG neurons (Vriens et al. 2011). Satisfyingly, the vast majority of biophysical and pharmacological properties that have been worked out for heterologously expressed channels have also been replicated in their endogenously expressed counterparts.

Already the first publications on TRPM3 noticed that after heterologous overexpression of TRPM3 proteins, a small but significant constitutively active conductance can be observed (Grimm et al. 2003; Lee et al. 2003; Naylor

et al. 2010; Oberwinkler et al. 2005). Subsequently, several chemical substances have been identified that affect the activity of TRPM3 channels. As often seen with TRP channels, these substances are chemically surprisingly diverse (e.g., Vriens et al. 2008). However, due to the non-negligible basal activity of TRPM3 channels, it is unclear whether the effect of these substances should be viewed as gating of the channels or as modulating the open probability of already "gated" channels.

5.1 Small Chemical Compounds that Increase the Activity of TRPM3 Channels

Grimm et al. (2005) described D-erythro-sphingosine (DeSPH) as agonist of TRPM3 channels. DeSPH is a rather lipophilic substance endogenously produced by the human body. In Ca^{2+}-imaging experiments, it activates human TRPM3 channels overexpressed in HEK293 cells with an apparent EC_{50} of 12 μM. As this substance is known to accumulate in the plasma membrane (Mathes et al. 1998), its effects may be only slowly reversible. Accordingly, the reversibility of DeSPH effects on TRPM3 channels has not yet been tested. Furthermore, effects of this substance have been difficult to reproduce in certain cell types (e.g., Wagner et al. 2008). The closely related substances *N*,*N*-dimethyl-D-erythro-sphingosine and dihydro-D-erythro-Sphingosine also activate TRPM3 channels, but with reduced efficacy. D-erythro-sphingosine-1-phosphate proved to be ineffective (Grimm et al. 2005).

The steroidal compound pregnenolone sulfate (PS) was subsequently identified as agonist of TRPM3 channels. PS activates TRPM3 channels quickly (<100 ms) and reversibly (Wagner et al. 2008). Also, the current amplitude obtained with PS is much larger than with DeSPH. These properties have made PS the most popular choice for experimentally activating TRPM3 channels, especially when working with endogenous TRPM3 channels (Ciurtin et al. 2010; Klose et al. 2011; Naylor et al. 2010; Straub et al. 2013a, b; Vriens et al. 2011; Wagner et al. 2008). Nevertheless, PS needs to be applied at high concentrations to activate TRPM3 channels ($EC_{50} = 23$ μM at room temperature; Wagner et al. 2008). PS is also an endogenous substance produced in considerable amounts by the human body, and it has been reasoned, despite the micromolar EC_{50} value, that the PS concentrations encountered physiologically in the human body may be sufficient to activate TRPM3 channels, especially at body temperature (Harteneck 2013; Vriens et al. 2011). Like DeSPH, PS is known to quickly partition into the plasma membrane, a process that can be monitored by measuring the concomitant increase in membrane capacity (Mennerick et al. 2008). Remarkably, however, PS quickly moves out of the plasma membrane again when it is removed from the extracellular medium, which might explain why TRPM3 currents quickly subside after washout of PS.

Meanwhile, several studies attempted to define the structure–activity relationship of steroidal compounds on TRPM3 channels (Drews et al. 2014; Majeed et al. 2010, 2012; Wagner et al. 2008). Early on, it was found that already minor

modifications of pregnenolone sulfate resulted in dramatically reduced potency or efficacy in activating TRPM3 channels. Changing PS to DHEA-S, which represents a minor modification at the C17 position, shifted the dose–response curve by at least a factor of 10 to higher values (Majeed et al. 2010; Wagner et al. 2008). On the other hand, removing the sulfate group from the C3 position did not shift the apparent EC_{50}, but the resulting pregnenolone, while still active, had much less effect on TRPM3 channels. Very similar data were obtained when DHEA was compared to DHEA-S (Majeed et al. 2010; Wagner et al. 2008). Subsequent studies established that a negatively charged moiety in 3β orientation was important at the C3 position to efficiently activate TRPM3 channels (Drews et al. 2014; Majeed et al. 2010). Several other, very closely related steroidal analogues of PS have been identified in the course of these studies that also activate TRPM3 channels, like pregnenolone glucuronidate, pregnenolone hemisuccinate, epiandrosterone sulfate, epipregnanolone sulfate, and epiallopregnanolone sulfate (Drews et al. 2014; Majeed et al. 2010). None of these compounds is a stronger agonist than PS, although the effects of epiallopregnanolone sulfate are close to those of PS. Importantly, all other steroidal compounds tested did not activate TRPM3, including the bile acid taurochenodeoxycholate (Düfer et al. 2012). Together these data argue for a surprisingly sharp structure–activity relationship for the steroid action on TRPM3, despite the low affinity with an EC_{50} value in the micromolar concentration range.

It came as a surprise that nifedipine, an inhibitor of L-type voltage-gated Ca^{2+} channels, was found to activate TRPM3 channels (Wagner et al. 2008). To do this, nifedipine needs to be applied in supratherapeutic, micromolar concentrations ($EC_{50} = 30$ μM). PS and nifedipine appear not to bind to the same binding site, since these compounds are capable of synergistically activating TRPM3 channels (Drews et al. 2014). Analogues of nifedipine, like nitrendipine, nicardipine, and nimodipine, did not activate TRPM3 channels (Drews et al. 2014; Wagner et al. 2008). Interestingly, these substances however activate TRPA1 channels (Fajardo et al. 2008).

The pharmacology of TRPM3 agonists for human (Majeed et al. 2010, 2012) and mouse TRPM3 (Drews et al. 2014; Wagner et al. 2008) is remarkably similar, as no significant species differences have been reported so far. The known pharmacological activators of TRPM3 are summarized in Table 2.

It should be noted that none of the known TRPM3 agonists is highly specific for TRPM3. At the concentration necessary to affect TRPM3 channels (i.e., in the micromolar concentration range), all TRPM3 agonists are known to have important effects on other ion channels from several ion channel families. A few examples illustrating this point are voltage-gated Ca^{2+} channels (Hige et al. 2006), NMDA receptors (Jang et al. 2004; Wu et al. 1991), and potassium channels (Kobayashi et al. 2009; Wang et al. 1998) that are activated or potentiated by PS. PS also has known inhibitory actions on $GABA_A$ receptors (Majewska et al. 1988; Majewska and Schwartz 1987). On the other hand, store-operated CRAC channels are inhibited by DeSPH (Mathes et al. 1998), as are, probably even more importantly,

the ubiquitously expressed TRPM7 channels and their close relatives TRPM6 (Qin et al. 2013).

5.2 Physical Stimuli Activating TRPM3

Exposing TRPM3-expressing HEK293 cells to hypotonic solutions induced an increase of the intracellular Ca^{2+} concentration, which was not seen in untransfected control cells (Grimm et al. 2003). Also, in perforated whole-cell patch-clamp recordings, but not in whole-cell patch-clamp recordings with a ruptured patch, exposure to a hypotonic solution induced, in a subset of cells, weakly rectifying currents with a reversal potential consistent with nonselective cation channels (Grimm et al. 2003). In this study, it was not attempted to elucidate whether TRPM3 channels are directly responding to the mechanical membrane stretch due to the hypotonicity-induced cell swelling or if the channels indirectly react to some unidentified intracellular signal induced by hypotonicity. No follow-up studies investigating the effect of hypotonicity on TRPM3 channel function have been published, leaving the mechanism underlying this effect still unresolved.

Many TRP channels, collectively termed "thermoTRPs," respond to changes in temperature (Patapoutian et al. 2003). The temperature sensitivity of these channels has been functionally and mechanistically linked to the voltage sensitivity that these channels display concomitantly to their temperature sensitivity (Nilius et al. 2005; Voets et al. 2004a, 2005). Recently, TRPM3 channels have been shown to be temperature and voltage sensitive as well and therefore should be considered to belong to the group of "thermoTRPs" (Vriens et al. 2011). While TRPV1, the prototypical heat-activated TRP channel, shows a sharp increase in activity at temperatures above 42 °C (Caterina et al. 1997), TRPM3 channel activity increases more gradually over a lower and broader range of temperatures. For instance, increasing the temperature from 15 to 35 °C increased the current amplitude through TRPM3 channels stimulated by 5 μM PS more than sevenfold (Vriens et al. 2011). Increasing the temperature shifted the dose–response curve of the chemical agonist to lower values, effectively increasing the potency of PS [although this finding has been contested recently (Majeed et al. 2012)]. Thus, at the physiological body temperature of 37 °C, responses to PS concentrations as low as 100 nM reliably activated TRPM3 channels (Vriens et al. 2011). Such low PS concentrations are considered to be within the physiological range of plasma PS concentrations (Harteneck 2013).

5.3 Inhibitors of TRPM3 Channels

Trivalent lanthanides have been in use as TRP channel blockers since long before mammalian TRP channels have been discovered (Hardie and Minke 1992; Hochstrate 1989) and have meanwhile been tried on every TRP channel. Therefore, the first studies on TRPM3 used La^{3+} or Gd^{3+} to inhibit TRPM3 activity (Grimm

Table 2 Pharmacological activators of TRPM3 channels

Class of substance	Substance	EC_{50} [μM]	References
Sphingolipid	D-erythro-Sphingosine	12 (CI)	Grimm et al. (2005)
	N,*N*-Dimethyl-D-erythro-Sphingosine	n.d.	Grimm et al. (2005)
	Dihydro-D-erythro-Sphingosine	n.d.	Grimm et al. (2005)
1,4-Dihydropyridine	Nifedipine	30–32 (PC)	Wagner et al. (2008)
Steroid	Pregnenolone sulfate	12–32 (PC)	Wagner et al. (2008)
		1–5(CI)	Majeed et al. (2010)
			Naylor et al. (2010)
	Pregnenolone	14–15 (PC)	Wagner et al. (2008)
	Pregnenolone glucuronidate	n.d.	Drews et al. (2014)
	Pregnenolone hemisuccinate	n.d.	Drews et al. (2014)
	Epiallopregnanolone sulfate	n.d.	Drews et al. (2014)
	Epipregnanolone sulfate	14 (CI)	Majeed et al. (2010)
	DHEA sulfate	299–303 (PC)	Wagner et al. (2008)
		10–33 (CI)	Majeed et al. (2010)
			Naylor et al. (2010)
	DHEA	62–72 (PC)	Wagner et al. (2008)
	Epiandrosterone sulfate	n.d.	Majeed et al. (2010)

Note that only TRPM3 isoforms with a short pore (e.g., TRPM3α2) have been investigated
CI calcium imaging, *PC* patch-clamp, n.d. not determined

et al. 2003; Lee et al. 2003). Other cations reported to inhibit TRPM3 channels are Pb^{2+} (Sukumar and Beech 2010) and, surprisingly, Na^+ (Oberwinkler et al. 2005). Na^+ ions seem to affect the splice variant TRPM3α2 (but not the splice variant TRPM3α1; Sect. 5.5). Although the block by Na^+ at physiological concentrations is rather strong, it is incomplete, thus allowing for TRPM3 channel activity under physiological conditions. Equally, 2-aminoethoxydiphenyl borate (2-APB), which inhibits many TRP channels, was also found to inhibit TRPM3 channels (Xu et al. 2005).

From the intracellular side, elevated concentrations of Mg^{2+} inhibit both splice variants TRPM3α1 and TRPM3α2 (Oberwinkler et al. 2005). This is similar to the situation in the related channels TRPM7 (Nadler et al. 2001; Prakriya and Lewis 2002) and TRPM6 (Voets et al. 2004b).

Naylor et al. (2008) reported the generation of an antibody, termed T3M3, that binds to the putative third extracellular loop of TRPM3 close to the ion-conducting pore and inhibits the activity of TRPM3 channels. TRPM3 inhibition displayed comparatively slow kinetics taking several minutes. Therefore, these antibodies were mostly used after preincubation of the cells for several hours (Ciurtin et al. 2010; Naylor et al. 2010). The reported inhibition of TRPM3 activity was only partial, and it was suggested that the effects of the antibody were at least partly due to the internalization of antibody-bound TRPM3 channels (Ciurtin et al. 2010).

After the discovery of PS as steroidal agonist of TRPM3, other steroids that only weakly activate TRPM3 channels (pregnenolone, DHEA, and DHEA sulfate) have been found to reduce PS-induced TRPM3 activity (Majeed et al. 2010), consistent with their proposed action as partial agonists. In a subsequent study other steroids with no known agonistic effect on TRPM3 channels were also found to be weak inhibitors of TRPM3 channels: pregnanolone, progesterone, 17OH-progesterone, 21OH-progesterone, dihydrotestosterone, and estradiol (Majeed et al. 2012). The effect of dihydrotestosterone was remarkable, as it inhibited PS-induced TRPM3 activity, but not nifedipine-induced TRPM3 signals, while progesterone inhibited active TRPM3 channels regardless of the agonist used to stimulate them. Also, progesterone inhibited the agonist-independent constitutive activity of TRPM3 channels, while dihydrotestosterone did not (Majeed et al. 2012). Overall, the inhibition of TRPM3 channels caused by these steroids required high doses (>10 μM), and even at these doses, inhibition still was only partial.

Using methyl-β-cyclodextrin to increase or reduce the amount of cholesterol in the plasma membrane, Naylor et al. (2010) found that cholesterol itself has an inhibitory effect on the activity of human TRPM3 channels, a finding that was recently reproduced with mouse TRPM3α2 channels (Drews et al. 2014). Furthermore, from a set of 32 substances that have similar structures as steroids, but are themselves not steroids ("steroid look-alikes"), one substance, N-CPOTPC (*N*-(3-chloro-4-morpholinophenyl)-6-oxo-1,4,5,6-tetrahydro-3-pyridazinecarboxamide), was found at 10 μM to inhibit 1 μM PS-induced Ca^{2+} signals by approximately 50 % (Majeed et al. 2010). Another of these substances, 2-CMNPBC (2-chloro-4-(methylsulfonyl)-*N*-[4-(1-pyrrolidinyl)phenyl]benzenecarboxamide), was found, similar to dihydrotestosterone, to specifically inhibit TRPM3 channels when activated by PS but not when activated by nifedipine (Majeed et al. 2012).

The PPARγ- (peroxisome proliferator-activated receptors γ-) agonists rosiglitazone, troglitazone, and pioglitazone were found to inhibit TRPM3 channels. Rosiglitazone was the most potent of these substances (IC_{50} between 4.6 and 9.5 μM, depending on assay). It inhibited TRPM3 activity completely at concentrations of 100 μM (Majeed et al. 2011). *N*-(6-Aminohexyl)-5-chlor-1-naphthalinsulfonamid, also known as W7, a substance traditionally used to inhibit calmodulin, was shown to inhibit PS-activated TRPM3 channels with an IC_{50} of 15 μM (Harteneck and Gollasch 2011). Since calmodulin has been shown to bind to TRPM3 (Holakovska et al. 2012), it is formally possible that the effect of W7 on TRPM3 is indirect. Furthermore, some substances used as ligands of σ1-receptors (antagonists: BD1407, BD1063; agonist: 4-IBP) at high concentrations (100 μM) all partially inhibited nifedipine or PS-induced activity of TRPM3 channels expressed in HEK293 cells (Amer et al. 2013). So far, none of the substances from the groups of steroids, thiazolidinediones, and σ1-receptor ligands have shown properties that indicate that those substances could be of use as TRPM3 channel antagonists for pharmacological in vivo studies.

Searching for more specific and potent inhibitors of TRPM3, fenamates were identified as TRPM3 antagonists (Klose et al. 2011). Mefenamate was identified as the most potent of these substances ($IC_{50} = 6.6$ μM). The effect of mefenamate on

TRPM3 currents decreased when the extracellular solution was alkalinized, indicating that mefenamate acts in its protonized, uncharged form on the channels. Mefenamate had little effects on TRPC6, TRPM2, and TRPV4 channels (Klose et al. 2011), but it should be kept in mind that this substance is capable of activating TRPA1 channels (Hu et al. 2010). Recently, a screening approach was used to identify flavanones like naringenin, hesperetin, and isosakuranetin as inhibitors of TRPM3 channels, as well as the deoxybenzoin compound ononetin (Straub et al. 2013a, b). Of these substances, isosakuranetin was the most potent inhibitor ($IC_{50} = 50$ nM in Ca^{2+}-imaging experiments and 80–120 nM in electrophysiological recordings). Isosakuranetin had no or only minor effects on other TRP channels involved in nociception (TRPV1, TRPA1, and TRPM8) heterologously overexpressed as well as endogenously expressed in dorsal root ganglia at concentrations up to 10 μM. Even the closely related channel TRPM1 was only weakly inhibited by 10 μM isosakuranetin. Importantly, isosakuranetin also inhibited heat-evoked TRPM3 currents. Because hesperetin and isosakuranetin displayed little cell toxicity, these compounds could be tested in vivo in mice and were thus found to reduce pain induced by heat or the TRPM3 agonist PS (Straub et al. 2013a).

While nifedipine activates TRPM3 channels, nicardipine, nimodipine, and nitrendipine—other 1,4-dihydropyridines—were found to inhibit PS-activated TRPM3 channels (Drews et al. 2014). While these substances probably are of limited use for in vivo studies regarding TRPM3 due to their potent inhibition of L-type voltage-gated Ca^{2+} channels, it is interesting to compare their effect on TRPM3 with their action on TRPA1, as TRPA1 channels—in marked contrast to TRPM3 channels—are activated by all four dihydropyridines mentioned (Fajardo et al. 2008). Table 3 lists the known chemical inhibitors of TRPM3 channels.

5.4 Properties of the Steroid Binding Site of TRPM3 Channels

PS inserts readily into the plasma membrane and this likely affects biophysical parameters of the lipid bilayer (such as fluidity and membrane tension). It was therefore an open question whether TRPM3 channels are activated by PS through such changes in biophysical parameters or whether PS binds to a specific binding site in order to activate TRPM3 channels. The enantiomer of PS was used to settle this question, because pairs of enantiomers have exactly the same biophysical properties. But the natural enantiomer of PS turned out to be a much more potent agonist of TRPM3 channels compared to its synthetic enantiomer, thereby showing that PS exerts its effect on TRPM3 channels by binding to a specific chiral, and thus proteinaceous, binding site (Drews et al. 2014). Data from Majeed et al. (2012) also support the notion that TRPM3 channels possess a specific binding site for PS, because overexpressing TRPM3 proteins in HEK293 cells was shown to increase the capacity of membrane preparations to bind PS in a dot blot assay.

Since PS only affects TRPM3 channel activity when it is applied from the extracellular side (Wagner et al. 2008), the PS-binding site must be accessible

Table 3 Pharmacological inhibitors of TRPM3 channels

Class of substance	Substance	IC_{50} [μM]	References
Cation (extracellular)	La^{3+}, Gd^{3+}	n.d.	Lee et al. (2003)
			Grimm et al. (2003)
	Pb^{2+}	n.d.	Sukumar and Beech (2010)
	Na^{+}, (Li^{+}, K^{+})	<10.000 (PC)	Oberwinkler et al. (2005)
Cation (intracellular)	Mg^{2+}	n.d.	Oberwinkler et al. (2005)
1,4-Dihydropyridine	Nimodipine, Nicardipine, Nitrendipine	n.d.	Drews et al. (2014)
Fenamate	Mefenamate	6.6 (CI)	Klose et al. (2011)
	DCDPC	7.5 (CI)	Klose et al. (2011)
	Tolfenamate	11.1 (CI)	Klose et al. (2011)
	Meclofenamate	13.3 (CI)	Klose et al. (2011)
	Flufenamate	33.1 (CI)	Klose et al. (2011)
	Niflumate	123.5 (CI)	Klose et al. (2011)
Steroid	Pregnanolone, Progesterone	n.d.	Majeed et al. (2012)
	17OH-Progesterone, 21OH-Progesterone	n.d.	Majeed et al. (2012)
	Dihydrotestosterone	n.d.	Majeed et al. (2012)
	Estradiol, Mifepristone	n.d.	Majeed et al. (2012)
	Cholesterol	n.d.	Drews et al. (2014); Naylor et al. (2010)
"Steroid look-alike"	N-CPOTPC, 2-CMNPBC	n.d.	Majeed et al. (2010, 2012)
PPARγ agonist	Rosiglitazone	4.6–9.5 (CI)	Majeed et al. (2011)
	Troglitazone, Pioglitazone	12 (CI)	Majeed et al. (2011)
σ1-Receptor ligand	BD1407, BD1063, 4-IBP	n.d.	Amer et al. (2013)
Flavanone	Naringenin	0.5 (CI)	Straub et al. (2013b)
		0.3–0.5 (PC)[a]	
	Hesperetin	2 (CI)	Straub et al. (2013b)
	Eriodictyol	1 (CI)	Straub et al. (2013b)
	Isosakuranetin	0.05 (CI)	Straub et al. (2013a)
		0.08–0.12 (PC)	
	Liquiritigenin	0.5 (CI)	Straub et al. (2013a)
Deoxybenzoin	Ononetin	0.3 (CI)	Straub et al. (2013b)
		0.3–2 (PC)[a]	
Others	2-APB	n.d.	Xu et al. (2005)
	W7	15 (CI)	Harteneck and Gollasch (2011)

[a]Depending on the concentration of pregnenolone sulfate used to activate the channels. The block of nifedipine-induced currents was weaker for naringenin and ononetin (Straub et al. 2013b)
Note that only TRPM3 isoforms with a short pore (e.g., TRPM3α2) have been investigated
CI calcium imaging, *PC* patch-clamp, n.d. not determined

from the outside of the cells, as PS is unlikely to cross the plasma membrane in any significant amount due to its negatively charged sulfate moiety.

Studies investigating the structure–activity relationship (see above) established that a negatively charged group at the C3 position of the steroid is necessary and that it must be in the 3β orientation (Drews et al. 2014; Majeed et al. 2010). Two pairs of substances differing only in the stereochemical orientation at the C3 position have been investigated so far (Majeed et al. 2010): epipregnanolone sulfate (3β) and pregnanolone sulfate (3α), and epiandrosterone sulfate (3β) and androsterone sulfate (3α). It was found that the 3β-oriented compounds were much stronger agonists compared to their 3α stereoisomers. Equally, the stereochemical orientation at position C5 was investigated with the pair epipregnanolone sulfate (5β) and epiallopregnanolone sulfate (5α). Here the 5α-reduced steroid activated TRPM3 channels more strongly, in fact almost as strong as PS (Drews et al. 2014). This can be understood by comparing the three-dimensional structure of these compounds. Both PS and epiallopregnanolone sulfate (3β, 5α) are flat, elongated structures, while all other isomers are bent. Consequently, it appears that the binding site for PS is especially suited for accepting a flat, elongated molecule and is tight enough for rejecting the other conformations. Also, it is reasonable to assume that the binding site contains a positively charged group that helps accommodating the negatively charged substituent at the C3 position.

The earlier finding that DHEA-S, which differs from PS only at the position C17 of the steroid D-ring, is nevertheless approximately tenfold less potent than PS (Wagner et al. 2008) reinforces the concept that the structure–activity relationship of PS activating TRPM3 is exceptionally sharp, despite the low apparent affinity. Therefore, the binding site for PS seems to provide an adequate fit for this agonist.

5.5 Permeation Through TRPM3 Channels

Alternative splicing in exon 24 leads to two different pores in TRPM3 channels, differing by 13 amino acids (Sect. 1). The properties of these two pores have been studied by comparing directly TRPM3α1 and TRPM3α2 isoforms that have an identical primary amino acid sequence except the aforementioned difference in the pore region. The biophysical properties of these two isoforms are strikingly different: TRPM3α1 channels are much less permeable to divalent ions than TRPM3α2 channels (shown for Ca^{2+} and Mg^{2+}). Furthermore, Na^{+} ions inhibit TRPM3α2 channels strongly, but incompletely, without a similar effect on TRPM3α1 channels (Oberwinkler et al. 2005). This remarkable block by Na^{+} ions persists also when TRPM3α2 channels are activated with PS (Wagner et al. 2008). Interestingly, the closely related TRPM1 channels have—at exactly the position where the splicing occurs in TRPM3—an insertion of seven amino acids which dramatically alters the permeability to Zn^{2+} ions (Lambert et al. 2011).

Only the permeation profile of TRPM3α2 channels has been described quantitatively (Wagner et al. 2010). TRPM3α2 channels are approximately ten times more permeable to a variety of divalent cations compared to monovalent cations, which

all have comparable permeability. TRPM3α2 channels are well permeated by Ni^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} ions. For some divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}), the high permeability was also shown for endogenous TRPM3 channels in pancreatic β-cells (Wagner et al. 2008, 2010). Under more physiological conditions, the fractional Ca^{2+} current through PS-activated TRPM3α2 channels was estimated to be 24 % (Drews et al. 2010), a value that is higher than those reported for other mammalian TRP channels (Egan and Khakh 2004; Karashima et al. 2010; Samways et al. 2008; Zeilhofer et al. 1997). Thus, under physiological conditions activation of short-pore TRPM3 channels is suspected to significantly increase the intracellular Ca^{2+} concentration.

6 Physiological Functions in Native Cells, Organs, or Organ Systems

So far, in endogenously expressed TRPM3 channels, only Ca^{2+}-permeable variants have been studied. Possibly, this is due to methodological considerations, because Ca^{2+}-permeable channels are much easier to detect experimentally. However, this restriction means that no information about TRPM3 channels with a long-pore region (e.g., TRPM3α1 channels) is available from native systems.

Endogenous TRPM3 channels were first investigated in the insulinoma cell line Ins1 and mouse pancreatic β-cells in primary culture (Wagner et al. 2008). Identification of TRPM3 channels in these cells was achieved with pharmacological (PS and nifedipine) and biophysical (rectification, Na^+ block) means. Furthermore, shRNA-mediated knockdown decreased TRPM3 protein expression and PS-induced Ca^{2+} signals concomitantly. Subsequent studies found that TRPM3 channels in pancreatic β-cells are, like their heterologously expressed counterparts, highly permeable to Zn^{2+} (Lambert et al. 2011; Wagner et al. 2010) and sensitive to mefenamate (Klose et al. 2011). Stimulating pancreatic β-cells with high (50 μM) doses of PS increased glucose-induced insulin release (Klose et al. 2011; Wagner et al. 2008) and Zn^{2+} uptake (Wagner et al. 2010).

Naylor et al. (2010) employed a TRPM3-specific antibody (Naylor et al. 2008) and siRNA to determine that functional TRPM3 channels are expressed in vascular smooth muscle cells from human saphenous veins and mouse aorta. Blockade of TRPM3 channels with the functional antibodies increased IL-6 cytokine secretion, while applying PS to activate TRPM3 decreased cytokine liberation through the subsequent Ca^{2+} influx. Furthermore, applying PS contracted the vascular smooth muscle cells, and this effect was blocked by pre-exposure to the TRPM3-blocking antibody (Naylor et al. 2010). From these data, a relevance of TRPM3 channels for blood vessel contraction and proliferation of smooth muscle cells was deduced.

In a subclass of small nociceptor neurons from dorsal root and trigeminal ganglia from mice, TRPM3 proteins are expressed and form PS-activated channels with similar pharmacology and biophysical properties as heterologously expressed channels (Vriens et al. 2011). Importantly, however, the number of PS-sensitive nociceptors was strongly reduced in TRPM3-deficient (knockout) mice. On the

behavioral level, these knockout mice did not respond to injections of PS with nocifensive behavior. As TRPM3 channels were also identified to be activated by increased temperatures, and TRPM3-deficient animals displayed reduced thermal nociception, a function of TRPM3 channels as detectors of noxious thermal stimuli was inferred (Vriens et al. 2011). The nocifensive behavior of mice after injection of PS into a paw or after exposure to heat was diminished by treating the animals with the TRPM3 antagonists hesperetin and isosakuranetin (Straub et al. 2013a).

Unlike pharmacological manipulation of TRPV1, which causes robust changes in body temperature [reviewed by Romanovsky et al. (2009)], neither did activation of TRPM3 channels with PS decrease core body temperature (Vriens et al. 2011) nor did pharmacological blockade of TRPM3 raise the body temperature of mice (Straub et al. 2013a). Therefore, TRPM3 channels appear to be less implicated in the homeostasis of body temperature than TRPV1.

Studies relating to endogenously expressed TRPM3 have been reported for further cell types, in particular for synovial fibroblasts (Ciurtin et al. 2010), oligodendrocytes (Hoffmann et al. 2010), and cerebellar Purkinje neurons (Zamudio-Bulcock et al. 2011). In these studies, functional TRPM3 channels were identified with antibodies and pharmacological tools (PS and/or DeSPH). Ciurtin et al. (2010) also used the functional antibody T3M3. However, none of these antibodies was validated against tissue from TRPM3-deficient mice, a practice increasingly considered as mandatory (Everaerts et al. 2009; Flockerzi et al. 2005). Since also the pharmacological tools employed may not be selective for TRPM3 (see above), the certainty of identification of TRPM3 channels may be less than in the other studies that employed additional biophysical criteria, knockdown approaches, or the gold standard use of TRPM3-deficient animals. The putative TRPM3 channels were found to inhibit release of hyaluran from synovial fibroblasts (Ciurtin et al. 2010) and to increase glutamate release onto cerebellar Purkinje cells in developing rat brains (Zamudio-Bulcock et al. 2011). In oligodendrocytes, the physiological function of putative TRPM3 channels is unknown, but was discussed to be related to oligodendrocyte maturation and CNS myelination (Hoffmann et al. 2010).

7 Lessons from Knockouts: The Phenotype of TRPM3-Deficient Mice

Only two published reports have employed TRPM3-deficient (knockout) mice (Hughes et al. 2012; Vriens et al. 2011). These mice have a constitutively deleted *Trpm3* gene due to a targeted insertion of a lacZ/neomycin cassette. These TRPM3-deficient mice appear to be normal regarding fertility, gross anatomy, locomotion, and exploratory behavior. Interestingly, they also have a normal resting blood glucose level, indicating that the TRPM3 channels in pancreatic β-cells are not involved in the regulation of basal glucose levels or that their contribution can be compensated (Vriens et al. 2011).

But TRPM3-deficient mice demonstrated that TRPM3 channels are indeed the major receptor for PS in vivo, as TRPM3-deficient mice, in contrast to wild-type mice, did not react to PS injection into the paw (Vriens et al. 2011). Also, TRPM3-deficient mice did not share the aversion to drink PS-containing water with their wild-type littermates. Further analysis indicated that TRPM3-deficient mice have a specific deficit in sensing elevated temperatures in a variety of behavioral assays (tail immersion, hot plate, thermal preference assay). TRPM3-deficient mice also displayed a deficit in inflammation-induced hyperalgesia toward noxious heat provoked by injection of complete Freud's adjuvant. However, the hyperalgesia upon exposure to cold stimuli was preserved. TRPM3-deficient and wild-type mice have the same body temperature, also when they are treated with either PS, which did not change the body temperature, or with capsaicin, which induced a pronounced transient hypothermia in both genotypes (Vriens et al. 2011).

TRPM3-deficient mice have reduced consensual pupillary responses to light, although their pupils contracted with normal strength when exposed to a muscarinic agonist (Hughes et al. 2012). Interestingly, the reduction in pupillary constriction was observed in response to dim and to bright stimuli, indicating that TRPM3 deficiency does not affect a single retinal signal transduction pathway, which originate from the three classes of photosensitive cells—rods, cones, or intrinsically photoreceptive retinal ganglion cells. Rather TRPM3 appears to act downstream of these photoreceptive cell types. The strong expression of TRPM3 in the ciliary body (Karali et al. 2007) led to the speculation that TRPM3 may play a role directly in this effector organ of the pupillary light response (Hughes et al. 2012).

8 Role in Hereditary and Acquired Diseases

No human diseases have convincingly been described as a consequence of mutations in the *Trpm3* gene or an acquired dysfunction of TRPM3 channels, as witnessed by the fact that TRPM3 is not even mentioned in pertinent reviews (e.g., Nilius and Owsianik 2010). Possible involvement in human pathophysiological processes has therefore been inferred only from the function of TRPM3 channels in various tissues and organs (see Sect. 6) or from the phenotype of TRPM3-deficient mice (Sect. 7).

Hence, TRPM3 channels have been implicated in (inflammatory) pain syndromes (Straub et al. 2013a; Vriens et al. 2011), reduced insulin secretion (Klose et al. 2011; Wagner et al. 2008), rheumatoid arthritis (Ciurtin et al. 2010), and proinflammatory cytokine secretion (Naylor et al. 2010). From this list of conditions, the involvement of TRPM3 in pain sensation is momentarily best established, because of the clear phenotype of TRPM3-deficient mice. However, even there, the preclinical findings obtained from cellular and animal models still need to be translated to human in vivo studies, in which the efficacy of TRPM3 inhibitors in reducing pain is tested. Before this is attempted, however, it seems advisable to concentrate the efforts to elucidate the properties and functions of the channels formed by the diverse isoforms of TRPM3 proteins in much deeper detail.

We have only started to obtain the first glimpse of the intricacies of these remarkably unusual ion channels.

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TRPM4

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Contents

Abstract

TRPM4 is a Ca^{2+}-activated nonselective cation channel. The channel is activated by an increase of intracellular Ca^{2+} and is regulated by several factors including temperature and $Pi(4,5)P_2$. TRPM4 allows Na^+ entry into the cell upon activation, but is completely impermeable to Ca^{2+}. Unlike TRPM5, its closest relative in the transient receptor potential family, TRPM4 proteins are widely expressed in the body. Currents with properties that are reminiscent of TRPM4 have been described in a variety of tissues since the advent of the patch clamp technology, but their physiological role is only beginning to be clarified with the increasing characterization of knockout mouse models for TRPM4.

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Furthermore, mutations in the TRPM4 gene have been associated with cardiac conduction disorders in human patients. This review aims to overview the currently available data on the functional properties of TRPM4 and the current understanding of its physiological role in healthy and diseased tissue.

Keywords
TRPM4 • Ca^{2+}-activated nonselective cation channel • Immune system • Cardiovascular system • Central nervous system • Cardiac arrhythmias

The characterization of different members of the transient receptor potential (TRP) superfamily of ion channels provided novel insights into the molecular identity of calcium-activated nonselective cation channels (so-called CAN channel, for Ca^{2+}-activated nonselective), which have been described since the advent of patch clamp technique. TRPM4 and its closest relative TRPM5 are to date the only molecular candidates for this type of ion channels. They are activated by intracellular Ca^{2+} and display weak voltage sensitivity and temperature dependence. Since the last version of this Handbook of Experimental Pharmacology (Vennekens and Nilius 2007), the physiological role of TRPM4 in native systems has become increasingly clarified, especially after the generation and characterization of TRPM4-deficient mice. Among others, TRPM4 was shown to play an important role in the cardiovascular system, the immune system, and the central nervous system.

1 Gene Cloning, Expression, and Protein Structure

TRPM4 is a 1214 amino acid (aa) protein encoded by a gene located in chromosome 19 in the human genome (Ensembl: ENSG00000130529) (Launay et al. 2002; Nilius et al. 2003). In mice, the TRPM4 gene is located on chromosome 7 and encodes a 1213 aa protein (Ensembl: ENSMUSG00000038260) (Murakami et al. 2003; Nilius et al. 2003). In rats, the gene is located on chromosome 1 and encodes a 1208 aa protein (Ensembl: ENSRNOG00000020714) (Yoo et al. 2010). Initial cloning of TRPM4 cDNA succeeded from human tissue (Xu et al. 2001), and subsequent efforts indicated that splice variants of the TRPM4 gene exist in human and mouse tissue (Launay et al. 2002; Nilius et al. 2003). Accordingly, the full-length human clone is designated as TRPM4b and a short variant, lacking 174 N-terminal amino acids, as TRPM4a. In mice, two additional splice variants were identified. Recently, Yoo et al. (2010) cloned the rat transient receptor potential-melastatin 4, rTRPM4a, a nonfunctional channel lacking the N-terminal region, and rTRPM4b, which is the orthologue of human TRPM4b. Analysis of the ENSEMBL database suggests that several other splice variants also could exist, but the physiological significance of these is still unclear. The majority of the functional characterization of the TRPM4 protein has been performed with the full-length clones (TRPM4b).

CAN channels with properties reminiscent of TRPM4 (or TRPM5) were first described in cardiac myocytes (Colquhoun et al. 1981) but have since been described in a wide range of excitable cells including several neuron types (El-Sherif et al. 2001; Liman 2003; Magistretti and Alonso 2002; Mironov 2008; Partridge and Swandulla 1987; Shalinsky et al. 2002; Swandulla and Lux 1985), vascular and smooth muscle cells (Eto et al. 2003; Kim et al. 1998; Miyoshi et al. 2004), and in pancreatic tissue (Sturgess et al. 1987). They have also been found in non-excitable cells such as red blood cells (Kaestner and Bernhardt 2002; Rodighiero et al. 2004), exocrine cells from the pancreas (Gogelein and Pfannmuller 1989; Gray and Argent 1990; Maruyama and Petersen 1984; Suzuki and Petersen 1988), white and brown adipocytes (Halonen and Nedergaard 2002; Ringer et al. 2000), mammalian renal tubule (Hurwitz et al. 2002), cochlear hair cells (Van den Abbeele et al. 1996), mast cells (Vennekens et al. 2007), T cells (Launay et al. 2004), and vascular endothelial cells (Csanady and Adam-Vizi 2003; Popp and Gogelein 1992; Suh et al. 2002; Watanabe et al. 2002). Likewise, the TRPM4 gene is expressed in most of these tissue types. For a detailed overview of TRPM4 gene expression data available in the literature, see Table 1. TRPM4 is also endogenously expressed in HEK-293 cells (Amarouch et al. 2013; Launay et al. 2002) and CHO cells (Yarishkin et al. 2008).

The TRPM4 protein consists of six TM domains with a pore region between TM regions 5 and 6. Likely four subunits are required to form a functional channel, although the stoichiometry of the channel is not formally known (Murakami et al. 2003). Within the TRPM subfamily, TRPM4 is most closely related to TRPM5, sharing approximately 50 % homology. Unlike other members of the TRP channel family, no ankyrin repeats are present in the N terminus of TRPM4. Several protein domains were identified in the TRPM4b protein sequence, including putative calmodulin binding sites in the N and C terminus, as well as phosphorylation sites for protein kinases PKA and PKC, four Walker B motifs, a phosphatidylinositol bisphosphate (PiP_2) binding site with homology to a pleckstrin homology domain (PH), and comprising a decavanadate binding site and two ABC transporter-like signature motifs (Nilius et al. 2005b). A coiled-coil domain is predicted in the C terminus. It is likely that TRPM4 proteins in vivo are part of a channelosome. Interaction partners have been identified for the TRPM4 channel, including TRPC3 and the sulfonylurea receptor, SUR1. The physiological relevance of these interactions is however still unclear. Interaction between TRPM4 and TRPC3 was shown in a heterologous overexpression system and might be important for TRPM4-dependent suppression of store-operated Ca^{2+} entry (Park et al. 2008), although another interpretation seems more likely (see below). The interaction of TRPM4 with SUR1 is controversial. Woo et al. (2013) clearly implicate physical interaction between TRPM4 and SUR1 in overexpressing cells, which also has functional consequences for TRPM4 channel activity, while Sala-Rabanal et al. (2012) deem a functional and physical interaction unlikely.

Table 1 Main properties of full-length TRPM4 derived from overexpression studies

Structure	Sic transmembrane domains, pore between fifth and sixth TM domain			
		hTRPM4	mTRPM4	rTRPM4
	Chromosomal location	19q13.33	7B3	1q22
	Translation length (aa)	1214	1213	1208
	Ensembl #	ENSG00000130529	ENSMUSG00000038260	ENSRNOG00000020714
Permeability	$Na^+ \approx K^+ > Cs^+ > Li^+$ Non-permeable for Ca^{2+} and Mg^{2+}			
Conductance	~23 pS			
Ca^{2+} sensitivity (EC_{50})	Whole cell: 0.5–20 μM Cell-free: 140–370 μM			
Voltage dependence	Slow inactivation at positive potentials Fast inactivation at negative potentials			
Physiological modulation	Positive	Ca^{2+}, PIP_2, temperature (>30 °C), H_2O_2		
	Negative	Intracellular adenine nucleotides, spermine		
Pharmacological modulation	Positive	Decavanadate, BTP2		
	Negative	9-phenanthrol, flufenamic acid, quinine, quinidine, glibenclamide		

2 Functional Properties of TRPM4

2.1 Lessons from Overexpression Studies

Thus far, the majority of the functional data available on TRPM4 has been gathered using heterologous expression of the full length, TRPM4b, mostly in HEK293 cells after transient or stable transfection. For an overview see Table 2. Extensive electrophysiological analysis, using the patch clamp technique, showed that this protein functions as a Ca^{2+}-activated cation channel (Launay et al. 2002; Nilius et al. 2003; Ullrich et al. 2005). Ca^{2+} release from the ER through the IP_3 receptor can activate TRPM4 directly (Gonzales et al. 2010a). The pore of TRPM4 is selective for monovalent cations (permeability sequence: $Na^+ \sim K^+ > Cs^+ > Li^+$) and is virtually impermeable to divalent cations such as Ca^{2+} or Mg^{2+} (Launay et al. 2002; Nilius et al. 2005a; Ullrich et al. 2005). The region between the fifth and the sixth transmembrane domain of TRPM4 contains a stretch of 5–6 acidic amino acid residues which are critical for ion selectivity. This was formally shown in a study using chimeric TRPM4 proteins containing the pore sequence of TRPV6, a distantly related, Ca^{2+}-permeable ion channel. The chimeric channel provides the same gating properties as TRPM4 but is Ca^{2+} permeable (Nilius et al. 2005a; Owsianik et al. 2006). There is general consensus about the sensitivity of TRPM4 to $[Ca^{2+}]_{cyt}$, but there are discrepancies between studies regarding more specific properties. In the whole-cell mode of the patch clamp technique, Ca^{2+}-activated TRPM4-dependent currents are reported to be stable for at least 1 min (Launay et al. 2002) or inactivating within 30–120 s (Nilius et al. 2003). TRPM4 shows a second phase of activation after a variable delay in the range of 250 s, which might be due to recruitment of TRPM4 to the plasma membrane (Cheng et al. 2007; Ullrich et al. 2005). In excised, cell-free patches, TRPM4 currents inactivate slower and not complete, which might be explained by washout of intracellular regulators in this configuration (Nilius et al. 2003). The steady-state current–voltage relationship is reported to be strongly outwardly rectifying (Nilius et al. 2003, 2004a, 2005b; Ullrich et al. 2005) or quasi-linear (Launay et al. 2002). The single-channel conductance amounts approximately 23pS (Launay et al. 2002; Nilius et al. 2003). The EC_{50} value for activation by Ca^{2+} varies in the whole-cell measurements between 500 nM and 20 μM. In excised inside-out patches, an EC_{50} value of 374 μM was reported. When an EC_{50} is determined immediately after current activation, or after incomplete inactivation to a steady-state current level, values of respectively 4.4 μM compared to 140 μM were found (Nilius et al. 2003, 2005b). Part of the explanation of these discrepancies probably lies in the modulatory input (see below), which regulates TRPM4 function and which might be different between cell lines overexpressing TRPM4.

TRPM4 channels display weak voltage sensitivity, as was shown also for other TRP channels. It is clear that increased Ca^{2+} is a necessary requirement to open the channel, but Nilius et al. extensively documented that at positive potentials the open probability of the channel is higher than at negative potentials (Nilius et al. 2003). The voltage sensitivity of the channel can be expressed as the $V_{1/2}$ for half-maximal

Table 2 Tissue expression of TRPM4

Tissue	Species/cells	Method	References
Adrenal gland	Mouse	Western blot	Mathar et al. (2010)
Bladder	Mouse bladder Mouse urothelium	RT-PCR Western blot	Yu et al. (2011)
	Rat detrusor smooth muscle	RT-PCR, Western blot, immunocytochemistry, immunohistochemistry	Smith et al. (2013b)
	Guinea pig detrusor smooth muscle	Western blot, immunocytochemistry	Smith et al. (2013a)
Brain	Human spinal cord	Immunohistochemistry	Schattling et al. (2012)
	Human cerebral cortex	In situ hybridization, RT-PCR	
	Human cerebral cortex	RT-PCR	Malhotra et al. (2013)
	Mouse whole brain	RT-PCR	Schattling et al. (2012)
	Mouse hippocampal neurons		
	Mouse spinal cord motor neurons	Immunohistochemistry	
	Mouse spinal cord	Immunohistochemistry	Gerzanich et al. (2009)
	Mouse substantia nigra	RT-PCR	Mrejeru et al. (2011)
	Mouse pre-Bötzinger region	RT-PCR	Crowder et al. (2007)
	Rat hypothalamus	Immunocytochemistry, RT-PCR	Teruyama et al. (2011)
	Rat whole brain	RT-PCR	Yoo et al. (2010)
	Rat cerebral artery smooth muscle cells	RT-PCR, immunocytochemistry, Western blot	Crnich et al. (2010), Earley et al. (2004), Gonzales and Earley (2012), Reading and Brayden (2007)
Endothelium	Mouse aorta	Western blot	Mathar et al. (2010)
		Northern blot	Nilius et al. (2003)
Heart	Human whole heart	Northern blot	Launay et al. (2004)
	Human atrium	RT-PCR	Guinamard et al. (2004)
	Human Purkinje fibers, septum, right atrium, ventricles	RT-PCR	Kruse et al. (2009)
	Human and mouse	Northern blot	Nilius et al. (2003)
	Mouse atrium and ventricle	Western blot	Mathar et al. (2010)
	Mouse sinoatrial node	RT-PCR Western blot	Demion et al. (2007)
	Rat ventricular myocytes from SHR	RT-PCR	Guinamard and Bois (2007)
	Bovine heart slices	Immunocytochemistry	Liu et al. (2010)

(continued)

Table 2 (continued)

Tissue	Species/cells	Method	References
Hematopoietic cells	Bone marrow-derived dendritic cells (mouse)	RT-PCR	Barbet et al. (2008), Serafini et al. (2012)
	Bone marrow-derived macrophages (mouse)	RT-PCR	Serafini et al. (2012)
	Mast cells (mouse)	Western blot, RT-PCR, immunostaining	Vennekens et al. (2007)
	Monocytes (mouse)	RT-PCR	Serafini et al. (2012)
	Peripheral blood mononuclear cells (human)	RT-PCR	Malhotra et al. (2013), Schattling et al. (2012)
	Neutrophils (mouse)	RT-PCR	Serafini et al. (2012)
	Th2 and Th1 cells (mouse)	Microarray, RT-PCR	Weber et al. (2010)
Intestine	Human and mouse	Northern blot	Nilius et al. (2003)
	Human and monkey colonic smooth muscle	RT-PCR	Dwyer et al. (2011)
Kidney	Human	Northern blot	Launay et al. (2002)
	Human and mouse	Northern blot	Nilius et al. (2003)
	Mouse	RT-PCR	Kunert-Keil et al. (2006)
	Mouse	Western blot	Vennekens et al. (2007)
	Mouse	Western blot	Mathar et al. (2010)
Liver	Human	Northern blot	Launay et al. (2002)
Pancreas	Human	Northern blot	Launay et al. (2002)
	Human and mouse	Northern blot	Nilius et al. (2003)
	Mouse whole pancreas and mouse islets	Western blot	Vennekens et al. (2007)
Placenta	Human	Northern blot	Launay et al. (2002)
	Human and mouse	Northern blot	Nilius et al. (2003)
Prostate	Human	RT-PCR	Fonfria et al. (2006)
	Human	Northern blot	Nilius et al. (2003)
Skeletal muscle	Human	Northern blot	Launay et al. (2002)
	Mouse	RT-PCR	Kunert-Keil et al. (2006)
Spleen	Human	Northern blot	Launay et al. (2002)
	Mouse	RT-PCR	Kunert-Keil et al. (2006)
	Rat	RT-PCR	Yoo et al. (2010)
Testis	Human	Northern blot	Nilius et al. (2003)
	Mouse	RT-PCR	Kunert-Keil et al. (2006)
	Rat	RT-PCR	Yoo et al. (2010)
Thymus	Human	Northern blot	Launay et al. (2002)
	Rat	RT-PCR	Yoo et al. (2010)
Dental follicle stem cells	Rat	RT-PCR	Nelson et al. (2013)

(continued)

Table 2 (continued)

Tissue	Species/cells	Method	References
Cell lines	Human basophil cell line: Ku812	Northern blot	Launay et al. (2002)
	Human umbilical vein endothelial cells: HUVEC	RT-PCR	Becerra et al. (2011)
	Human B-lymphocyte cell line: Ramos	Northern blot	Launay et al. (2002)
	Human embryonic kidney cell line: HEK-293	Northern blot	Launay et al. (2002)
		Western blot	Amarouch et al. (2013)
	Human epithelial cervical cancer-derived cells: HeLa	RT-PCR	Prawitt et al. (2003)
	Human melanoma cell line: G361	Northern blot	Launay et al. (2002)
	Human T-lymphocyte cell line: Jurkat	Northern blot	Launay et al. (2002)
		RT-PCR	Cheng et al. (2007), Prawitt et al. (2003)
	Human monocyte cell line: U937	Northern blot	Launay et al. (2002)
	Human and mouse osteoblast cell lines	RT-PCR	Abed et al. (2009)
	α-TC1-6 (mouse)	RT-PCR, Immunocytochemistry	Nelson et al. (2011)
	Mouse B-lymphoma cell line: A20	RT-PCR	Prawitt et al. (2003)
	β-TC3 (mouse)	RT-PCR	Marigo et al. (2008)
	MIN-6 (mouse)	RT-PCR	
		RT-PCR	Prawitt et al. (2003)
	INS-1 (rat)	RT-PCR	Cheng et al. (2007)
	RINm5F (rat)	RT-PCR	Cheng et al. (2007), Marigo et al. (2008)
	α-cell line: INR1G9 (hamster)	RT-PCR	Marigo et al. (2008)
	HIT-T15 (hamster)	RT-PCR	Cheng et al. (2007), Marigo et al. (2008)
	CHO cells (hamster)	RT-PCR Western blot	Yarishkin et al. (2008)

current activation, which ranges between −20 and +60 mV, depending on a variety of factors, including $[Ca^{2+}]_{cyt}$, temperature, phosphatidylinositol 4,5-bisphosphate content, and the presence of calmodulin (Nilius et al. 2003, 2005b, 2006; Talavera et al. 2005). In the presence of high $[Ca^{2+}]_{cyt}$, the voltage sensitivity will be shifted towards more negative values. Likewise temperatures above 30 °C and application of PIP_2 will increase the open probability of TRPM4 at physiologically relevant,

negative membrane potentials. Since the rectification of the steady-state IV curve and the maximal open probability of the channel are obviously dependent on the voltage sensitivity of the channel, which by itself can be shifted by variation in PIP_2 levels, temperature, and calmodulin, it is obvious that variations in recording conditions can lead to the reported variability of TRPM4 properties from overexpression studies.

The molecular determinants for Ca^{2+} and voltage dependence of TRPM4b are not completely elucidated. Overexpression of a calmodulin mutant unable to bind Ca^{2+} dramatically reduced TRPM4b activation by Ca^{2+} (Nilius et al. 2005b). Concomitantly, mutation of any of the three putative calmodulin binding sites in the C terminus of TRPM4b strongly impaired current activation by reducing the Ca^{2+} sensitivity of TRPM4b and shifting the voltage dependence of activation to very positive potentials (Nilius et al. 2005b). This indicates a crucial role of calmodulin in inferring Ca^{2+} sensitivity to TRPM4b. However, since Ca^{2+} sensitivity is never completely lost in TRPM4b mutants unable to bind calmodulin, it is conceivable that another mechanism also plays a role (Nilius et al. 2005b). Neutralizing a positive charge in the linker between TM domains 4 and 5 of TRPM4b significantly reduces voltage sensitivity and shifts the activation curve dramatically to more positive potentials (Nilius et al. 2005c), indicating that the voltage sensor of TRPM4 is located in the S4 region, analogous to voltage-gated K^+ channels, as was also shown for other TRP channels (Voets et al. 2007).

2.2 Lessons from Overexpression Studies: Modulation

$PI(4,5)P_2$ has been indicated as an important cofactor in the activation of TRPM4 (Nilius et al. 2006). Depletion of PIP_2 leads to rapid rundown of the TRPM4 current, which can be rescued by application of PIP_2 to cell-free patches and inhibition of PIP_2 hydrolysis. TRPM4 is not activated by PIP_2, but application of PIP_2 increases the Ca^{2+} sensitivity and shifts the voltage dependence of activation towards negative potentials, strongly increasing the open probability at a physiological membrane potential. Two putative PIP_2-binding pleckstrin homology domains were identified in the C terminus of TRPM4b. Neutralization of all four positively charged amino acids in one of these stretches resulted in a channel exhibiting very rapid desensitization and highly reduced sensitivity to PIP_2.

ATP influences the Ca^{2+} sensitivity of TRPM4, in inside-out patches when MgATP is applied to the cytosolic side of the membrane (Nilius et al. 2004b). ATP has also been found to block TRPM4 channel activity (Nilius et al. 2004a). It is unclear whether the inhibitory ATP binding site and the facilitating binding site on TRPM4 are identical (Nilius et al. 2004b). Multiple ATP binding sites can be predicted from the amino acid sequence of TRPM4b, including two Walker B motifs in the N terminus and two more in the cytoplasmic loop between TM3 and TM4. When either of these motifs was mutated, the ATP-induced recovery was strongly reduced in all mutants. Moreover, these mutations drastically accelerated the channel desensitization to Ca^{2+}. Thus, these findings indicate that ATP plays a

crucial role in maintaining Ca^{2+} sensitivity of TRPM4b through direct binding to the channel protein (Nilius et al. 2005b). Surprisingly, decavanadate, a compound known to interfere with ATP binding in ATP-dependent transporters, does not have opposite effects on TRPM4b function compared with ATP. Instead, decavanadate is a strong modulator of voltage-dependent gating of the TRPM4b. In the presence of decavanadate on the cytosolic side of excised inside-out patches, TRPM4b currents are sustained, not desensitizing, and linear over a voltage range from −180 to +140 mV. The binding site for decavanadate to the TRPM4b channel was identified and located to the C-terminal tail of TRPM4b (Nilius et al. 2004a).

TRPM4b is also a heat-activated channel. Ion channels, as all other types of enzymes, are temperature dependent to some extent, quantified by the 10° temperature coefficient Q10 value, defined as rate(T + 10)/rate(T) (Hille 1992). Ion channels regarded as temperature independent display Q10 values in the range of 1–4. TRPM4b current amplitude at +25 mV showed a Q10 of 8.5 ± 0.6 between 15 and 25 °C, indicating strong temperature dependence. Heating shifted the activation curve for voltage-dependent opening of the channel towards negative and increased the rate of current relaxation at every potential between −100 and +180 mV (Talavera et al. 2005).

Oxidative stress might also modulate TRPM4 activity. It was reported that H_2O_2-induced necrotic cell death in HEK293 and HeLa cells is dependent on the expression and activity of TRPM4. Simon et al. suggest that H_2O_2 abolishes the desensitization usually observed for heterologously expressed TRPM4 channels, giving rise to a sustained current (Simon et al. 2010).

Finally, trafficking of TRPM4 proteins likely represents another aspect of channel modulation. Several groups have shown that the number of TRPM4 channels can be dynamically regulated (Cheng et al. 2007; Crnich et al. 2010). Kruse et al. showed that the TRPM4 protein is SUMOylated. Protein SUMOylation is a regulation mechanism that controls the trafficking towards the membrane of, e.g., AMPA receptors and K^+ channels (Rougier et al. 2010). A specific mutation in the TRPM4 protein prevents its SUMOylation and leads to an increase of functional channels at the plasma membrane in an overexpression system. This mechanism has been implicated in the pathology of familial heart block (Kruse et al. 2009).

2.3 Endogenous TRPM4 Currents: Lessons from Trpm4$^{-/-}$ Mice

TRPM4 is broadly expressed (see Table 1), and Ca^{2+}-activated currents with properties reminiscent of TRPM4 have also been described in several tissues (for a detailed overview see Vennekens and Nilius 2007). Most recently, endogenous TRPM4-like currents were described in HEK cells and CHO cells. In HEK cells Amarouch et al. described that endogenous TRPM4 currents are very similar to those obtained from cells overexpressing TRPM4, in terms of voltage dependence and cation selectivity. Overexpressed TRPM4 channels seemed somewhat less Ca^{2+} sensitive, and the time course of activation upon application of a high

$[Ca^{2+}]_{cyt}$ was faster for the endogenous channel compared to overexpressed channels (Amarouch et al. 2013).

In the rest of this section, we will focus on currents that were derived from a comparison of WT and *Trpm4*$^{-/-}$ mice. In mast cells a TRPM4-dependent current was functionally identified, by comparing results of wild-type and *Trpm4*$^{-/-}$ animals (Vennekens et al. 2007). The properties of this current are somewhat different from those described in an overexpression system. Most strikingly, the current–voltage relation was linear, showing no apparent voltage dependence, and the current was stable upon activation for at least 5 min. Ca^{2+} dependence and ion selectivity are similar to TRPM4 as assessed in TRPM4-overexpressing HEK cells. The single-channel conductance was 25.3 ± 0.7pS. In dendritic cells Barbet et al. (2008) showed an outwardly rectified, Ca^{2+}-activated current, which was absent in *Trpm4*$^{-/-}$ cells. Single-channel conductance was 22.1 ± 0.6pS, and there was no apparent selectivity for K^+ compared to Na^+. Similar results were obtained in bone marrow-derived macrophages (Serafini et al. 2012). Guinamard et al. reported a TRPM4-like single-channel current in atrial myocytes in cell-free patches, which is absent from *Trpm4*$^{-/-}$ myocytes (Simard et al. 2013). Unitary conductance was 25 ± 0.6 pS, and the current displayed Ca^{2+}-dependent activation and ion selectivity comparable to overexpressed TRPM4. The current was blocked upon addition of 10 μM 9-phenanthrol or flufenamic acid (Simard et al. 2013).

3 Pharmacology

Unfortunately, a major difficulty in the determination of the physiological implications of TRPM4 is the poor specificity of available pharmacological tools. However, several molecules have been described to modulate TRPM4 currents. Potent blockers of TRPM4 activity include intracellular spermine and flufenamic acid applied from the extracellular side, both with IC_{50} values in the range of 1–10 μM (Nilius et al. 2004b). The bitter compounds quinine and quinidine inhibit TRPM4 in the range of 100 μM (Talavera et al. 2008). These compounds are poorly selective among other ion channels and thus provide only a limited pharmacological basis for current dissection in primary cells. Furthermore, TRPM4 is inhibited by intracellular adenine nucleotides, including ATP, ADP, AMP, and AMP-PNP with an IC_{50} value between 2 and 19 μM. Adenosine also blocked TRPM4 at 630 μM. GTP, UTP, and CTP do not exert any effect at concentrations up to 1 mM. ATP^{4-}, which is the ionic form of ATP, inhibits currents with an IC_{50} value of 1.3 μM (Nilius et al. 2004b). Recently, 9-phenanthrol has received much attention as a selective TRPM4 blocker. Its use for the dissection of TRPM4 dependent currents in primary cells will be discussed below. 9-phenanthrol blocks TRPM4 with an IC_{50} of 20 μM in transfected HEK cells (Grand et al. 2008). Glibenclamide blocks TRPM4-like currents in sinoatrial node cells completely at a concentration of 100 μM (Demion et al. 2007). Upon coexpression of TRPM4 with SUR1 in HEK cells, the IC_{50} of glibenclamide shifts to 850 nM (Woo et al. 2013). Decavanadate (see also above), applied intracellularly, leads to

immediate current activation followed by rapid current decay with an EC_{50} of 2 μM (Nilius et al. 2006). 10 μM 3,5-bis(trifluoromethyl)pyrazole (BTP2) was shown to enhance TRPM4 currents through an unclear mechanism, after pretreating TRPM4-overexpressing HEK cells for several minutes (Takezawa et al. 2006).

4 Functional Role of TRPM4

4.1 Conjectures from Knockdown and Pharmacological Inhibition Studies

4.1.1 Smooth Muscle Cells

Local control of cerebral blood flow is regulated by myogenic constriction of resistance arteries, which was originally described as the Bayliss effect. Stretch of the muscle membrane opens a stretch-activated ion channel. The cells become depolarized, and this results in a Ca^{2+} signal. It is generally hypothesized that TRPC6 and TRPM4 are involved in the vascular smooth muscle depolarization as mechanosensitive ion channels (Brayden et al. 2008; Earley et al. 2004; Morita et al. 2007). Earley and coworkers presented a detailed analysis of the role of TRPM4 in vascular smooth muscle (VSM) in several studies. Specifically, they found that pressure-induced VSM cell depolarization was attenuated by TRPM4 antisense oligodeoxynucleotides. Moreover, 9-phenanthrol hyperpolarized the membrane from −40 to −70 mV, leading to abolishment of the myogenic tone (Gonzales et al. 2010b). Furthermore, in vivo suppression of TRPM4 using antisense technology decreases myogenic constriction in cerebral arteries and leads to a higher cerebral blood flow both at resting and at elevated mean arterial pressure, confirming a role for TRPM4 in myogenic constriction and cerebral blood flow regulation (Earley et al. 2004). Later it was shown that TRPM4 activation during pressure-induced depolarization is protein kinase C (PKC) dependent. Indeed, stimulation of PKC activity increased the intracellular Ca^{2+} sensitivity of TRPM4 (Earley et al. 2007), as was found previously by others in HEK293 cells overexpressing TRPM4 (Nilius et al. 2005b). PKC activation also might induce translocation of TRPM4 to the plasma membrane. Using a green fluorescent protein (GFP) tagged TRPM4 being overexpressed in HEK cells, it was shown that PKC activation with phorbol 12-myristate 13-acetate (PMA) increased the amount of TRPM4-GFP protein on the cell surface about threefold. Furthermore, data indicates that this translocation was independent of PKC-alpha and PKC-beta activity but was inhibited by blockade of PKC delta with rottlerin (Crnich et al. 2010). Taken together, it has become clear that TRPM4 has a critical role in vascular smooth muscle cell depolarization as a mechanosensitive ion channel, together with PKC-delta activation. The link between mechanical stress and TRPM4 activation is however still unresolved. It should also be mentioned that the Bayliss effect was not changed in *Trpm4*$^{-/-}$ mice (Mathar et al. 2010).

Spontaneous transient outward currents in smooth muscle cells result from activation of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels following

transient release of Ca^{2+} from ryanodine receptors located on the sarcoplasmic reticulum (Nelson et al. 1995). It was shown that transient inward cation currents (TICCs) are the result of activation of TRPM4 channels by SR Ca^{2+} released from inositol trisphosphate (IP_3) receptors, since TICC activity is inhibited by flufenamic acid and 9-phenanthrol and also attenuated by downregulation of TRPM4 expression in cerebral artery myocytes (Gonzales et al. 2010a). Besides the analysis of the role of TRPM4 in cerebral arteries, TRPM4 was also shown to be present and functional in several types of smooth muscle outside of the vasculature. TICCs similar to those described in cerebral artery myocytes have been recorded in human- and monkey-isolated colonic smooth muscle cells. These currents were attenuated using by 9-phenanthrol (Dwyer et al. 2011). Furthermore, TRPM4 channels have been described to be present in rat and guinea pig detrusor smooth muscle cells. Also here, TICC-like currents that were sensitive to application of 9-phenanthrol were recorded. In addition, it reduced contractility of isolated bladder strips (Smith et al. 2013a, b). Collectively, the mentioned studies suggest that TRPM4 may be important for the function of several types of smooth muscle cells.

4.1.2 Cardiomyocytes

Cardiac hypertrophy is an adaptive process that occurs in response to increased physical stress on the heart. A common reason of cardiac hypertrophy is high blood pressure or heart valve stenosis. Recently, several TRP channels including TRPC1, TRPC3, and TRPC6 have been shown to be related to various aspects of cardiac hypertrophy (Guinamard and Bois 2007). Guinamard et al. showed a higher expression of this channel in cardiomyocytes of spontaneously hypertensive rats (SHR), a model of hypertension and cardiac hypertrophy. They also found an increase in the TRPM4 current activity in myocytes from SHR rats (Guinamard et al. 2006). While mechanisms underlying the onset of hypertrophy are still not clear, intracellular Ca^{2+} levels appear to be a major component of the process. The mechanism how TRPM4 may modify this Ca^{2+} signaling or modify the known signaling cascades (NFAT, JNK, ERK) involved in triggering hypertrophy still remains to be determined.

The physiological and pathological role of TRPM4 in heart function is poorly understood (see below). Two reports investigate the arrhythmic and/or cardioprotective effect of 9-phenanthrol. Simard et al. (2013) showed that application of 9-phenanthrol abolishes arrhythmias induced by hypoxia and reoxygenation in spontaneously beating right ventricle preparations from mouse. Additionally, Wang et al. show that pretreatment of rat hearts with 9-phenanthrol reduces infarct size and increases contractile function after ischemia-reperfusion-induced injury. However, a direct role of TRPM4 in this process is unclear since 9-phenanthrol was applied before, but not during, the induction of ischemia (Wang et al. 2013).

4.1.3 Neurons

TRPM4 transcripts are frequently detected in the central nervous system (see Table 2). RT-PCR experiments showed TRPM4 and TRPM5 expression in brain and spinal cord extracts from mouse and rat (Crowder et al. 2007; Launay

et al. 2002; Yoo et al. 2010). The Allen Brain Atlas confirms these results in mouse by in situ *hybridization* and showed different levels of expression according to the structures: TRPM4 is expressed at a quite high level in hippocampus and hypothalamus and at a reduced level in the olfactory bulb, cerebellum, pons, and cortex.

The general hypothesis that Ca^{2+}-activated nonselective cation channels, like TRPM4, could support bursts of action potentials has been put forward already many years ago. Some work that associates TRPM4 with this process has been performed in pre-Bötzinger complex neurons in mice. The pre-Bötzinger complex encompasses a set of neurons, which are rhythmically active and regulate breathing rhythm in mammals. Only 20 % of these neurons present pacemaker activity, meaning that most of the neurons generate inspiratory drive potentials in response to postsynaptic currents evoked by a glutamatergic input (Del Negro et al. 2005; Mironov 2008; Pace et al. 2007). CAN channels have been proposed to be the candidate to amplify glutamatergic synaptic drive by transforming the glutamatergic synaptic inputs to long-lasting membrane depolarization. Pace et al. showed that Ca^{2+} influx was able to induce plateau potentials, and external sodium substitution and flufenamic acid exposure attenuated those plateau potentials. They also proposed that CAN channel can be activated by glutamatergic inputs directly (via NMDA receptor-mediated calcium influx) or indirectly (via mGluR-induced IP_3-dependent calcium release or AMPA receptor-dependent activation of voltage-gated calcium channels). Crowder et al. detected by RT-PCR TRPM4 and TRPM5 expression in preBötC neurons and showed that excess of PIP_2 augmented the inspiratory drive potential and the effect was attenuated by flufenamic acid application (Crowder et al. 2007), which fits to some extent with features that have been published before on TRPM4 after overexpression in cell lines. It should be mentioned however that flufenamic acid is not at all a specific TRPM4 blocker (Guinamard et al. 2013), and many ion channels and transporters are influenced by PIP_2 levels (Logothetis et al. 2010). Also, to date no abnormalities concerning breathing rhythm has been reported in *Trpm4*$^{-/-}$ mice. A similar role for TRPM4 has been suggested in neurons from the entorhinal cortex and in dopamine neurons of the substantia nigra pars compacta (Egorov et al. 2002; Mrejeru et al. 2011). The idea of TRPM4 supporting burst potentials was explicitly tested in cerebellar Purkinje cells using *Trpm4*$^{-/-}$, *Trpm5*$^{-/-}$, and TRPM4–TRPM5 double KO mice (Kim et al. 2013). In Purkinje cells of the posterior cerebellum, a slow inward cation current (depolarization-induced slow current, DISC) is present, which shares on first sight properties with TRPM4 (and TRPM5 for that matter). The current is Ca^{2+} sensitive, monovalent cation selective, and strongly attenuated by nonselective blockers of TRPM4 and TRPM5. However, measurement of DISC currents in Purkinje cells derived from *Trpm4*$^{-/-}$, *Trpm5*$^{-/-}$, and double KO mice as well as wild-type mice with TRPM4 short hairpin RNA knockdown showed only a partial attenuation with 35–46 % of current remaining. Thus, while the DISC conductance shares some properties with TRPM4 and TRPM5, these ion channels are not absolutely required for DISC (Kim et al. 2013). This example illustrates that knockout mice are an absolutely required control to assign a protein to a relatively poorly characterized current.

4.1.4 Other

In rat dental follicle stem cells, knockdown of TRPM4 expression led to enhanced osteogenesis, but decreased adipogenesis, suggesting that TRPM4-mediated modulation of Ca^{2+} signals has a differential effect on cell proliferation according to cell fate. How this occurs is unclear (Nelson et al. 2013). Using a dominant-negative construct, ΔN-TRPM4, the same group has shown that Ca^{2+} signals are generally significantly reduced in multiple cell line models of pancreatic ß-cells and pancreatic α-cells (Cheng et al. 2007; Marigo et al. 2008; Nelson et al. 2011). Indeed, arginine-vasopressin, glibenclamide, and glucose-induced Ca^{2+} signaling and insulin secretion are reduced in ß-cell lines from rat, hamster, and mouse origin when TRPM4 activity is inhibited. In an α-cell line shRNA-mediated knockdown of TRPM4 expression decreased Ca^{2+} responses and glucagon secretion in response to arginine-vasopressin, KCl, L-arginine, and the voltage-gated Ca^{2+} channel agonist BayK 8644 (Cheng et al. 2007; Nelson et al. 2011). However, in *Trpm4*$^{-/-}$ mice no phenotype was apparent in a blood-glucose homeostasis after glucose administration and in glucose-induced insulin secretion from primary pancreatic islets (Vennekens et al. 2007). Furthermore, pancreatic ß-cells also express TRPM5 (Colsoul et al. 2010), which has similar properties to TRPM4. It remains to be shown how the shRNA strategy and overexpression of ΔN-TRPM4 affect TRPM5 expression and activity in the abovementioned cell lines (Marigo et al. 2008; Nelson et al. 2011).

4.2 Insights from TRPM4-Deficient Mice

TRPM4 knockout mice have been described by two independent groups (Barbet et al. 2008; Vennekens et al. 2007). In general, both these mouse lines are viable and fertile and reach a normal age in standard laboratory conditions. No anatomical or gross behavioral abnormalities have been reported to date. Below we summarize the data that has been gathered through the analysis of different organ systems in wild-type and *Trpm4*$^{-/-}$ mice.

4.2.1 TRPM4 in the Cardiovascular System

A role of TRPM4 in blood pressure regulation has been suggested in several studies (see above). *Trpm4* is expressed in several organs involved in blood pressure regulation such as kidney, heart, adrenal glands, and vascular smooth muscle. Mathar et al. (2010) showed a mild but stable blood pressure increase in *Trpm4*$^{-/-}$ mice: an average increase of about 10 mmHg is present during both the resting and active period and is not due to changes in locomotor activity. Heart rate, cardiac output, ejection fraction, and cardiac contractility are not changed in *Trpm4*$^{-/-}$ mice under basal conditions. However, *Trpm4*$^{-/-}$ mice show increased plasma epinephrine levels and increased urinary excretion of catecholamine metabolites, which is likely due to increased catecholamine release from chromaffin cells of the adrenal gland. Indeed, isolated chromaffin cells from *Trpm4*$^{-/-}$ mice hypersecrete catecholamines, but the cellular mechanism still needs to be clarified. Aged

Trpm4$^{-/-}$ mice (>6 months) show a mild hypertrophy, which is likely due to the hypertension (Mathar et al. 2010).

In atrial myocytes, it was suggested that TRPM4 contributes to the action potential and regulates sinus rhythm (Hof et al. 2013; Simard et al. 2013). *Trpm4*$^{-/-}$ atrial myocytes have a shorter action potential compared to WT atrial myocytes. In *Trpm4*$^{-/-}$ atrial myocytes the repolarization of the action potential is faster, leading to a reduction of the time to 50 and 90 % repolarization of the action potential. Action potential duration (APD) in atrial myocytes can also be decreased by 9-phenanthrol, which has no effect on *Trpm4*$^{-/-}$ atrial myocytes (Simard et al. 2013). In a follow-up study, the same group suggested that TRPM4 also contributes to the diastolic depolarization and thereby regulates sinus automaticity, though the heart rate in living mice is not different between WT and *Trpm4*$^{-/-}$ mice. Surprisingly, in the latter study 9-phenanthrol had no effect on APD in WT atrial myocytes, which is attributed to a frequency-dependent effect of 9-phenanthrol on APD, but which was not explicitly shown (Hof et al. 2013). These results illustrate that TRPM4 might have a profound role in cardiac atrial myocytes, but important questions remain. TRPM4 activity in atrial myocytes per se has never been shown, apart from cell-free patches. Thus it is unclear how the activity of TRPM4 correlates with the time course of intracellular Ca^{2+} events in atrial myocytes. To influence the sinus rate, TRPM4 should be active before the action potential and the main Ca^{2+} transient, while an effect on the late phase of the action potential can only be envisaged when TRPM4 is activated during the main Ca^{2+} transient. Considering that the affinity of TRPM4 for Ca^{2+} is in the range of 20–100 μM at a positive membrane potential (+40 mV) in atrial myocytes (Demion et al. 2007), this value would even be lower at a negative resting potential during the diastolic phase, which raises the question whether local Ca^{2+} sparks during the diastolic phase would be sufficient to open TRPM4 channels (Little and Mohler 2013). Thus it remains unclear at what time point TRPM4 is actually activated in normal-beating myocytes, i.e., during the main Ca^{2+} transient in the systolic phase or even before the action potential. Also, the apparent role of TRPM4 in atrial myocytes has no obvious consequences for the function of the heart. The basal sinus rate is the same in WT and *Trpm4*$^{-/-}$ mice (Hof et al. 2013), and there are no apparent abnormalities in basal cardiac contractility or heart rate reported in *Trpm4*$^{-/-}$ mice. Finally, the specificity of 9-phenanthrol is not established enough to exclude that the drug has any effects on the activity of the Na^+/Ca^{2+} exchanger, hyperpolarization-activated ion channels (HCN), and voltage-gated T-type Ca^{2+} channels which play a distinct role in the control of sinus rate (Mangoni and Nargeot 2008).

4.2.2 TRPM4 in the Immune System

From the first characterization of TRPM4, it was already suggested that TRPM4 could control Ca^{2+} influx through depolarizing the membrane potential and thus limiting the driving force for Ca^{2+} entry through voltage-independent Ca^{2+} channels such as store-operated Ca^{2+} channels (Launay et al. 2002). Indeed, the amount of Ca^{2+} influx through these channels is largely dependent on the level of

the membrane potential, and Ca^{2+} signals in non-excitable cells thus often completely mirror changes in V_m. This idea was originally elaborated in Jurkat T cells using RNAi-mediated knockdown of TRPM4 expression. Additionally, TRPM4 was functionally inhibited through expression of TRPM4a, which supposedly functions as a dominant-negative isoform (Launay et al. 2004). Though the selectivity of this approach is likely limited, it was shown that endogenous CAN currents could be inhibited to 25 % of control values with both methods. When Jurkat T cells were stimulated using phytohemagglutinins (PHA), the oscillatory Ca^{2+} signal, which is apparent in WT cells, was transformed in a prolonged, sustained, and larger Ca^{2+} increase in TRPM4 knockdown cells. Concomitantly, interleukin-2 production in TRPM4-downregulated cells was significantly increased (Launay et al. 2004).

The concept of TRPM4 being a physiologically meaningful regulator of driving force for Ca^{2+} entry was further developed using TRPM4 knockout mice. In mast cells, FcεRI-mediated activation leads to a PLC-dependent increase of intracellular Ca^{2+} levels, dependent on Ca^{2+} release from IP_3-dependent stores and Ca^{2+} influx via store-operated Ca^{2+} channels (Vennekens et al. 2007). In *Trpm4*$^{-/-}$ mast cells, Ca^{2+} increase is drastically elevated after receptor activation, due to the fact that V_m remains very negative for a prolonged period after receptor activation. In contrast, in WT cells, V_m shortly hyperpolarizes and quickly depolarizes, leading to a relatively short peak of the intracellular Ca^{2+} level upon receptor activation. These data support the hypothesis that Ca^{2+} influx in mast cells is controlled through the interplay between a Ca^{2+}-activated hyperpolarizing current (likely SK4) and a Ca^{2+}-activated depolarizing current (TRPM4). Removal of the depolarizing TRPM4-dependent component leads to increased histamine release and aggravated anaphylactic reactions in sensitized animals (Vennekens et al. 2007). This concept of TRPM4 was further tested in dendritic cells. This cell type is essential for the initiation of adaptive immune responses. The absence of TRPM4 led to Ca^{2+} overload in these cells, which did not influence dendritic cell maturation, but considerably impaired chemokine-dependent dendritic cell migration (Barbet et al. 2008). Notably, also in mast cells, deletion of TRPM4 leads to impaired migration of cells triggered by dinitrophenylated human serum albumin (DNP-HSA) or stem cell factor (SCF) (Shimizu et al. 2009).

In a recent study, the importance of TRPM4 in other immune cells was tested to determine its relevance in the control of inflammation. In this study, it was reported that the ablation of the *Trpm4* gene dramatically increased mouse mortality in a model of sepsis induced by cecal ligation and puncture (Serafini et al. 2012). The lack of the TRPM4 channel significantly impaired the phagocytotic activity of macrophages in bacteria-infected peritoneal cavities and increased the systemic level of Ly6C(+) monocytes and proinflammatory cytokine production. Since Ca^{2+} signals were actually reduced in macrophages isolated from the peritoneum when stimulated with *Escherichia coli*, it was hypothesized that Ca^{2+} overload occurs at an earlier time point in the maturation of *Trpm4*$^{-/-}$ macrophages, during blood monocyte extravasation within the peritoneal cavity, and that this event could drive *Trpm4*$^{-/-}$ macrophages in an unresponsive state (Serafini et al. 2012). Notably,

from this study it was also shown that Ca^{2+} signaling in neutrophils was unchanged in the absence of a functional *Trpm4* gene, indicating that the contribution of TRPM4 to the regulation of Ca^{2+} signaling cannot be generalized. A similar finding appeared in mouse Th1 and Th2 cells, which both express TRPM4. Inhibition of TRPM4 expression increased Ca^{2+} influx in Th2 cells, whereas the opposite was observed in Th1 cells. Concomitantly Th2 cells show increased NFATc1 nuclear localization, in contrast to Th1 cells, after an identical stimulus (Weber et al. 2010).

4.2.3 TRPM4 and Disease

TRPM4 has recently been associated with disease conditions that might be relevant to humans. The human chromosome location where the *Trpm4* gene is located has been associated with autosomal dominant hearing loss (Pusch et al. 2004). Other genes in this area include voltage-gated K^+ channels (KCNJ14, KCNA7, and KCNC3), sodium-dependent inorganic phosphate co-transporter (SLC17A7), synaptotagmin (SYT3), and myosin-type motor molecules (MYH14 and MYBPC2). Further analysis to identify disease-causing mutations showed that the TRPM4 gene is likely not involved in this pathology (Yang et al. 2005). Increased expression of *Trpm4* was shown in prostate cancer and in an aggressive form of large B-cell non-Hodgkin lymphoma. Upregulation of *Trpm4* transcripts was also observed in cervical uterus cancer (Armisen et al. 2011).

In three branches of a South African Afrikaner pedigree with an autosomal dominant form of Progressive Familial Heart Block Type 1, a specific mutation in the TRPM4 gene was found, which leads to an amino acid substitution (E7K) in the N terminus of the TRPM4 protein (Brink et al. 1995; Kruse et al. 2009). Subsequently, three more mutations were shown in a French-Lebanese pedigree with isolated cardiac conduction disease (Liu et al. 2010) and additional six mutations in patients with right ventricular bundle branch block and atrioventricular block (Stallmeyer et al. 2012). The latter study was performed in random patients, with only limited familial relation. The phenotype of carriers with specific TRPM4 mutations can be variable, and the penetrance of the mutations (the chance that a carrier will actually have the disease) is in some cases less than 50 %, indicating that in human cardiac conduction disease clearly also additional factors will play a role (Stallmeyer et al. 2012). Analysis of the first described mutation, E7K, suggested that the amino acid substitution found in PFHB Type 1 is originating from a gain-of-function of TRPM4 channel activity. It was reported that the mutated amino acid leads to increased SUMOylation of mutant channels, a posttranslational mechanism crucial in determining the degradation and/or regulation of proteins. Alteration of the SUMOylation/deSUMOylation process may impair endocytosis and stabilize the mutant channels at the cell membrane, leading to a higher number of functional channels at the membrane of cells carrying this mutation (Kruse et al. 2009). It is conceivable that larger TRPM4 activity might lead to a long-lasting depolarization of the cardiac conduction fibers, which could result in slowing down or even block of action potential propagation, leading to uncoordinated contraction of the ventricular muscle. However the gain-of-function nature of these mutations has only been formally shown in overexpression studies.

It is not shown how they influence TRPM4 channels endogenously expressed in cardiac myocytes. Recently, Liu et al. addressed whether Brugada syndrome could be attributed to TRPM4 mutations, since it is frequently associated with cardiac conduction anomalies, specifically in patients without known mutations in the SCN5a gene. Several mutations in the TRPM4 gene were identified, but the consequences of these mutations are wide-ranging on the level of channel electrophysiology and cellular expression (Liu et al. 2013). Indeed, "disease-causing" mutations were found to result in decreased, increased, and unchanged channel activity after overexpression in HEK cells.

TRPM4 seems to be critically involved in the process of cell death. Excessive Na^+ influx through TRPM4 channels, induced by, e.g., Ca^{2+} overload, could lead to cell volume increase and necrotic cell death. Indeed, knockdown or inhibition of TRPM4 prevents LPS-induced Na^+-dependent cell death in a human endothelial cell line (Becerra et al. 2011). Furthermore, this mechanism is now extensively associated with the pathology of spinal cord injury, experimental autoimmune encephalomyelitis, and multiple sclerosis.

Contusion of the CNS involving the brain or spinal cord injury (SCI) is frequently complicated by expansion of the hemorrhage (Guth et al. 1999). Although sometimes erroneously attributed to continued bleeding of vessels fractured by the original trauma, this phenomenon likely represents a secondary pathological process. Expansion of secondary hemorrhage results from progressive catastrophic failure of the structural integrity of capillaries (Griffiths et al. 1978). Secondary hemorrhage is particularly damaging because it greatly expands the volume of neural tissue destroyed by the primary injury. The capillary dysfunction implicit with secondary hemorrhage causes tissue ischemia and hypoxia, and the blood is exquisitely toxic to CNS cells (Wang et al. 2002), further injuring neural tissues owing to oxidative stress and inflammation. Together, these processes render secondary hemorrhage the most destructive mechanism of secondary injury involving the CNS. In rat model of spinal cord injury, Gerzanich et al. (2009) show that the expression level of TRPM4 is strongly upregulated in tissue surrounding the injury, especially in microvessels. Treatment of rats with TRPM4-antisense nucleotides strongly reduced the occurrence of secondary hemorrhages. This was further supported by data in *Trpm4*$^{-/-}$ mice. Also in these animals secondary hemorrhages after spinal cord injury are reduced, and neurobehavioral performance was improved compared to wild-type animals after spinal cord injury. The hypothesis is that sustained opening of TRPM4 channels, as would arise with the severe ATP depletion associated with SCI, could lead to continuous influx of Na^+, which, if unchecked, could induce oncotic swelling and oncotic death of endothelial cells, resulting in capillary fragmentation (Gerzanich et al. 2009). Indeed, COS-7 cells that overexpress TRPM4 are more prone to ATP depletion-induced oncotic cell death (Gerzanich et al. 2009). A similar hypothesis was put forward when the clinical development of experimental autoimmune encephalomyelitis was analyzed in WT and *Trpm4*$^{-/-}$ mice (Schattling et al. 2012). In *Trpm4*$^{-/-}$ mice, reduced axonal and neuronal degeneration was observed, which correlated with improved clinical disease scores in *Trpm4*$^{-/-}$ mice. EAE is an inflammatory demyelinating

disease which shares some features of and is often used as a disease model for multiple sclerosis in humans (Baker et al. 2011; Kuerten and Lehmann 2011). In MS and EAE progressive demyelination of neurons leads to dysfunction of especially motor neurons, but an important component of the disease includes also cell death and progressive development of irreversible neurological damage, which correlates best with clinical disability during the progressive course of multiple sclerosis. It is this aspect of the disease that is apparently missing in *Trpm4*$^{-/-}$ mice (Schattling et al. 2012). Upon induction of EAE, WT, and *Trpm4*$^{-/-}$, mice initially develop neurological problems to the same extent, but *Trpm4*$^{-/-}$ animals recover to some degree after the initial outbreak and show overall reduced disease severity and a significantly better recovery from weight loss following the induction of EAE compared with WT mice. Somewhat surprisingly, the same effect was obtained by treating mice with a nonspecific blocker of TRPM4, glibenclamide, which had no additional effect in *Trpm4*$^{-/-}$ animals (Schattling et al. 2012). Although functional TRPM4 channels are also expressed in immune cells (see above), the improved clinical score in EAE mice was not due to changes in the EAE-induced autoimmune response. Rather, TRPM4 seems to be involved in the process of neuronal cell death upon excitotoxic stress and energy depletion due to glutamatergic stress which is a major contributor to neurodegeneration in multiple sclerosis. High glutamate levels cause excitotoxic neurodegeneration through ionotropic glutamate receptors by eliciting Ca^{2+} and Na^{+} influx (Lau and Tymianski 2010). Since the rise of intracellular Ca^{2+} will activate TRPM4 channels in neurons, this process seems likely to be aggravated by the presence of TRPM4 (Schattling et al. 2012).

Concluding Remarks

An obvious lack in the characterization of the physiological role of TRPM4 is a highly selective blocking compound, which would allow acute and reversible, selective blockade of TRPM4 channel activity in a living organism. A recurring theme in the TRP channel literature is the apparent discrepancy between the effect of gene knockout in a mouse and gene knockdown or pharmacological inhibition of a TRP channel on the properties of a cell type or tissue. For instance, in TRPM4 knockout mice, blood pressure is constitutively increased, and myogenic constriction of resistance vessel smooth muscle cells is unimpaired (Mathar et al. 2010). In studies using isolated cerebral arteries, TRPM4 knockdown and inhibition of TRPM4 with 9-phenanthrol lead to decreased myogenic tone and smooth muscle cell hyperpolarization, which would promote vasodilation and hypotension in vivo (Brayden et al. 2008; Earley et al. 2004). This apparent discrepancy leaves several interpretations. First, and most obvious, properties of distinct smooth muscle cell types can be very different. Second, pharmacological inhibitors and siRNA constructs might be unspecific and have off-target effects. A necessary control would be to test their efficacy in knockout mice. Third, in a knockout mouse model there is the inherent possibility that the function of the knocked-out gene is being compensated by the organism through upregulation of a redundant gene with more or less the same function. Time- and tissue-specific knockout mouse strategies are to date rare in

the TRP field and could clarify this type of problems in the literature. Finally, one should also consider that acute blockade of an ion channel current does not necessarily change the physiological properties of a tissue to the same extent as observed in a knockout mouse. A knockout mouse basically equals the complete blockade and/or removal of an ion channel from conception of the organism until adulthood, which is a time span rarely covered by application of a drug. With this in mind one should also be cautious to deduce off-target effects of channel blocking drugs from the phenotype of classic knockout mice. TRPM4 knockout mice display hypertension (Mathar et al. 2010), aggravated anaphylactic reactions (Vennekens et al. 2007), increased sensitivity to sepsis (Serafini et al. 2012), and deficits in dendritic cell migration (Barbet et al. 2008). On the other side of the spectrum, TRPM4 knockout mice show decreased vascular permeability and secondary bleedings upon spinal cord injury (Gerzanich et al. 2009) and are resistant to certain aspects of experimental allergic encephalomyelitis (a mouse model of multiple sclerosis) (Schattling et al. 2012). Additionally, data suggests that TRPM4 blockers might be beneficial for the treatment of cardiac arrhythmias (Simard et al. 2012). Thus from first sight systemic application of a TRPM4 blocker could have beneficial but also grave adverse effects. Yet it remains to be shown whether all of these effects are also present when a specific blocking agent would be applied to a living organism for a relatively short time span.

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TRPM5

Emily R. Liman

Contents

Abstract

TRPM5 is a Ca^{2+}-activated cation channel that mediates signaling in taste and other chemosensory cells. Within taste cells, TRPM5 is the final element in a signaling cascade that starts with the activation of G protein-coupled receptors by bitter, sweet, or umami taste molecules and that requires the enzyme PLCβ2. PLCβ2 breaks down PIP_2 into DAG and IP_3, and the ensuing release of Ca^{2+} from intracellular stores activates TRPM5. Since its initial discovery in the taste system, TRPM5 has been found to be distributed in sparse chemosensory cells located throughout the digestive track, in the respiratory system, and in the olfactory system. It is also found in pancreatic islets, where it contributes to

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insulin secretion. This review highlights recent work on the mechanisms of the activation of the TRPM5 channel and its regulation by voltage, phosphoinositides, temperature, and pH. The distribution of the channel in the body and its functional contribution to various sensory and nonsensory processes are discussed.

Keywords
Transient receptor potential • Calcium • PI(4,5)P2 • Taste • Bitter • Sweet • Insulin

1 Gene and Protein Structure of TRPM5

TRPM5 was first identified in an effort to find genes associated with the tumor producing condition known as Beckwith–Wiedemann syndrome (BWS), and although failing to link TRPM5 with BWS, these initial studies defined the basic structure of the gene (Prawitt et al. 2000; Enklaar et al. 2000). The human TRPM5 gene comprises 24 exons on chromosome 11, and it contains an open reading frame of 3,495 bp which predicts a protein of 1,165 amino acids (Prawitt et al. 2000). The orthologous mouse gene is located on the syntenic distal end of chromosome 7, and it contains an open reading frame that predicts a protein of 1,158 amino acids (Enklaar et al. 2000). TRPM5 shows highest homology to TRPM4 (40 % identity at the amino acid level), and it is more distantly related to other TRPM channels, such as the cold and menthol receptor TRPM8 (McKemy et al. 2002; Peier et al. 2002). Like other TRP channels, TRPM5 is thought to contain six transmembrane domains and to assemble as a tetramer (Montell et al. 2002; Clapham 2003). There is no evidence that it can co-assemble with any other TRP channel subunits, and no interacting proteins have been reported.

2 Expression Pattern

TRPM5 was first reported to be expressed in mammalian taste buds, in a subset of cells that co-express receptors for bitter, sweet, and umami tastes (Perez et al. 2002; Zhang et al. 2003). Subsequent studies showed a more wide-spread distribution of the channel (Kaske et al. 2007). Using a mouse in which the TRPM5 promoter drives expression of GFP (Clapp et al. 2006) (Fig. 1a), or direct antibody labeling, TRPM5 expression has been detected in two distinct subsets of olfactory neurons, one ciliated (Lin et al. 2007) and another microvillus (Lin et al. 2008a; Hansen and Finger 2008), in the vomeronasal organ, in the gastrointestinal tract, and in the respiratory system (Kaske et al. 2007). In most of these tissues, TRPM5 is expressed in solitary cells. Within the gastrointestinal system, TRPM5 is expressed in the stoma, small intestine, and colon in sparely distributed solitary cells. In both

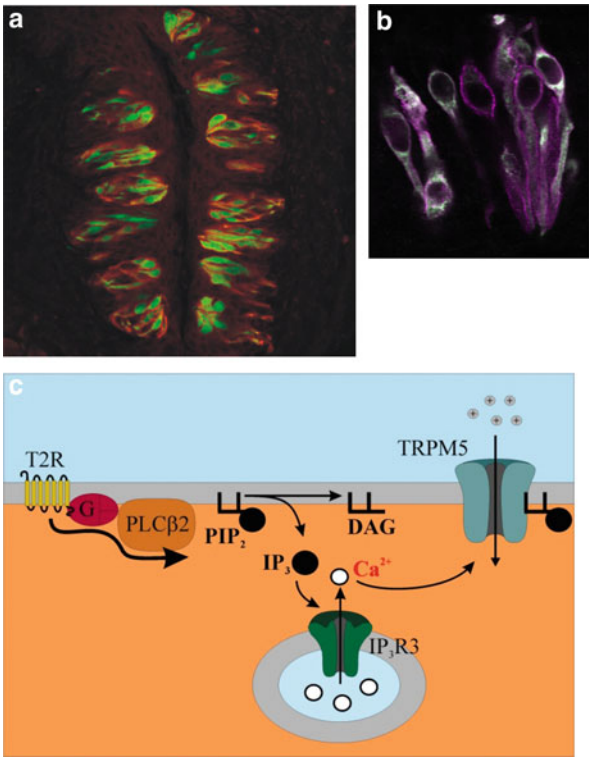

Fig. 1 (**a**, **b**) Distribution of TRPM5 in taste buds of the mouse circumvallate papillae, showing (**a**) co-expression of TRPM5 (*red*) with GFP expressed under the TRPM5 promoter (*green*) and (**b**) co-localization of TRPM5 with PLCβ2 [from Zhang et al. (2007), with permission]. (**c**) A model for taste transduction. Binding of taste stimuli to G protein-coupled taste receptors (R) leads to dissociation of the heterotrimeric G protein. βγ subunits of the G protein activate PLCβ2, which in turn hydrolyzes PIP_2 into DAG and IP_3. IP_3 activates IP_3 receptors, which release Ca^{2+} from intracellular stores. Intracellular Ca^{2+} opens TRPM5 channels, leading to an influx of Na^+ and depolarization of the cell. Note that TRPM5 is not permeable to Ca^{2+} and, therefore, there is no positive feedback [from Liu and Liman (2003), with permission]

the respiratory system and the digestive system, the cells that express TRPM5 have been shown to be brush cells based on morphological criteria, including co-localization with villin, and CK18 (Kaske et al. 2007; Bezencon et al. 2008). TRPM5 is also expressed in pancreatic islets, where it is localized to insulin-secreting β-cells (Brixel et al. 2010; Colsoul et al. 2010). In addition, there are a few reports that TRPM5 may be expressed in the central nervous system (Dehkordi et al. 2012; Kim et al. 2012). In taste cells, the channel protein is present along the basolateral surface of the cell (Zhang et al. 2007; Kaske et al. 2007) where it may be inaccessible to apically delivered chemicals (Fig. 1b).

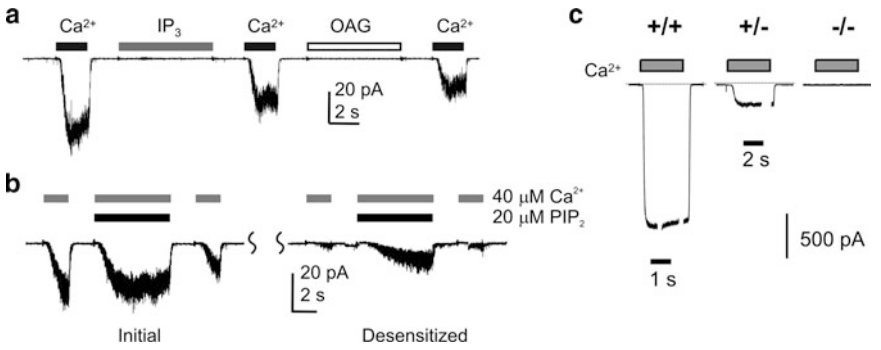

Fig. 2 TRPM5 is activated by intracellular calcium. (**a**) Activation by 40 μM Ca^{2+} of an inward current in a patch excised from a TRPM5-transfected CHO-K1 cell ($V_m = -80$ mV). Neither 10 μM IP_3 nor 100 μM OAG elicited a current in the same patch. (**b**) PIP_2 partially restores TRPM5 channel activity following desensitization. Responses to 40 μM Ca^{2+} in the presence and absence of 20 μM PIP_2 before and after desensitization. Desensitization was induced by a 30 s exposure to 40 μM Ca^{2+} ($V_m = -80$ mV). (**c**) Activation of TRPM5 currents by 50 μM Ca^{2+} in patches excised from GFP^+ taste cells isolated from TRPM5-GFP mice. WT and heterozygote mice show a large Ca^{2+}-activated current that is absent in $TRPM5^{-/-}$ mice [from Liu and Liman (2003) and Zhang et al (2007), with permission]

3 Ion Channel Properties

3.1 Activation by Ca^{2+}

The expression pattern of TRPM5 in a subset of taste cells suggests that the channel is activated downstream of a PLC-mediated signaling cascade (Perez et al. 2002; Zhang et al. 2003). Consistent with this prediction, TRPM5 currents can be gated in heterologous cell types by stimulation of Gq-coupled receptors that activate PLC (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003; Zhang et al. 2003). PLC hydrolyzes $PI(4,5)P_2$ into DAG and IP_3, and IP_3 causes release of Ca^{2+} from intracellular stores, and presumably one or more of these small molecules activate TRPM5. Although an initial report suggested that store depletion or elevation of IP3 directly gated TRPM5 (Perez et al. 2002; Zhang et al. 2003), subsequent studies by three laboratories independently showed that instead elevation of intracellular Ca^{2+} can directly gate TRPM5 channels (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003) (see Fig. 2a). That intracellular calcium is the physiological activator of TRPM5 channels is supported by several independent observations. First, activation of TRPM5 by Gq-coupled receptors is abolished when intracellular Ca^{2+} is strongly buffered (Liu and Liman 2003; Prawitt et al. 2003) or when IP_3 receptors are inhibited with heparin (Hofmann et al. 2003). In addition, elevation of IP_3 through UV-uncaging activates TRPM5 channels, and this activation is abolished if calcium is buffered (Zhang et al. 2007). Activation of native TRPM5

channels in taste cells has also been demonstrated, and the native channel display many of the same features observed for heterologously expressed channels (Zhang et al. 2007). Overall, these data support a model for the activation of TRPM5 shown in Fig. 1c. In this model, taste receptors (or other G protein-coupled receptors) signal through PLCβ2 to release Ca^{2+} from intracellular stores, which rapidly activates TRPM5 channels (Liu and Liman 2003). This is consistent with physiological data from taste cells and with results from targeted deletion of taste transduction molecules (Akabas et al. 1988; Hwang et al. 1990; Bernhardt et al. 1996; Wong et al. 1996; Ogura et al. 1997, 2002; Huang et al. 1999; Zhang et al. 2003).

Ca^{2+} signals can be generated from a number of different sources, and they can vary in magnitude and temporal properties (Hille 2001). For example, release of Ca^{2+} through ryanodine receptors generates a rapid elevation of local Ca^{2+} ("spark") that can reach levels as high as 20–30 μM, a concentration that is able to activate closely opposed plasma membrane Ca^{2+}-activated K^+ channels (Wellman and Nelson 2003). On the other hand, global changes in Ca^{2+} concentration rarely exceed 1 μmol and can last for many seconds (Hille 2001). In understanding how TRPM5 channels are gated under physiological conditions, two questions must be answered: (1) Are the channels localized in close proximity to a Ca^{2+} source? (2) How sensitive to Ca^{2+} is the gating of the channels? As an answer to the first question, we know that TRPM5 channels are distributed across the entire plasma membrane of taste cells (Perez et al. 2002; Zhang et al. 2007; Kaske et al. 2007) (Fig. 1a). This is in striking contrast to the distribution of the pheromone-transduction channel TRPC2 which is localized to sensory microvilli of vomeronasal sensory neurons (Liman et al. 1999). The IP_3 receptor and PLCβ2 show a similarly diffuse expression pattern in taste cells (Clapp et al. 2001), and therefore it is conceivable that the three molecules are localized in a signaling complex, like that which organizes signaling components of fly phototransduction (Montell et al. 2002).

Determination of the Ca^{2+} sensitivity of TRPM5, and of the related channel TRPM4, has been more difficult than might be expected, and there is a great deal of variation in the values for half activation of the channels by Ca^{2+} reported by different groups. This may be in part due to the fact that the Ca^{2+} sensitivity of these channels is subject to modulatory influences that are not completely understood. Perhaps the most robust and reproducible way to measure intracellular Ca^{2+} sensitivity is in inside-out patches (Fig. 2a). In this mode, immediately after patch excision, TRPM5 channels are activated by intracellular Ca^{2+} with an EC_{50} of 20–30 μM (Liu and Liman 2003; Ullrich et al. 2005). A similar value (8 μM) was measured for the activation of native TRPM5 channels in taste cells (Zhang et al. 2007). Ca^{2+} sensitivity decreases over time, possibly as a result of loss of PI(4,5)P_2 from the channels, to 80 μM (Liu and Liman 2003). Under the same conditions, the structurally related channel TRPM4 is five times less sensitive to activation by intracellular Ca^{2+} (Zhang et al. 2005; Ullrich et al. 2005). The low sensitivity of TRPM5 channels in this recording mode argues that to be activated by

physiological stimuli, the channels are most likely localized in close proximity to a Ca^{2+} source.

Somewhat mysteriously, the sensitivity of TRPM5 channels to activation by Ca^{2+} in whole cell recording mode is several orders of magnitude higher than it is in excised inside-out patches. While dose–response data is more difficult to obtain in this mode due to rundown of the current (Fig. 2c) and the need to use population data, there is nonetheless general consensus that TRPM5 can be near-maximally activated by intracellular dialysis of 1 μmol Ca^{2+} (Ullrich et al. 2005; Prawitt et al. 2003) [but see also Hofmann et al. (2003)]. This might reflect the loss of a factor that enhances the sensitivity of the channels to Ca^{2+}. In addition, it is possible that perfusion of the cells with relatively low concentrations of Ca^{2+} elicits Ca^{2+} release in the vicinity of the TRPM5 channels, which further augments their activation. The high sensitivity to Ca^{2+} of TRPM5 channels in whole cell recoding mode could be used to argue that the channels detect global changes in Ca^{2+} (Prawitt et al. 2003).

3.2 Ion Selectivity

TRPM5 channels show little discrimination among the monovalent cations Na^{+}, K^{+}, and Cs^{+} and do not conduct divalent cations (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003). It is probably not coincidence that the two Ca^{2+}-activated TRP channels are also the only TRP channels that are impermeable to Ca^{2+}. The structural basis for the differential Ca^{2+} permeability of TRP channels is not known. In a detailed set of experiments, residues in the putative pore of TRPM4 were changed to the corresponding residues in a Ca^{2+}-permeable TRP channel, TRPV6, and this conferred moderate Ca^{2+} permeability to the chimeric channel (Nilius et al. 2005a). However the fact that these authors were not able to identify a mutant that could confer more substantial Ca^{2+} permeability suggests that multiple residues or regions of the channel contribute to this process. Nonetheless, these experiments have provided experimental evidence that the region between the fifth and sixth transmembrane domain of TRPM4, and by homology TRPM5, contains the pore of the channel (Owsianik et al. 2006).

3.3 Unitary Properties

Single TRPM5 channels expressed in heterologous cells show a conductance of ~16–25 pS (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003). A similar conductance (17–21 pS) was measured for native TRPM5 channels in mouse taste cells (Zhang et al. 2007). Channel openings are short lived and flickery (Liu and Liman 2003; Zhang et al. 2007), which has precluded a detailed analysis of gating properties. This is in contrast to the long-lived openings of TRPM4 channels (bursts can last several seconds) (Launay et al. 2002; Zhang et al. 2005) and may serve as a defining feature in categorizing native channels.

3.4 Voltage-Dependent Activation

Although the activation of TRPM5 requires elevated Ca^{2+} levels, gating of the channel is also strongly affected by voltage (Hofmann et al. 2003; Liu and Liman 2003; Talavera et al. 2005). This is apparent in the outward rectification of TRPM5 currents in response to a voltage ramp, despite a linear *I–V* for the single channel conductance, and in the time-dependent relaxation of the current following a voltage step (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003; Talavera et al. 2005). Voltage-dependent activation has also been reported for TRPM8 and TRPM4 and thus may be a common feature of TRPM channels (Hofmann et al. 2003; Nilius et al. 2003, 2005b; Rohacs et al. 2005). Why should channels whose primary role is to transduce sensory signals be voltage dependent? While the answer to this question is not known, it has been hypothesized that the weak voltage dependence of these channels allows their gating to be easily modulated (Nilius et al. 2005b), a hypothesis that is supported by work on cold regulation of TRPM8, TRPV1, and TRPM5 (Voets et al. 2004; Talavera et al. 2005), decavanadate modulation of TRPM4 (Nilius et al. 2004), and PI(4,5)P_2 regulation of TRPM4 and TRPM8 (Rohacs et al. 2005; Zhang et al. 2005) (see Sect. 4.2). At present the structural mechanism of voltage sensing of any of the TRP channels is not known. The fourth transmembrane of these channels contains several charged residues which might act as the voltage sensor, by analogy to voltage activation of K^+ channels (Nilius et al. 2005b; Jiang et al. 2003).

3.5 Lipid Regulation

TRPM5 currents rapidly desensitize after activation (Hofmann et al. 2003; Liu and Liman 2003; Prawitt et al. 2003), a process that may play a role in the sensory adaptation of taste cells. In whole cell recording mode, rundown is observed following dialysis of intracellular Ca^{2+} (Fig. 2c), and similar rundown is observed in perforated patch recording following activation by bath-applied Ca^{2+} ionophore, arguing that rundown is not due to washout of signaling components (Liu and Liman 2003). In excised inside-out patches, rundown of TRPM5 currents is accompanied by both a change in the Ca^{2+} sensitivity and in the maximal magnitude of the current (Liu and Liman 2003). The phosphoinositide PI(4,5)P_2 has emerged as an important cofactor in the activation of ion channels, and hydrolysis of PI(4,5)P_2 has been proposed to underlie rundown of many types of PI(4,5)P_2-sensitive ion channels (Suh and Hille 2005). PI(4,5)P_2 is likewise a cofactor for the activation of TRPM5 (Liu and Liman 2003). Exogenous PI(4,5)P_2 enhances both the Ca^{2+} sensitivity and magnitude of TRPM5 currents following rundown, but is ineffective prior to rundown suggesting that loss of this signaling molecule underlies desensitization (Liu and Liman 2003). Another lipid that appears to regulate TRPM5 is arachidonic acid (Oike et al. 2006). Arachidonic acid may be generated in taste cells from DAG by the actions of PLA2-IIA which is expressed in

these cells, although there is presently no evidence that this pathway contributes to or is required for taste sensation.

3.6 Temperature Modulation of TRPM5

TRPM5 is structurally related to the cold-activated channel TRPM8 and more distantly related to heat-activated TRPV channels, suggesting the possibility that its activity is also thermal sensitive. Indeed warm temperatures promote activation of TRPM5, similar to the effects of heat on the TRPV channels (Talavera et al. 2005). An elegant theoretical framework has been developed to explain heat and cold activation of TRP channels which postulates that their extreme thermal sensitivity derives from their small voltage dependence (Nilius et al. 2005b). Heat acts by shifting the midpoint for voltage-dependent activation to negative voltages for TRPV1 and TRPM5 and positive voltages for TRPM8, leading to opposing thermal sensitivities of the channels (Voets et al. 2004; Talavera et al. 2005). However, unlike TRPV1, heat is not sufficient to activate TRPM5, which even at warm temperatures requires elevated Ca^{2+} (Talavera et al. 2005). The thermal sensitivity of TRPM5 suggests that sensation of bitter, sweet, and umami tastes might be reduced at cold temperatures. Electrophysiological recordings from mice indeed show that sweet taste is highly sensitive to temperature, although bitter and umami are unaffected (Talavera et al. 2005). Thus while these data support a role for TRPM5 in the thermal sensitivity of sweet taste, there are likely other factors that contribute to the thermal sensitivity of this process.

3.7 Pharmacology

Blockers of TRPM5 have the potential to alter taste sensation, and therefore the identification of these molecules is of great interest. One molecule that blocks TRPM5 is the bitter chemical, quinine, which is known to be a general inhibitor of ion channels. Quinine blocks TRPM5 with an EC50 of 50 μmol. The block of TRPM5 by quinine predicts that co-delivery of quinine with an agonist of the sweet receptors, which also act upstream of TRPM5, will blunt the sweet response. This is indeed what is observed, leading to the conclusion that TRPM5 is a locus for interaction between sweet and bitter tastes (Talavera et al. 2008). In a directed high-throughput screen for specific blockers of TRPM5, the chemical triphenylphosphine oxide, TPPO, was identified (Palmer et al. 2010). TPPO blocks human TRPM5 with an IC50 of 12 μmol and murine TRPM5 with an IC50 of ~30 μmol. A related compound, triphenylphosphine, was without effect, indicating that the oxygen is absolutely required for the block. TPPO appears to be specific for TRPM5, and no effect of TPPO on the activity of TRPA1, TRPV1, or TRPM4 was observed (Palmer et al. 2010).

TRPM5 is sensitive to extracellular pH level below 7.0 and is completely blocked by pH 5.9 (Liu et al. 2005). By comparison, TRPM4 is insensitive to pH

levels as low as 5.4. Two residues account for most of the pH sensitivity of TRPM5—a glutamate residue in the S3–S4 linker and a His residue in the pore region (S5–S6 linker) (Liu et al. 2005). It is tempting to speculate that acid block of TRPM5 may play a functional role in taste sensation, possibly decreasing responses of taste cells to activation by bitter, sweet, or umami when consumed at acid pH.

4 Physiological Function

4.1 Taste Cells

A major advance in understanding the physiological significance of TRPM5 came with the discovery that its expression is high in the subset of taste receptor cells that mediate bitter, sweet, and umami taste (Perez et al. 2002; Zhang et al. 2003). There are five modalities of taste, of which three, bitter, sweet, and umami, are mediated by G protein-coupled receptors that bind their respective tastant (Lindemann 2001; Margolskee 2002). These receptors activate the G protein gustducin and phopholipase C (PLC) β2 (Lindemann 2001; Margolskee 2002), thereby initiating an intracellular signaling cascade that leads to membrane depolarization and release of the neurotransmitter ATP. TRPM5 is co-expressed with receptors for all three taste qualities and with gustducin and PLCβ2 indicating that it is part of the signaling pathway (Perez et al. 2002; Zhang et al. 2003) (Fig. 1a). This is supported by patch clamp recording from taste cells, which shows that a TRPM5-dependent current is activated by the elevation of intracellular IP_3 or Ca^{2+} by UV-uncaging (Zhang et al. 2007). Moreover, membrane depolarization and ATP release by taste cells in response to tastants are dependent on a functional TRPM5 gene (Huang and Roper 2010). TRPM5 is required for normal taste, as $TRPM5^{-/-}$ mice are dramatically less sensitive to bitter, sweet, and umami, although they retain their ability to detect sour and salty (Zhang et al. 2003). More recently it has been shown that TRPM5 may also be an element in the transduction of fat and high-salt tastes (Liu et al. 2011; Oka et al. 2013; Ren et al. 2013).

4.2 Gut and Other Chemosensory Tissues

TRPM5 is now recognized to be expressed in the small intestine and stomach, where it may play a role in post-ingestive chemosensation (Perez et al. 2002; Kokrashvili et al. 2009). Interestingly, TRPM5 is expressed in a type of enteroendocrine cell in the duodenum that expresses the endogenous opioids, beta-endorphin and Met-enkephalin. Beta-endorphin appears to be released in response to hypertonic stimuli, and release is dependent on an intact TRPM5 gene. The mechanism by which TRPM5 is activated in this context and how signaling by these cells acts to regulate digestive function remains to be determined. TRPM5 has also been found to play a role, downstream of GPCRs, in linoleic acid-induced release of cholecystokinin by enteroendocrine cells (Shah et al. 2011).

Within the respiratory and olfactory epithelium, TRPM5 is expressed in at least two morphologically and functionally distinct populations of cells. In solitary chemoreceptors, it may function downstream of bitter receptors to detect inhaled irritants (Lin et al. 2008a, b). Goblet cells of the respiratory tract secrete Mucin 5AC, and secretion is dependent on a functional TRPM5 channel (Mitrovic et al. 2013). The subset of olfactory neurons that express TRPM5 appears to be specialized to detect pheromones and other semiochemicals (Lin et al. 2007).

4.3 Pancreatic β-Cells

TRPM5 is also expressed in pancreatic β-cells, where it plays a role in insulin secretion (Prawitt et al. 2003; Brixel et al. 2010; Colsoul et al. 2010). Insulin secretion is initiated when rising levels of glucose in the bloodstream cause its uptake into beta cells, producing a change in the ATP/ADP ratio. Elevated levels of ATP cause a block of a specific class of potassium channel (KATP) composed of KIR/SUR subunits, which depolarizes the membrane potential (Ashcroft et al. 1984; Cook and Hales 1984). Voltage-gated calcium channels open upon depolarization, leading to an elevation of intracellular Ca^{2+}. From there, Ca^{2+} levels and the membrane potential oscillate, which drives pulsatile secretion of insulin (Gilon et al. 1993). In addition to the known conductances, it has been hypothesized that there must be Na^{+}-permeable "background" current in β-cells to drive membrane depolarization upon closure of KATP channels (Ashcroft and Rorsman 1989). Ca^{2+}-gated Na^{+}-permeable currents have been described in pancreatic beta cells and in related cell lines where they are referred to as Ca-NS (Sturgess et al. 1987). Candidates to mediate Ca-NS and to regulate insulin secretion include TRPM4, TRPM2, and TRPM5. However, animals that carry a targeted deletion of TRPM4 do not have any defects in glucose tolerance or insulin secretion (Vennekens et al. 2007). In contrast, two groups have reported that TRPM5 channels are essential for normal insulin release. In response to glucose challenge by i.p. injection (IPGTT) or by oral administration (OGTT), $TRPM5^{-/-}$ animals show both a decrease in insulin secretion and a reduced glucose clearance (tolerance) as compared with WT mice (Brixel et al. 2010; Colsoul et al. 2010). Corroborating this observation, isolated islets from $TRPM5^{-/-}$ animals show a reduced level of insulin release to glucose challenge. The involvement of TRPM5 in insulin secretion predicts that genetic variation in the gene could contribute to the propensity to develop diabetes, a possibility that has received some support (Ketterer et al. 2011).Thus, it can be concluded that TRPM5 is part of the network of finely tuned conductances that contribute to insulin secretion and that it is a potential drug target for the control of diabetes (Colsoul et al. 2011; Liman 2010).

A Bittersweet Conclusion

The identification of a robust mechanism for the activation of TRPM5 in heterologous cells has facilitated the discovery of basic features of the channel and novel regulatory mechanisms that are likely to be of physiological

significance. PI(4,5)P_2 hydrolysis has been proposed to play an important role in desensitization of TRPM5 and may mediate sensory adaptation of taste. Thermal sensitivity of TRPM5 has been shown to contribute to the temperature dependence of sweet sensation, and acid inhibition of TRPM5 may also modulate sensory responses to taste. Finally, in the future we can look forward to structural information that will allow the design of rational chemicals to block or enhance TRPM5 function and thereby remove some of the bitterness or enhance some of the sweetness of pharmaceuticals and foods we consume.

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TRPM6

Vladimir Chubanov and Thomas Gudermann

Contents

Abstract

TRPM6 is a bifunctional protein comprising a TRP cation channel segment covalently linked to an α-type serine/threonine protein kinase. TRPM6 is expressed in the intestinal and renal epithelial cells. Loss-of-function mutations in the human *TRPM6* gene give rise to hypomagnesemia with secondary hypocalcemia (HSH), suggesting that the TRPM6 channel kinase plays a central role in systemic Mg^{2+} homeostasis. In contrast, *Trpm6* null mice show a delay in prenatal development, neural tube defects, and prenatal death. Possible functions of TRPM6 in prenatal and adult organisms will be discussed in this chapter.

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TRPM6 • TRPM7 • Magnesium • Epithelial cells

1 Gene

*T*ransient *r*eceptor *p*otential cation channel, subfamily *M*, member *6* (TRPM6) is a member of a large gene family of TRP cation channels (Nilius and Owsianik 2011; Nilius et al. 2007; Ramsey et al. 2006; Venkatachalam and Montell 2007). In mammals, the melastatin-related subfamily comprises eight genes (*TRPM1–8*, respectively) which were named after the first cloned member, melastatin or TRPM1. TRPM6 is also known as channel kinase 2 (Chak2) due to the presence of an α-type kinase domain fused to the C-terminal end of the TRP channel segment (Ryazanov 2002). TRPM channels are highly conserved throughout the animal kingdom (Hofmann et al. 2010; Mederos y Schnitzler et al. 2008). However, only TRPM6 and TRPM7 (or channel kinase 1, Chak1) are known ion channels covalently linked to α-kinase domains. α-Kinases are atypical serine/threonine protein kinases that have no obvious amino acid sequence homology to conventional protein kinases (Ryazanov et al. 1988, 1997). Mammals have six distinct proteins containing α-kinase domains including the relatively well-studied elongation factor 2 kinase (Ryazanov et al. 1988, 1997).

The human *TRPM6* gene (NCBI ID 140803) is located on chromosome 9 (9q21.13) and comprises 39 exons (Fig. 1). Its orthologs are found in all vertebrate species, including genetically tractable organisms *Mus musculus* (ID 225997), *Rattus norvegicus* (ID 293874), and *Danio rerio* (ID 100149353).

2 Expression

The human gene expresses multiple mRNA isoforms (Table 1). For instance, alternative 5′ exons of the gene can be spliced in-frame to a common second exon resulting in three full-length mRNA variants, *TRPM6a*, *TRPM6b*, and *TRPM6c* (Table 1, Fig. 1) (Chubanov et al. 2004). In addition, several alternatively spliced transcripts lacking exons coding for the channel segment have been cloned. Accordingly, these gene products were named M6-kinases 1, 2, and 3 (Table 1) (Chubanov et al. 2004). Finally, a testis-specific variant *TRPM6t* has been identified. This gene product contains an alternative exon 36B, which harbors a STOP codon and, therefore, encodes a channel subunit devoid of the kinase domain (Table 1) (Chubanov et al. 2004). So far, the human variant *TRPM6a* was mainly studied in recombinant expression systems, whereas functional properties of the other TRPM6 gene products remain unknown.

Several independent studies characterized the expression pattern of TRPM6 in different tissues. Thus, TRPM6 expression in rat organs was assessed by RT-PCR

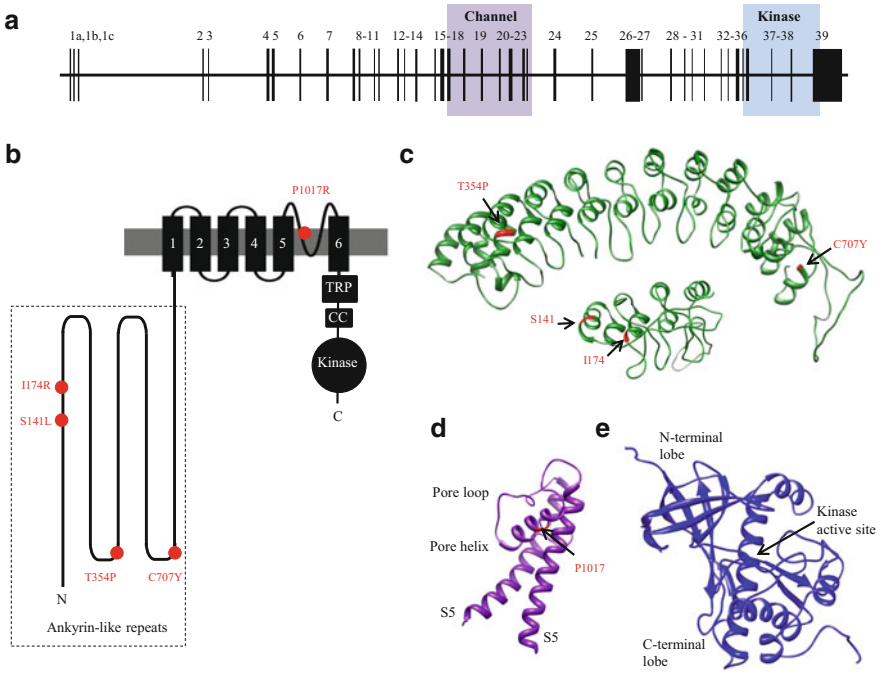

Fig. 1 Human *TRPM6* gene encodes a kinase-linked TRP channel subunit. (**a**) Intron-exon structure of the human *TRPM6* gene. The coding sequence of *TRPM6* comprises 39 exons (numbered *1–39*) including three alternative first exons (*1a*, *1b*, and *1c*) (Chubanov et al. 2004). *Magenta* and *blue* backgrounds highlight exons encoding the channel and kinase domains, respectively. (**b**) Domain topology of TRPM6 protein. A large N-terminus of TRPM6 harbors predicted ankyrin-like repeats. The channel segment of TRPM6 comprising six transmembrane helices (*1–6*) is fused to the kinase domain (*kinase*). Positions of five missense mutations identified in HSH families are indicated by *red dots*. (**c**) A three-dimensional (3D) model of two N-terminal segments (residues 406–783 and 119–268 of human TRPM6) was generated by MODELLER (Yang et al. 2011) and UCSF Chimera (Yang et al. 2011) using the annotated coordinates of the human ankyrinR protein [PDB code 1N11 (Michaely et al. 2002)] and "idealized" ankyrin repeat protein [PDB code 1N0R (Mosavi et al. 2002)], respectively. Positions of four HSH missense mutations are indicated by *red*. (**d**) 3D model of the pore-forming region TRPM6 was generated as reported previously (Chubanov et al. 2007). A short stretch between transmembrane helices 5 (*S5*) and 6 (*S6*) contains a putative pore-forming loop and pore helix. A position of P1017 affected in HSH is indicated in *red*. (**e**) 3D model of the TRPM6 kinase domain generated as described in (**c**) using annotated coordinates of the mouse TRPM7 kinase [PDB code 1IA9 (Yamaguchi et al. 2001)]. Similar to TRPM7, the TRPM6 kinase harbors a catalytic site in the cavity formed by N-terminal and C-terminal lobes

(Schlingmann et al. 2002). TRPM6 transcripts were detected in the intestine and kidney. Further experiments on microdissected rat nephrons revealed high expression levels of TRPM6 in the distal convoluted tubule (DCT) and low levels in the proximal tubule and collecting duct. Expression of human *TRPM6* was also assessed by in situ hybridization (ISH) (Schlingmann et al. 2002). In line with

Table 1 Known human TRPM6 isoforms

Name	Differences in splicing	ORF, amino acids	NCBI accession number
TRPM6a	Alternative first exon 1a	2,022	AY333282
TRPM6b	Alternative first exon 1b	2,017	AY333283
TRPM6c	Alternative first exon 1c	2,017	AY333284
TRPM6t	Testis-specific variant containing an exon 36B harboring a STOP codon	1,943	AY333285
M6 kinase 1	In-frame splicing of exon 14 to exon 28	973	AY333286
M6 kinase 2	In-frame splicing of exon 13 to exon 30	855	AY333287
M6 kinase 3	In-frame splicing of exon 7 to exon 34	569	AY333288

RT-PCR data, TRPM6 was detected in epithelial cells of the colon and DCT segments of the kidney (Schlingmann et al. 2002).

Northern-blot analysis of multiple human tissues showed that TRPM6 is highly expressed in colon, kidney, and testis, whereas a faint signal was detected in lung and leukocytes (Walder et al. 2002). Northern-blot experiments using RNA samples isolated from normal mouse embryos revealed high TRPM6 mRNA expression at embryonic day 4.5 (e4.5) and moderate levels of TRPM6 transcripts from e5.5 to 14.5 (Walder et al. 2009).

Immunolocalization of mouse TRPM6 using a polyclonal TRPM6-specific antibody demonstrated that the protein is predominantly localized at the apical surface of DCT cells in the kidney and in the brush border of epithelial cells in the duodenum (Voets et al. 2004). It has been suggested that TRPM6 protein levels on the apical surface of DCT cells are tightly regulated by blood EGF levels (Groenestege et al. 2007; Thebault et al. 2009).

RT-PCR and Western-blot approaches were also used to monitor expression of TRPM6 in HEK 293 cells, small cell lung carcinoma cells H69 and H510, human bronchial epithelial cells (BEAS-2B), human bronchial smooth muscle cells (Chubanov et al. 2004), primary mouse smooth muscle cells (Touyz et al. 2006), human breast cancer cells MCF-7 (Guilbert et al. 2009), mammary epithelial cells HC11 (Wolf et al. 2009), intestinal epithelial cells Caco2 (Gouadon et al. 2012), rat renal epithelial cells NRK-52E (Ikari et al. 2010), rat hepatocellular cells RLC-18 (Lam et al. 2012), primary human monocytes (Wuensch et al. 2010), and mouse trigeminal and dorsal root ganglia (Vandewauw et al. 2013). To summarize, TRPM6 appears to be predominantly expressed in transporting epithelia of the kidney and intestine; however TRPM6 transcripts are also detected in other tissues and in a growing number of cell lines.

A recent study suggests that EGF upregulates TRPM6 mRNA expression (Ikari et al. 2009). The promoter region responsible for EGF regulation of human TRPM6 was identified in the 5′-flanking region of the TRPM6 coding sequence. This genomic segment harbors three AP-1-binding sites which can be occupied by c-Fos and c-Jun upon stimulation of HEK293 cells by recombinant EGF (Ikari et al. 2009).

Expression levels of TRPM6 were changed in several animal models of asthma and drug-induced hypomagnesemia implying that TRPM6 plays a prominent role in acquired forms of hypomagnesemia (Bai et al. 2013; da Silva et al. 2009; Dimke et al. 2010; Famularo et al. 2013; Hodgkinson et al. 2006; Jiang et al. 2011; Lameris et al. 2013; Ledeganck et al. 2013; Perazella 2013; van Angelen et al. 2012).

3 The Channel Protein Including Structural Aspects

The full-length TRPM6 isoforms are large (2017–2022 residues, Table 1) proteins comprising multiple membrane and cytosolic domains as depicted in Fig. 1. It is commonly accepted that the overall architecture of the pore-forming segment of TRP channels is similar to that of tetrameric voltage-gated K^+ channels (Nilius et al. 2007; Ramsey et al. 2006; Venkatachalam and Montell 2007). TRP channels function as homo- as well as heterotetramers (Hofmann et al. 2002). Similar to K^+ channels, the channel segment of TRPM6 comprises six transmembrane helixes (S1–S6) (Fig. 1b). A short stretch of amino acids between S5 and S6 contains a predicted hydrophobic pore helix followed by a pore loop (Fig. 1b, d). Like in K^+ channels, it is assumed that the loops of four channel subunits contribute to a common ion selectivity filter. Functional analysis of recombinant TRPM6 and TRPM7 carrying point mutations in the predicted pore loop is consistent with this model and supports the notion that a short sequence motif within the pore loop (^{1024}EVY^{1026} in human TRPM6a and ^{1047}EVY^{1049} in mouse TRPM7) determines the permeability of divalent cations (Li et al. 2007; Mederos y Schnitzler et al. 2008).

Apart from the transmembrane channel domain, TRPM6 has large cytosolic N- and C-terminal segments (Fig. 1b). As opposed to the TRPM6 N-terminus, the architecture of the C-terminal segment is fairly well understood. Similar to most TRP channels, a highly conserved TRP domain [including the EFKWAR "TRP box" amino acid sequence (Nilius et al. 2007; Ramsey et al. 2006; Venkatachalam and Montell 2007)] is located immediately downstream of the TRPM6 S6 helix. C-terminal to the TRP domain, TRPM6 contains a coiled-coil domain (Fujiwara and Minor 2008) followed by a poorly conserved "linker sequence" [also known as the "Ser/Thr-rich" domain (Clark et al. 2008b; Matsushita et al. 2005)], which then entails the kinase moiety (Fig. 1b, e). Structural biology and biochemistry approaches showed that the isolated coiled-coil segment of TRPM7 assembles into a four-stranded antiparallel coiled-coil complex (Fujiwara and Minor 2008). Consequently, it was proposed that the coiled-coil domains govern homo- and heterotetrameric associations of full-length TRPM6 and TRPM7 subunits (Fujiwara and Minor 2008).

The kinase domain of TRPM6, like other α-kinases, displays no apparent primary sequence homology to conventional protein kinases. However, the crystal structure of the murine TRPM7 kinase domain complexed with nucleotides (ADP and AMP-PNP) revealed that the architecture of TRPM7 kinase is remarkably similar to that of conventional kinases and metabolic enzymes harboring

ATP-grasp folds (Yamaguchi et al. 2001). Furthermore, catalytically relevant residues are well conserved between TRPM7 and classical protein kinases (Yamaguchi et al. 2001). Given the high degree of primary sequence identity (77 %) between TRPM6 and TRPM7 kinases, a 3D model of the TRPM6 kinase domain can be reliably predicted, including the localization of residues contributing to the active site (Fig. 1e).

The N-terminal portion of human TRPM6 comprises more than 700 amino acid residues and is highly conserved in vertebrate and non-vertebrate TRPM channels and does not exhibit any noteworthy primary sequence homology to other domains. Nevertheless, attempts to model the 3D topology of these regions in TRPM6 or TRPM7 invariably predict the presence of multiple "ankyrin-like" repeats (Fig. 1b, c). This prediction does not come as a surprise, because many TRP channels contain multiple ankyrin repeats in similar positions (Phelps et al. 2007). Interestingly, the four point mutations identified in HSH families (Fig. 1b) appear to be located in putative helixes of the predicted ankyrin-like domains suggesting that these distinct mutations give rise to a common pathomechanism compromising TRPM6 channel assembly and function.

4 Interacting Proteins

TRPM6 appears to form functional heteromeric channel complexes together with TRPM7. At least four protein domains may be directly involved in the assembly of TRPM6/TRPM7 complexes. Thus, co-expression of wild-type TRPM7 with a TRPM6 variant carrying a dominant-negative point mutation in its pore-forming region (P1017R, Fig. 1b) resulted in a suppression of TRPM7 currents, indicating that the transmembrane segments of TRPM6 and TRPM7 contribute to a common channel pore (Chubanov et al. 2007). Another point mutation, S141L in the intracellular N-terminus of human TRPM6 (S138L in mouse TRPM7), disrupts the assembly of heteromeric TRPM6/TRPM7 complexes underscoring a critical role of the N-terminus for the formation of functional channel complexes (Chubanov et al. 2004). In addition, it was postulated that the coiled-coil domains of TRPM7 and TRPM6 are directly involved in the assembly of the channel-kinase complexes (Fujiwara and Minor 2008). Finally, structural and biochemical experiments revealed that the kinase domains of TRPM6 and TRPM7 form functional homo- and heterodimers and that such dimerization is required for kinase activity (Crawley and Cote 2009; Fujiwara and Minor 2008).

Little is known about accessory subunits of TRPM6 (updated information is available at http://www.trpchannel.org/summaries/TRPM6). The kinase domain of TRPM6 may interact with the repressor of estrogen receptor activity (REA), the receptor of activated protein kinase C 1 (RACK1), and methionine sulfoxide reductase B1 (MsrB1) (Cao et al. 2008, 2009, 2010). Co-expression of recombinant REA, RACK1, and MsrB1 with human TRPM6 resulted in modest suppression of TRPM6 channel activity (Cao et al. 2008, 2009, 2010).

5 Biophysical Aspects of Channel Function, Permeation, and Gating

TRPM7 was one of the first TRPM channels to be functionally characterized in heterologous expression systems where it behaves as a constitutively active cation channel highly permeable to a broad range of divalent cations including Ca^{2+} and Mg^{2+} (Monteilh-Zoller et al. 2003; Nadler et al. 2001; Runnels et al. 2001; Schmitz et al. 2003). Intracellular and extracellular Mg^{2+} tightly regulates TRPM7 channel activity. External Mg^{2+} is a permeant blocker of TRPM7. Internal Mg^{2+} (as well as Mg^{2+}-ATP) inhibits TRPM7 via a nucleotide-binding site in the kinase domain synergistically with another as yet unidentified Mg^{2+}-binding site extrinsic to the α-kinase moiety. TRPM7 currents are also modulated by PIP_2, intra- and extracellular pH, and other factors (Demeuse et al. 2006; Jiang et al. 2005; Li et al. 2006; Nadler et al. 2001; Runnels et al. 2001, 2002; Schmitz et al. 2003).

The functional characteristics of recombinant TRPM6 were described controversially. Two groups used several expression systems to provide evidence to show that TRPM6 displays distinct features as compared to TRPM7 (Chubanov et al. 2004, 2007; Schmitz et al. 2005). Specifically, the researchers noted that TRPM6 does not efficiently form homomultimeric channel complexes in the plasma membrane, but requires TRPM7 to be co-targeted to the cell surface (Chubanov et al. 2004, 2007; Schmitz et al. 2005). Within the heterooligomeric channels formed, TRPM6 alters the biophysical properties of the heteromultimer as compared to homomeric TRPM7 channel properties (Chubanov et al. 2004, 2007; Li et al. 2006; Schmitz et al. 2005). Interestingly, transient expression of human TRPM6 specifically using the pCINeo-IRES-GFP vector allows to detect functional homooligomers with biophysical characteristics similar to TRPM7 channel (Li et al. 2006; Voets et al. 2004; Xie et al. 2011). However, TRPM6 was found to be slightly more permeable to Mg^{2+} than to Ca^{2+} and, like TRPM7, sensitive to intracellular Mg^{2+}, ATP, $PI(4,5)P_2$, hydrogen peroxide, 17β-estradiol, and sphingosine (Cao et al. 2010; Li et al. 2006; Qin et al. 2012; Voets et al. 2004; Xie et al. 2011). Furthermore, insulin and FGF acting via Rac1 may increase the cell surface expression of recombinant TRPM6 (Nair et al. 2012; Thebault et al. 2009). It remains unknown why different heterologous expression systems yield disparate results.

6 Physiological Functions in Native Cells, Organs, and the Organism

The expression of TRPM6 is confined to a few cell types, while TRPM7 is a ubiquitously expressed protein. Thus, on a cellular level, TRPM6 always coexists with TRPM7, implying that a lack of TRPM6 results in either quantitative or qualitative alterations of TRPM7 function. To this end, the functional interplay of TRPM6 channel and kinase activities with those of TRPM7 in native cells is a central issue in the field.

6.1 Channel Activity

Endogenous divalent cation-selective TRPM7-like currents have been detected in all cell types examined. These ubiquitous currents were referred to as *m*agnesium-*i*nhibited *c*urrents (MIC) or *mag*nesium *nu*cleotide-regulated *m*etal ion currents (MagNuM) (Kozak et al. 2002; Nadler et al. 2001; Prakriya and Lewis 2002). There are only two systematic attempts to delineate the role of TRPM6 in endogenous MIC/MagNuM currents. In one study, Li et al. (2006) took advantage of immortalized mouse distal convoluted tubule (MDCT) cells, a broadly used in vitro model of renal DCT. MDCT cells co-express TRPM6 and TRPM7 and elicit characteristic MIC/MagNuM currents (Li et al. 2006). Accordingly, Li et al. (2006) attempted to dissect the role of TRPM6 and TRPM7 for these currents using 2-aminoethoxydiphenyl borate (2-APB). Recombinant TRPM7 channel can be blocked by micromolar concentrations of 2-APB, while the activity of recombinant TRPM6 was found to be enhanced by 2-APB irrespective of the concentration administered. However, micromolar concentrations of 2-APB could fully suppress endogenous currents.

In a second study, Ryazanova et al. (2010) investigated mouse embryonic stem (ES) cells derived from wild-type and *Trpm7* null mutant mice. Interestingly, ES cells express both TRPM7 and TRPM6. ES cells lacking TRPM7 exhibited a proliferation arrest that could be rescued by Mg^{2+} supplementation, supporting the notion that *Trpm7* controls cellular Mg^{2+} levels. Moreover, the solitary deletion of *Trpm7* completely ablated endogenous MIC/MagNuM currents suggesting that TRPM6 cannot compensate for the absence of TRPM7 (Ryazanova et al. 2010).

6.2 Kinase Activity

Physiological substrates of the TRPM6 kinase remain unknown. Ryazanov and colleagues suggested that α-kinases prefer to phosphorylate serine/threonine residues located within α-helices, in contrast to conventional protein kinases that phosphorylate their physiological substrates within β-turns, loops, or irregular structures (Dorovkov et al. 2011; Drennan and Ryazanov 2004; Ryazanov et al. 1988, 1997). In vitro studies showed that the TRPM6 and TRPM7 kinases can phosphorylate myosin IIA, IIB, and IIC on identical residues (Clark et al. 2006, 2008a). Therefore, it is possible that the TRPM6 kinase also phosphorylates other in vitro substrates of the TRPM7 kinase, annexin A1, elongation factor 2 kinase, and phospholipase Cγ2 (PLCγ2) (Deason-Towne et al. 2012; Dorovkov and Ryazanov 2004; Perraud et al. 2010). However, it is not yet established whether the latter proteins are phosphorylated in vivo either by TRPM6 or TRPM7. Similar to TRPM7, TRPM6 kinase can autophosphorylate its own Ser/Thr residues (Clark et al. 2008b). Furthermore, upon co-expression with TRPM7, TRPM6 is able to cross-phosphorylate residues in the TRPM7 protein (Schmitz et al. 2005). Functional consequences of auto- and cross-phosphorylation processes are unclear.

7 Lessons from Knockouts

So far, there are only two studies explored genetically modified mice to elucidate a role of *Trpm6* in vivo. Thus, Walder et al. (2009) reported that *Trpm6* null mice die at e12.5. Those animals that survive beyond this stage display massive neural tube closure defects (NTD). In contrast to HSH patients, high-Mg^{2+} diet did not rescue the mortality of mutant embryos (Walder et al. 2009). The authors suggested that TRPM6 plays a direct role in neural tube closure resulting in embryonic mortality of mutant mice (Walder et al. 2009). The exact molecular mechanisms underlying NTD in *Trpm6* null mice still remain elusive.

More recently, Woudenberg-Vrenken et al. (2010) studied another *Trpm6* null mutant line and found that adult $Trpm6^{-/-}$ mice are not viable, while $Trpm6^{+/-}$ mice are characterized by moderately reduced serum Mg^{2+} levels. Unexpectedly, however, urinary Mg^{2+} excretion was unaffected in $Trpm6^{+/-}$ animals (Woudenberg-Vrenken et al. 2010). Thus, constitutive null mutations of *Trpm6* in mice resulted in unexpected phenotypic presentations suggesting that the physiological role of TRPM6 cannot be fully recapitulated from clinical symptoms of humans with loss-of-function mutations in *TRPM6*.

8 Role in Hereditary and Acquired Diseases

8.1 Hypomagnesemia with Secondary Hypocalcemia

Two groups discovered that mutations in the human *TRPM6* gene give rise to autosomal recessive hypomagnesemia with secondary hypocalcemia (HSH) (Schlingmann et al. 2002, 2005; Walder et al. 2002). HSH manifests in infancy with generalized convulsions and increased neuromuscular excitability leading to muscle spasms. Clinical assessment at the time of manifestation reveals very low serum levels of Mg^{2+} (0.1–0.3 mM) and moderately reduced Ca^{2+} concentrations (1–1.6 mM) as compared to values of healthy individuals (0.7–1.1 mM Mg^{2+} and 2.2–2.9 mM Ca^{2+}). Hypomagnesemia and other symptoms can be relieved by administration of high doses of Mg^{2+}. However, despite this treatment serum Mg^{2+} levels remain in the subnormal range (Konrad et al. 2004; Schlingmann et al. 2002, 2005, 2007; Walder et al. 2002).

However, several aspects of HSH still remain poorly understood. For instance, the mechanisms responsible for the development of hypocalcemia are unclear. Several explanations have been offered, including end organ unresponsiveness to parathyroid hormone (PTH), altered release of PTH, and impaired formation of 1,25-dihydroxy vitamin D3 (Konrad and Weber 2003; Schlingmann et al. 2005; Woodard et al. 1972). For reasons unknown several individuals homozygous for *TRPM6* mutations are asymptomatic, while other HSH patients present with additional symptoms such as mental retardation, osteoporosis, cardiac arrhythmia, severe failure to thrive, and bilateral basal ganglia calcification (Apa et al. 2008; Chubanov et al. 2004, 2007; Esteban-Oliva et al. 2009; Guran et al. 2011; Habeb

et al. 2012; Schlingmann et al. 2002, 2005; Walder et al. 2002; Zhao et al. 2013). In addition, a substantial predominance of male patients has been observed in several HSH families (Chery et al. 1994). Some authors speculated that male predominance is caused by genetic heterogeneity of the disease (Chery et al. 1994). Indeed, a high degree of consanguinity in the majority of HSH pedigrees complicates the identification of clear-cut genotype–phenotype relationships in affected individuals. Unfortunately, experiments with TRPM6 KO mice (Walder et al. 2009) could not further clarify the etiology of HSH, for instance, confirm the monogenic basis of HSH. Furthermore, studies with *Trpm6* gene-deficient mice (Walder et al. 2009) suggest that mutations in *TRPM6* may cause complications in the human fetus, thus offering an explanation for the fact that only few HSH families have been identified worldwide so far.

The large majority of HSH mutations in the *TRPM6* gene introduces stop and frameshift mutations and affects exon splicing, thus resulting in the complete lack of protein expression and a loss-of-function phenotype (Apa et al. 2008; Chubanov et al. 2004, 2007; Esteban-Oliva et al. 2009; Guran et al. 2011; Habeb et al. 2012; Schlingmann et al. 2002, 2005; Walder et al. 2002; Zhao et al. 2013). In addition, five point mutations in *TRPM6* have been described (Fig. 1b). Four of them are located in the N-terminus of the channel protein suggestive of a critical role of this domain for channel function. In fact, the $TRPM6^{S141L}$ mutant is unable to associate with TRPM7 (Chubanov et al. 2004). Consistently, introduction of the homologous mutation into TRPM7 also affects ion channel complex assembly (Chubanov et al. 2004). Another missense mutation, P1017R, is located in the putative pore-forming region of TRPM6 (Fig. 1b, d) (Chubanov et al. 2007). The latter mutation impairs channel activity of TRPM6/TRPM7 heteromers by dominant-negative suppression. This finding suggests that suppression of cation fluxes via TRPM6/7 channel-kinase complexes is sufficient for the development of HSH.

These studies support a model for the role TRPM6 in HSH as illustrated in Fig. 2. Two different Mg^{2+} transport systems exist in the kidney and intestinal epithelia: an active transcellular uptake and a passive paracellular transport pathway. Transcellular uptake consists of apical Mg^{2+} entry into epithelial cells and a basolateral extrusion step (Dai et al. 2001; Konrad et al. 2004; Quamme and de Rouffignac 2000; Schlingmann et al. 2007). In the kidney, the two transport systems are arranged sequentially along the nephron. Most of the filtered Mg^{2+} is reabsorbed in the thick ascending limb via the passive paracellular mechanisms driven by positive transepithelial voltage. Tight junctions containing claudin-16 and claudin-19 are involved in the paracellular transport. Only 5–10 % of the filtered Mg^{2+} is reabsorbed in the distal convoluted tubule (DCT) solely via the transcellular pathway. There is no Mg^{2+} reabsorption in more distal nephron segments suggesting that the DCT determines the final urinary Mg^{2+} content (Dai et al. 2001; Konrad et al. 2004; Quamme and de Rouffignac 2000; Schlingmann et al. 2007). RT-PCR and in situ hybridization and immunochemistry showed high expression levels of TRPM6 in the DCT (Schlingmann et al. 2002; Voets et al. 2004). On the contrary, TRPM7 mRNA is present at similar levels in all nephron segments examined (Chubanov et al. 2004). Thus, both ion channels

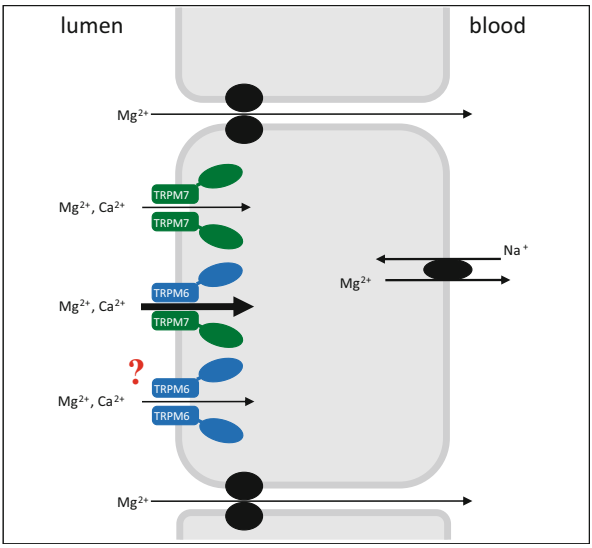

Fig. 2 Hypothetical role of TRPM6/M7 channel complexes in Mg^{2+} transport by renal and intestinal epithelial cells. For further explanation, see text

coexist in the DCT. Accordingly, at least two distinct pathomechanisms may cause HSH. Firstly, it is imaginable that TRPM6/M7 heterooligomers are the relevant molecular substrates *quantitatively* regulating Mg^{2+} uptake in DCT cells, while TRPM7 homomultimers are not sufficient for properly serving this function. Secondly, a still unidentified *qualitative* characteristic of heteromeric TRPM6/M7 complexes may be essential for DCT function that cannot be maintained by TRPM7 homomultimers (Fig. 2).

8.2 Isolated Autosomal Dominant Hypomagnesemia

Glaudemans et al. (2009) identified a new syndrome, autosomal dominant hypomagnesemia, which is caused by a point mutation (N255D) in human *KCNA1*, a gene encoding the voltage-gated potassium (K^+) channel Kv1.1. Affected individuals showed low serum Mg^{2+} levels, muscle cramps, tetanic episodes, tremor, and muscle weakness. Expression studies in HEK293 cells revealed that the $KCNA1^{N255D}$ variant is a nonfunctional channel, with a dominant-negative effect on wild-type Kv1.1 (Glaudemans et al. 2009). Kv1.1 protein was found to be colocalized with renal TRPM6 in DCT cells. In the DCT, Mg^{2+} reabsorption is primarily driven by the negative potential across the luminal membrane. Accordingly, it was proposed that Kv1.1 plays an essential role in maintaining a favorable luminal membrane potential in DCT cells and that KCNA1(N255D) reduces TRPM6-mediated Mg^{2+} reabsorption leading to hypomagnesemia (Glaudemans et al. 2009).

8.3 Isolated Recessive Renal Hypomagnesemia

Isolated recessive renal hypomagnesemia (IRH) is a new inherited form of hypomagnesemia caused by a point mutation in the *EGF* gene suggesting that EGF is a magnesiotropic hormone regulating systemic Mg^{2+} homeostasis (Groenestege et al. 2007). IRH patients present with low serum Mg^{2+} levels, mental retardation, and epileptic seizures. The identified mutation (P1070L) leads to impaired basolateral sorting of pro-EGF. Consequently, EGFR is inadequately stimulated, resulting in insufficient activation of TRPM6 and resultant Mg^{2+} loss (Groenestege et al. 2007).

8.4 Drug-Induced Hypomagnesemia

More than 40 % of the patients treated with cisplatin develop hypomagnesemia due to renal Mg^{2+} loss (Hodgkinson et al. 2006). Van Angelen et al. (2012) employed a mouse model to elucidate the mechanisms of cisplatin-induced hypomagnesemia. They found that renal mRNA levels of *Trpm6* were significantly reduced in the cisplatin-treated group supporting the notion that alterations in TRPM6 function may underline drug-induced hypomagnesemia. Ledeganck et al. (2013) used a rat model of cisplatin nephrotoxicity. It was established that the renal mRNA expression of TRPM6 and EGF showed a significant decrease after cisplatin treatment of rats, whereas levels of TRPM7, claudin-16, and EGFR transcripts remained unchanged, suggesting that a downregulation of EGF/TRPM6-dependent Mg^{2+} transport underlies the systemic depletion of Mg^{2+} in cisplatin-treated animals (Ledeganck et al. 2013).

Proton pump inhibitors (PPIs) are potent blockers of gastric acid secretion. PPIs are broadly used by patients suffering from gastric acid-related complaints. Severe hypomagnesemia is a well-documented side effect of PPIs (Bai et al. 2013; Famularo et al. 2013; Lameris et al. 2013; Perazella 2013). Since TRPM6 channel activity was found to be sensitive to extracellular protons (Li et al. 2007), it was proposed that PPI-induced alterations in luminal pH may reduce TRPM6 activity leading to hypomagnesemia (Bai et al. 2013; Famularo et al. 2013; Lameris et al. 2013; Perazella 2013).

Anticancer treatments with monoclonal antibodies (cetuximab) targeting EGF receptor (EGFR) induce hypomagnesemia in patients with colorectal cancer (Dimke et al. 2010; Groenestege et al. 2007). Recent studies suggested that EGF-induced change in TRPM6 activity is a likely mechanism of cetuximab-induced Mg^{2+} loss (Dimke et al. 2010; Groenestege et al. 2007).

8.5 TRPM6 Polymorphisms and Genome-Wide Associations

There is substantial evidence to suggest that systemic Mg^{2+} deficiency is associated with an increased risk of insulin resistance and type 2 diabetes (Sales and Pedrosa

Lde 2006). Recent studies employed genome-wide associations to test whether TRPM6 may be involved in this phenomenon. It was reported that two single nucleotide polymorphisms (SNPs) in the TRPM6 coding region, rs3750425 and rs2274924, might confer susceptibility to type 2 diabetes in women with low-Mg^{2+} intake (Song et al. 2009). Furthermore, these SNPs may be responsible for a higher likelihood of developing gestational diabetes mellitus (Nair et al. 2012). Genome-wide association studies with more than 15,000 individuals of European descent showed that rs11144134 in *TRPM6* is associated with lower serum Mg^{2+} levels (Meyer et al. 2010). Recently, genome-wide association studies to link Mg^{2+} intake and fasting glucose and insulin levels were carried out in more than 50,000 healthy Europeans (Hruby et al. 2013). The authors observed that higher Mg^{2+} intake is associated with lowered glucose and insulin concentrations, whereas rs2274924 in TRPM6 shows an association with higher glucose levels (Hruby et al. 2013). Taken together, TRPM6 emerges as a new diagnostic factor for insulin resistance and metabolic disorders and may be considered as a promising drug target.

Finally, Shuen et al. (2009) showed a significant association between estrogen receptor alpha (ESR1) polymorphisms and serum Mg^{2+} levels in line with previous studies linking physiologic changes in serum Mg^{2+} to estrogen status and TRPM6 activity (Cao et al. 2009).

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TRPM7

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Abstract

The channel kinases TRPM6 and TRPM7 are fusion proteins with an ion transport domain and an enzymatically active kinase domain. TRPM7 has been found in every mammalian tissue investigated to date. The two-in-one protein structure, the ubiquitous expression profile, and the protein's unique biophysical characteristics that enable divalent ion transport involve TRPM7 in a plethora of (patho)physiological processes. With its prominent role in cellular and systemic magnesium homeostasis, TRPM7 emerges as a key player in embryonic development, global ischemia, cardiovascular disease, and cancer.

Keywords

Magnesium • Calcium • Trace metals • Divalent cation • Transient Receptor Potential Channel • Adenosine triphosphate • Channel kinase • Magnesium homeostasis • Embryogenesis • Ischemia • Breast cancer

1 Gene

The official name of the TRPM7 gene is "transient receptor potential cation channel, subfamily M, member 7." In Homo sapiens, the TRPM7 gene is located on chromosome 15q21.2 (NCBI Gene ID 54822). Previous names for the gene include CHAK1, TRP-PLIK, and LTRPC7 (Ryazanov 2002; Runnels et al. 2001; Nadler et al. 2001). The human TRPM7 gene encodes an 1,865 amino acid protein (Schmitz et al. 2005). The molecule is unique in that it contains an ion transport domain (InterPro IPR005821) in its N-terminal section and an enzymatically active MHCK/EF2 kinase domain (InterPRo IPR004166) in the C-terminal section. Only two other mammalian genes (there are numerous channel enzymes in simple organisms) are known to code for ion channel and enzyme domain fusion proteins, namely, TRPM7's paralog genes TRPM6 and TRPM2 (Perraud et al. 2001; Schlingmann et al. 2002; Walder et al. 2002), described elsewhere in this book. TRPM7 gene orthologs with human amino acid sequence similarity of 94.3 % to 99.89 % have been identified in chimpanzee (*Pan troglodytes*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), cow (*Bos taurus*), and dog (*Canis familiaris*; GeneCards). In zebrafish (*Danio rerio*), an important model organism in biology, TRPM7 is located on chromosome 18 and shares 75 % amino acid sequence identity with the human TRPM7 gene.

Virtually nothing is known about whether the kinase and channel encoding portions of the TRPM7 gene domains can be expressed independently from each other. A splice variant in rat lacking the channel domain has been reported (Runnels et al. 2001). Human *Trpm7* has 9 predicted splice variants (http://www.ensembl.org: ENST00000561267, TRPM7-001 through TRPM7-009); however, aside from the 1,865 amino acid encoding protein, the function of any of these variants remains unexplored. Finally, no genes coding for TRPM7 auxiliary subunits are known (Chubanov et al. 2004; Schmitz et al. 2005).

2 Expression

Early investigations of human tissue and cell lines by PCR with reverse transcription indicated a ubiquitous distribution pattern of TRPM7 transcripts (Nadler et al. 2001; Runnels et al. 2001). A comprehensive quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of human tissue showed that TRPM7 is widely distributed in the central nervous system as well as in the periphery, with highest expression levels in the heart, pituitary, bone, and adipose tissue (Fonfria et al. 2006).

TRPM7 is also ubiquitously expressed across mouse organs as investigated by qRT-PCR. These data show that, compared to other members of the TRP gene family, TRPM7 is the most abundantly expressed TRP channel in the majority of adult mouse organs investigated (Kunert-Keil et al. 2006). Particularly, mouse intestine, lung, kidney, and brain have strong TRPM7 expression (Kunert-Keil et al. 2006), as well as testis (Jang et al. 2012). While TRPM7 levels can vary significantly between mouse strains (Kunert-Keil et al. 2006), they seem quite constant within a particular type of strain (Vandewauw et al. 2013). Along with TRPM2, TRPM4, and TRPM8, mouse trigeminal ganglia show very high expression of TRPM7, and this gene product has a stronger representation in dorsal root ganglia along the vertebral column compared to other members of the TRPM family (Vandewauw et al. 2013). Gene expression patterns of TRPM7 during mouse development seem to occur in two waves, peaking at embryonic day 18 (E12), raising again after postnatal day 4, and maintaining stable levels into adulthood (Staaf et al. 2010). Additional studies have confirmed TRPM7 RNA expression in adult rat prostate tissue (Wang et al. 2007) and intralobar pulmonary arterial and aortic smooth muscle (Yang et al. 2006), as well as rumen epithelial cells isolated from sheep (Schweigel et al. 2008).

The assessment of TRPM7 at the protein level has been more challenging due to the paucity of highly specific antibodies. Fortunately, due to the electrogenic nature of TRPM7's ion channel function, biophysical techniques such as whole-cell patch-clamp technique and single-channel measurements allow an estimate of the number of proteins in the plasma membrane of single live cells (Hamill et al. 1981). Endogenous TRPM7-like currents were first reported in renal cells (human HEK293), mast cells (rat RBL-2H3), and T lymphocytes (human Jurkat T) (Nadler et al. 2001). Due to the inhibition of these currents by magnesium (Mg), Mg·ATP, as well as their ability to conduct metal ions, native TRPM7-like currents were coined *mag*nesium-*nu*cleotide-regulated *m*etal ion currents [MagNuM (Nadler et al. 2001; Hermosura et al. 2002)] and also *m*agnesium-*i*nhibited *c*ation current [MIC (Kozak et al. 2002b)]. Subsequent investigation reported native currents with biophysical characteristics ascribed to TRPM7 in a wide variety of cell types, including the heart (Gwanyanya et al. 2004), brain (Aarts et al. 2003), and intestine (Kim et al. 2009).

Of the three cell types in which MagNuM currents were originally described, Jurkat T cells had the highest current density under the experimental conditions used (Nadler et al. 2001). Taking into account a single-channel conductance of

40 pS (Nadler et al. 2001; Li et al. 2006), this still amounts to only an estimated 30 active channels in the plasma membrane per T cell and 40 channels per HEK293 or RBL-2H3 cell. Subcellular location of TRPM7 protein in heterologous overexpression systems is to be expected (Chubanov et al. 2007), and evidence exists of native functional subcellular location in synaptic vesicles of sympathetic rat neurons (Krapivinsky et al. 2006), in tubulovesicular structures (Oancea et al. 2006), and reticular formations of vascular smooth muscle cells (Yogi et al. 2009).

3 The Channel Protein Including Structural Aspects

In analogy to other members of the TRP channel family, TRPM7 monomers are thought to form tetrameric units, modeled after voltage-gated potassium (K) channels (Mederos y Schnitzler et al. 2008; Jiang et al. 2003; Chubanov et al. 2007). The 1,865 amino acids of a human TRPM7 subunit can be subdivided into distinct domains with variable homology to TRPM7 subunits identified in other species. Four unique melastatin amino-terminal regions are linked to the 6 putative transmembrane spanning helices (Nadler et al. 2001), with the putative pore region linking segments 5 and 6. The "TRP box," unique to all identified TRP ion channels, is a highly conserved and proline-rich 24 amino acid region C-terminal to the transmembrane domains (Venkatachalam and Montell 2007), followed by a cytoplasmic coiled-coil (CC) domain thought to underlie channel assembly and trafficking (Fujiwara and Minor 2008). The CC domain is predicted to have a four-stranded antiparallel arrangement which seems aligned with the dimer-forming atypical α-kinase domain architecture just C-terminal of the CC (Fujiwara and Minor 2008). The latter is indeed one of the unique features of TRPM7, which it shares with its paralog TRPM6: the fusion of an N-terminal functional ion channel and a C-terminally located and active serine/threonine protein kinase (Ryazanov 2002; Runnels et al. 2001; Nadler et al. 2001). Activation of caspase leads to separation of TRPM7's kinase domain from the channel without affecting the functionality of the kinase but enhancing ion channel activity (Desai et al. 2012).

Both the CC and α-kinase domain are currently the only two TRPM7 regions where X-ray crystallography has provided structural information (Yamaguchi et al. 2001; Fujiwara and Minor 2008). The kinase domain's 300 amino acid residues fold into a cleft-forming structure containing the active ATP-binding site. In addition, one zinc (Zn) and two Mg binding sites have been reported. Sequence analysis reveals little primary amino acid sequence similarity between catalytic domains of conventional protein kinases and TRPM7's atypical kinase despite an overall similarity in the folding structure (Drennan and Ryazanov 2004; Yamaguchi et al. 2001). Analysis of truncation mutants using mouse TRPM7 identified two important regions in the kinase domain: residues 1553 to 1562 are essential for kinase phosphorylation activity and residues 1563 to 1670 are needed for dimer assembly (Crawley and Cote 2009).

Mass spectrometric proteomic techniques have been used on mouse and human TRPM7 to identify key phosphorylation sites. This resulted in the confirmation as well as new identification of several phosphorylation sites that are all located on the cytoplasmic C-terminus (Kim et al. 2012; Madsen et al. 2012; Matsushita et al. 2005): 3 in the CC region, 7 in a serine-threonine-rich (Ser/Thr) domain (Matsushita et al. 2005), and two P-sites of unknown function distal to the kinase domain (Kim et al. 2012). Furthermore, phosphomapping by mass spectrometry identified 47 autophosphorylation sites on TRPM7, the majority of which are located in the Ser/Thr-rich domain N-terminal of the kinase region. This part of the TRPM7 region is thought to control kinase substrate binding (Clark et al. 2008c).

4 Interacting Proteins

Information on proteins interacting with TRPM7 remains scarce, even for the kinase domain. A yeast two-hybrid screen of a rat library identified phospholipase C (PLC) as interacting partner of the TRPM7 kinase (Runnels et al. 2001). Subsequent work showed that receptor-stimulated activation of PLC causes inhibition of TRPM7 channel activity through localized phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (Runnels et al. 2002). Furthermore, hypomagnesemic conditions increase TRPM7-kinase-regulated Ser/Thr phosphorylation in the C2 domain of PLCγ2, leading to reduced Ca signaling (Deason-Towne et al. 2012).

Involvement of TRPM7 kinase in cell motility and adhesion has been linked to its ability to phosphorylate the assembly domains of non-muscle myosin IIA, IIB, and IIC and ATP-dependent motor proteins involved in actomyosin-based cell motility (Clark et al. 2006, 2008a, b). Annexin A1, a Ca-dependent membrane-binding protein with the ability to promote membrane fusion, is also phosphorylated by the TRPM7 kinase, providing a possible link to TRPM7's known involvement in cell growth and apoptosis (Dorovkov and Ryazanov 2004; Dorovkov et al. 2011). The TRPM7 kinase also mediates enhanced Thr phosphorylation at residue 56 of the eukaryotic elongation factor 2 (eEF2) through eEF2 kinase (Perraud et al. 2011). This specifically occurs under reduced dietary Mg and has been suggested to adjust protein translation rates to the availability of this important divalent ion.

To date, TRPM6 is possibly the best understood TRPM7-interacting protein as assessed in heterologous expression systems (Runnels 2011). Interestingly, recombinant and native TRPM6 seems to require TRPM7 for plasma membrane surface expression in mouse embryonic stem (ES), DT40, HEK293 cells, and *Xenopus* oocytes (Schmitz et al. 2005; Ryazanova et al. 2010; Chubanov et al. 2004, 2007), indicating that TRPM6 is inefficient in forming functional homomeric ion channels on its own. While this topic still remains somewhat controversial, supporting observations show that overexpression of TRPM6 cannot rescue cell growth arrest in chicken DT40 B cells lacking the TRPM7 protein (Schmitz et al. 2005) and, in

contrast to TRPM7, cannot alter motility and proliferation of HEK293 (Chubanov et al. 2004). Furthermore, a single-point mutation at amino acid residue S141 in TRPM6 disrupts heteromeric TRPM6/TRPM7 channel formation manifesting itself as hypomagnesemia with secondary hypocalcemia (Chubanov et al. 2004). Interestingly, when cloned into the pCINeo-IRES-GFP vector, TRPM6 can be overexpressed and forms functional homomeric channels in the plasma membrane (Voets et al. 2004; Li et al. 2006). While this seems to be the only vector able to do so for unknown reasons, it presents a valuable scientific tool to study the hypothetical behavior of TRPM6 if it were expressed natively. This may provide information as to why homomeric TRPM7 channels behave differently from the heterotetramer formed by TRPM6 and TRPM7 and as to what the underlying structural features might be. It would also be interesting to elucidate whether the noncoding sequence of the TRPM6-pCINeo-IRES-GFP expression construct can influence assembly and trafficking of TRPM6.

5 A Biophysical Description of the Channel Function, Permeation, and Gating

5.1 Channel Function

Aside from representing a fusion protein, TRPM7's most striking feature is its selectivity for divalent metal ions at hyperpolarized potentials (Monteilh-Zoller et al. 2003; Nadler et al. 2001). The strong outwardly rectifying current–voltage (I/V) signature of TRPM7 is due to voltage-dependent permeation block by extracellular divalent ions, mainly Ca and Mg (Kerschbaum et al. 2003; Nadler et al. 2001). Removal of divalent ions allows the assessment of TRPM7 single-channel characteristics at all physiological voltages, revealing a relatively large conductance of 40 pS and open times of several hundred milliseconds (Li et al. 2006). The channel itself shows no intrinsic voltage dependence, and the level of its constitutive activity is regulated by a surprising variety of intracellular and extracellular factors. Natively, most cells express only a few tens of TRPM7 proteins in the plasma membrane, and this can readily be assessed by the whole-cell patch-clamp method. However, the relative scarcity of endogenous TRPM7 in the cell's membrane hampers the use of other, less sensitive detection methods, such as immunofluorescence or biotinylation studies, or more global protein expression evaluations by Western blot.

5.2 Kinase Function

The identification of elongation factor-2 kinase revealed a new class of protein kinases with no sequence homology to conventional eukaryotic protein kinases in regard to their catalytic domains (Ryazanov et al. 1999). There are several members of this so-called atypical or α-kinase family in mammals, and two are fused to the

ion channels TRPM6 and TRPM7. The TRPM7 kinase specifically phosphorylates Ser and Thr residues in a Mg-dependent manner (Ryazanova et al. 2004). It autophosphorylates itself and phosphorylates myelin basic protein as well as histone H3. At least two of the identified autophosphorylation sites (S1511 and S1567) do not seem to influence channel behavior (Matsushita et al. 2005).

While manganese (Mn) can replace Mg to maintain kinase function, zinc (Zn) and cobalt (Co) inhibit kinase activity, while Ca plays no role (Matsushita et al. 2005; Ryazanova et al. 2004). Staurosporine, a common protein kinase inhibitor preventing ATP binding, does not interfere with TPRM7 kinase function, whereas rottlerin, a potent K^+ channel activator, suppresses kinase activity at high concentrations [$IC_{50} \sim 40$ μM (Ryazanova et al. 2004)].

5.3 Channel Permeation

The first indication that TRPM7 represents a bona fide divalent ion channel at negative voltages and allows monovalent ion flux only at depolarized voltages was published in one of the original reports on TRPM7 function (Nadler et al. 2001). Detailed studies followed confirming the channel's selectivity profile to be Zn = nickel (Ni) > barium (Ba) > Co > Mg > Mn > strontium (Sr) > cadmium (Cd) > Ca (Li et al. 2006; Monteilh-Zoller et al. 2003). Relatively large and complex structured polyamines can additionally act as permeant blockers of TRPM7 (Kerschbaum et al. 2003).

Several amino acid residues in the putative TRPM7 ion channel pore have been shown to control Ca and Mg permeability. Changing glutamic acid at residue 1047 or 1052 in the mouse channel into a neutral glutamine either strongly reduces (70 %) or even abolishes affinity to Ca or Mg, respectively (Li et al. 2007). On the other hand, changing residue E1047 into glutamine and Y1049 into proline results in linearized currents and loss of Ca permeation (Mederos y Schnitzler et al. 2008). Similar observations have been made for human TRPM7 (Numata and Okada 2008) with the corresponding key residues E1047 and E1052. In addition, aspartic acid at 1054 and 1059 also influences permeation block by divalent ions.

5.4 Channel Gating and Regulation

5.4.1 Magnesium

TRPM7 represents a constitutively active ion channel that is heavily regulated by a variety of physiological feedback mechanisms. One of the most important regulatory factors of channel activity is intracellular free Mg (Nadler et al. 2001), which can be mimicked by non-physiological Ba, Sr, Zn, and Mn (Kozak and Cahalan 2003). Detailed biophysical examination reveals that native TRPM7 in excised patches has two conductance states at 39 pS and 186 pS, with both reversibly inhibited by Mg (Chokshi et al. 2012c). The respective dose-response curves reveal

IC_{50} values of 25 μM and 91 μM. Mg seems to reduce the number of active channels rather than cause an overall reduction of single-channel conductance. Mg inhibition involves two separate binding sites on the protein (Chokshi et al. 2012b), one within the kinase domain and another on the channel proper (Schmitz et al. 2003). Mg inhibitory potency measured in excised patches is about 10- to 20-fold smaller than that seen in whole-cell patch-clamp experiments where 750 μM free Mg is needed to suppress channel activity by 50 %, both in overexpression and native cell systems (Nadler et al. 2001; Demeuse et al. 2006). This suggests that additional factors in the cellular environment of TRPM7 help regulate the channel's true physiological Mg sensitivity. Sites coordinating the Mg · ATP binding in the kinase domain are partially involved in regulating the overall affinity for Mg to the channel, since introduction of single-point mutations that abolish phosphotransferase activity (G1799D, K1648R) reduces TRPM7's sensitivity to intracellular Mg (Schmitz et al. 2003). In contrast, autophosphorylation does not seem to play a role here, since, at least for the single-point mutants investigated (S1511/S1567), no difference can be detected compared to the wild-type (wt) channel (Matsushita et al. 2005). Interestingly, intracellular Mg seems to synergize with a variety of factors regulating TRPM7 activity, including the highly specific TRPM7 inhibitor waixenicin A, intracellular chloride, and intracellular Mg·nucleotides (Zierler et al. 2011; Yu et al. 2013; Demeuse et al. 2006).

5.4.2 Mg·Nucleotides

There is general consensus that mammalian TRPM7 is regulated by free intracellular Mg (Penner and Fleig 2007). Evidence for intracellular adenosine triphosphate (ATP) as a feedback mechanism for TPRM7 was initially controversial. Runnels et al. reported facilitation of TPM7 activity by intracellular ATP (Runnels et al. 2001), whereas in a parallel study, Nadler et al. demonstrated an inhibitory effect of ATP in its physiologically relevant form bound to Mg (Mg·ATP) (Nadler et al. 2001). This issue has been resolved and the ATP-mediated activation of TRPM7 actually is due to a decrease in free Mg caused by supplemented Na·ATP. Subsequent analyses revealed that negative feedback inhibition by Mg·ATP requires an intact nucleotide-binding site of the kinase domain involving amino acid K1648 (Schmitz et al. 2003). The binding site also helps discriminate between Mg·nucleotide (Mg·NTP) species such as Mg·GTP or Mg·TTP, since point mutations of this residue or removal of the entire kinase domain renders the channel insensitive to intracellular nucleotide regulation (Demeuse et al. 2006). Mg·adenosine diphosphate (ADP), but not adenosine monophosphate (AMP), has similar inhibitory efficacy as Mg·ATP, indicating a protection against enhanced TRPM7 activation during variations of cell energy levels. Thus, Mg chelated to nucleotides seems key to interfere with TRPM7 gating. Furthermore, inhibition by Mg·nucleotides is synergistically enhanced by intracellular free Mg. In fact, nucleotides lose any efficacy below a minimal threshold of around 200 μM free Mg (Demeuse et al. 2006). The current model therefore postulates independent binding sites for Mg and Mg·ATP, synergistically regulating channel activity. Interestingly, kinase deletion at residue 1599 renders a nonfunctional channel

(Matsushita et al. 2005), while truncating the kinase domain at aa 1569 regains some channel function (Schmitz et al. 2003) and cutting the protein at residue 1510 fully recovers the ability to measure regular TRPM7 currents (Desai et al. 2012). Thus, it is tempting to speculate that the protein region between aa residues 1510 and 1599 is involved in coordinating the binding of Mg to the channel.

5.5 Receptor-Coupled TRPM7 Activity

Several studies have reported regulation of TRPM7 through PLC-dependent pathways. Co-overexpression of muscarinic receptor 1 and TRPM7 in HEK293 cells followed by charbacol stimulation leads to TRPM7 inactivation due to depletion of PIP_2 in the plasma membrane (Runnels et al. 2002). Endogenous TRPM7 in CA1 hippocampal neurons is sensitive to nerve growth factor via a PLC-dependent pathway (Tian et al. 2007), and in cardiac myocytes GTP analogues lead to TRPM7 inhibition through G-protein activity and PIP_2 metabolism (Macianskiene et al. 2008). In contrast, moderate overexpression of TRPM7 in neuroblastoma N1E-115 cells needs free intracellular Mg to fall below physiological levels for PLC-dependent inhibition to occur. Under normal Mg levels, TRPM7 currents are activated rather than inhibited following receptor stimulation through bradykinin, thrombin, or lysophosphatidic acid (Langeslag et al. 2007). Further evidence shows involvement of endogenous G_s/G_i-coupled receptors in TRPM7 regulation. Stimulation of acetylcholine receptors inhibits overexpressed TRPM7 currents in HEK293 cells. Isoproterenol stimulation of endogenous beta-adrenergic receptors, on the other hand, enhances TRPM7 activity and requires both a functional protein kinase A and an intact TRPM7 kinase domain (Takezawa et al. 2004).

5.6 Mechano-sensitivity and Volume

In vascular smooth muscle A7R5 cells overexpressing TRPM7, laminar flow-induced sheer stress causes channel translocation to the plasma membrane, implicating TPRM7 in cellular mechanotransduction of flow (Oancea et al. 2006). Endogenous TRPM7 in HeLa cells is directly activated by stretch or increased cell volume and does not involve exocytotic events for biomembrane incorporation (Numata et al. 2007). Exposing HEK293 cells expressing heterologous TPRM7 to varying osmotic gradients provides insight into the channel's osmo-sensitivity mediated by molecular crowding of solutes that affect channel activity without involvement of membrane stretch (Bessac and Fleig 2007). While results are currently controversial as to the exact mechanism, it seems safe to say that changes in cell volume will affect TRPM7 channel activity.

5.7 Acidity

Acidic extracellular conditions below pH 6 greatly potentiate TRPM7 currents at negative membrane potentials (Monteilh-Zoller et al. 2003). This is due to changes in selectivity of TRPM7 that enhance monovalent ion permeation caused by direct competition of protons with divalent ions for specific binding sites in the channel pore (Jiang et al. 2005). Specifically, mutating glutamic acid residues 1047 and 1052 into nonpolar glutamine in mouse TRPM7 decreases or abolishes not only divalent ion selectivity but also pH sensitivity (Li et al. 2007). In human TRPM7, overlapping glutamic acid or aspartic acid residues have been identified in the pore region with similar results [D1054, E1052, and D1059 (Numata and Okada 2008)]. The pH sensitivity of TRPM7's selectivity profile is an interesting biophysical feature that has to be taken into account under acidic pathological conditions.

5.8 TRPM7 Inhibitors

Several compounds have been reported to inhibit TRPM7, although most of them lack potency or specificity or both. Extracellular spermine blocks endogenous TRPM7-like currents in RBL-2H3 cells with an IC_{50} of 2.3 μM, whereas 20 μM of SKF-96365, a nonspecific TRP channel and voltage-gated Ca channel blocker (Singh et al. 2010), is needed for complete block (Kozak et al. 2002a). 2-Aminoethyl diphenylborinate (2-APB), a compound found to interfere with a variety of proteins involved in Ca signaling, inhibits overexpressed human TRPM7 currents with an IC_{50} of 174 μM (Li et al. 2006). Endogenous TRPM7-like currents can be reversibly inhibited at 50 μM 2-APB in Jurkat T lymphocytes (Prakriya and Lewis 2002). Interestingly, while 2-APB inhibits TRPM7, it activates its paralog TRPM6, making this compound a useful tool in discriminating between currents carried by these two proteins (Li et al. 2006). Furthermore, it is now known that 2-APB does not bind directly to TRPM7, but rather inhibits channel activity through an intracellular acidification mechanism (Chokshi et al. 2012a).

The first high-throughput drug-screening bioassay targeting TRPM7 was developed in 2010 using fluorescent-based Mn quench in HEK293 cells overexpressing human TRPM7 (Castillo et al. 2010). This led to the discovery of the first specific and highly potent TRPM7 inhibitor waixenicin A, a compound isolated from the soft coral *Sarcothelia edmondsoni* (Zierler et al. 2011). Waixenicin A blocks TRPM7 currents in a Mg-dependent manner with an IC_{50} of 16 nM, and TRPM7-dependent cell proliferation is inhibited with an IC_{50} of 3.2 μM in RBL-1 cells. Waixenicin A has no effects on other major pathways that regulate Ca influx such as TRPM2, TRPM4, and Ca release-activated Ca (CRAC) channels (Zierler et al. 2011), and the compound also does not inhibit TRPA1 at 10 μM concentrations (Zierler and Fleig unpublished data). Importantly, waixenicin A does not affect TRPM7's sister channel TRPM6, adding another pharmacological tool for differentiating between TRPM6 and TRPM7 (Zierler et al. 2011).

Using an aequorin bioluminescence-based assay, several small conductance Ca-activated K channel inhibitors were found to act on TRPM7 (Chubanov et al. 2012), including the antimalarial plant alkaloid quinine, CyPPA, dequalinium, NS8593, SKA31, and UCL1684. Of those, the most potent compound was NS8593 with an IC_{50} of 1.6 μM. NS8593 is thought to be a direct channel blocker and seems to interfere with the Mg-dependent regulation of TRPM7 while also inhibiting the mobility of HEK293 (Chubanov et al. 2012). The broad-spectrum serine protease inhibitor and anticoagulant nafamostat mesylate interfere with heterologous mammalian TRPM7 with an IC_{50} of 27 μM (Chen et al. 2010a), and several 5-lipoxygenase inhibitors (NDGA, AA861, and MK886) inhibit TRPM7 in the higher μM range (Chen et al. 2010a). Sphingosine, the primary component of sphingolipids in the plasma membrane, and fingolimod, a structural analogue of sphingosine and FDA-approved for treatment of multiple sclerosis, are inhibitors of TRPM7 with IC_{50}s of 600 nM and 720 nM, respectively (Qin et al. 2013). They act by reducing the open probability of the channel. Metabolites of sphingosine, such as sphingosine-1-phosphate or ceramides, have no effect. These properties are reminiscent of the sphingolipid effects reported for CRAC channels (Mathes et al. 1998). One known side effect of calcineurin inhibitors is hypomagnesemia. Cyclosporin A and FK506 (tacrolimus), important calcineurin inhibitors, affect Mg flux as assessed by MagFura measurements in the intestinal epithelial cell line CaCo (Gouadon et al. 2012). While cyclosporin A counteracts Mg accumulation, FK506 increases Mg influx without altering expression levels of TRPM6, TRPM7, or MagT1.

In conclusion, several compounds interfere with TRPM7 at various potencies or selectivity. Both 2-APB and waixenicin A could be useful tools to pharmacologically differentiate between TRPM6 and TRPM7.

6 Physiological Functions in Native Cells, Organs, and Organ systems

Early evidence pointed to TRPM7's possible involvement in cellular Ca and Mg homeostasis as well as cell viability and proliferation (Penner and Fleig 2007). Further studies identified central roles in cell migration, exocytosis, and development (Runnels 2011), and disruption of normal TRPM7 function has been associated with the progression of cancer, severity of brain ischemia, and cardiovascular disease.

6.1 Magnesium Homeostasis

The channel's involvement in cellular Mg homeostasis was shown through genetic knockout experiments in chicken B lymphocytes, leading to an arrest in cell proliferation and reduced intracellular Mg levels that could be rescued exclusively by high extracellular Mg supplementation (Schmitz et al. 2003). Interfering with

TRPM7 channel function by a genetic knockout of the kinase domain arrests mouse embryonic stem cell proliferation that again can be rescued by high external Mg (Ryazanova et al. 2010). Such rescue of TRPM7-deficient cells is now known to be mediated by endogenous expression of alternate Mg transporters such as SLC41A1 (Kolisek et al. 2008), SLC41A2 (Sahni et al. 2007), or MagT1 (Deason-Towne et al. 2011), depending on cell type.

TRPM7 is not only important for cellular Mg homeostasis but is also involved in maintaining systemic Mg levels (Ryazanova et al. 2010). Mice heterozygotic for a TRPM7 kinase domain deletion develop hypomagnesemia compared to control mice. This seems to be caused by a deficit in Mg absorption through the colon rather than reabsorption mechanisms through the kidney. On the other hand, tissue-specific deletion of TRPM7 in T lymphocytes of mice does not alter total Mg contents of these cells (Jin et al. 2008). This is not surprising, since selective tissue-specific deletion of TRPM7 is not expected to alter overall systemic Mg homeostasis, and T cells express compensating Mg transporters such as MagT1 (Li et al. 2011).

Amidst discussions of the importance of TRPM7 in cellular and systemic Mg homeostasis, it should be remembered that the channel represents a divalent ion influx mechanism for other divalent ion species, including Ca and trace metals (Monteilh-Zoller et al. 2003). Interestingly, TRPM7's ability to conduct Ca is currently linked to a disease-inducing role such as in neuronal ischemia or atrial fibrillation (Du et al. 2010; Aarts et al. 2003). The physiological role of Ca conductance by TRPM7 remains largely unexplored. Indeed, even TRPM7's role in cell migration seems to be linked to its ability to conduct Mg rather than Ca (Su et al. 2011), despite a close correlation between TRPM7 plasma membrane localization and cellular Ca hot spot microdomains thought to drive cell migration (Wei et al. 2009; Clark et al. 2006). Finally, recent work implicates TRPM7-mediated Cd uptake in osteoblast cytotoxicity (Martineau et al. 2010; Levesque et al. 2008), further emphasizing TRPM7's physiological role as a divalent ion channel mechanism.

6.2 Cell Proliferation, Cell Death, and Cell Differentiation

Genetic or pharmacological ablation of TRPM7 in proliferating tissue arrests cells at G0/G1 transition of the cell cycle (Zierler et al. 2011; Tani et al. 2007; Schmitz et al. 2003; Nadler et al. 2001; Abed and Moreau 2007; Sahni et al. 2010). When arresting RBL-2H3 cells at various stages of the cell cycle, endogenous TRPM7-like currents are significantly upregulated at the G0/G1 transition (Tani et al. 2007), further emphasizing the critical role of this channel at the transition stage from quiescence to proliferation. Differentiated mast cells, on the other hand, undergo apoptosis upon genetic suppression of TRPM7 (Ng et al. 2012), and similar observations are made in hepatic stellate cells, possibly involving the TNF-related apoptosis-inducing ligand (TRAIL) mechanism (Liu et al. 2012). Interestingly, proliferating rat embryonic hepatocytes and rat hepatoma show

higher TRPM7 expression levels than adult nondividing rat hepatocytes, indicating that downregulation of endogenous TRPM7 is linked to the differentiation process (Lam et al. 2012). Thus, it appears that TRPM7 is critical for the physiology of proliferating cells to maintain cell numbers, whereas differentiated cells reduce the expression of TRPM7 to levels that sustain supplementation of cells with Ca, Mg, and trace metals.

6.3 Migration

Early observations link TRPM7 activity to maintenance of cell structure, as TRPM7 overexpression in HEK293 cells leads to rounding and detachment of cells from the substrate, which requires functional m-calpain activity and depends on Ca influx through the TRPM7 channel domain (Nadler et al. 2001; Su et al. 2006). Activation of m-calpain by TRPM7 is thought to work through stress-dependent stimulation of p38 MAP kinase and JUN kinase, as inhibitors of these proteins inhibit the cell rounding and detachment caused by overexpressing TRPM7 (Su et al. 2010). TRPM7 seems to be partially responsible for supporting activated T cell migration as well as the velocity of migration (Kuras et al. 2012), and genetic suppression of TRPM7 in migrating WI-38 fibroblasts leads to a reduced number of Ca flickers accompanied by a disruption of normal cell migratory patterns (Wei et al. 2009). Interestingly, both intracellular Mg and Ca can influence m-calpain activity (Su et al. 2010), and indeed Rac- and Cdc42-dependent polarized cell movement of fibroblasts relies on the availability of intracellular Mg and not Ca (Su et al. 2011). Further studies identify TRPM7 as controlling actomyosin contractility and cell adhesion by increasing cellular Ca levels, and this involves a phosphorylation step utilizing the channel's kinase (Clark et al. 2006). Thus, it seems that divalent ion influx through TRPM7 is involved in cell adhesion and migration, whereas the protein's kinase domain supports actomyosin contractility.

7 Lessons from Knockouts

The role of TRPM7 in living organisms has been investigated in several genetically tractable animal models such as mouse (*M. musculus*), zebrafish (*D. rerio*), and frog (*Xenopus laevis*). The roles of TRPM7-related proteins have also been explored in invertebrate species such as fruit fly (*Drosophila melanogaster*) and roundworm (*Caenorhabditis elegans*).

7.1 Mouse *Trpm7*

Two *Trpm7* null mutant mice [$Trpm7^{\beta geo}$ and $Trpm7^{\Delta 17}$ (Table 1)] and mouse mutants lacking exons encoding the kinase domain [$Trpm7^{\Delta kinase/\Delta kinase}$ (Table 1)] die at embryonic day 6.5–7.5 (e6.5–e7.5) and e7.5, respectively (Ryazanova

Table 1 Mouse lines carrying mutant alleles in *Trpm7* gene

Allele	Targeting strategy	Functional outcome	References
$Trpm7^{\beta geo}$	Insertion of β-geo reporter sequence in the first intron of Trpm7	Constitutive loss of function and expression of β-galactosidase driven by Trpm7 promoter	Jin et al. (2008)
$Trpm7^{fl}$	LoxP sites flanking exon 17	Cre-mediated loss of function due to a frame shift	Jin et al. (2008, 2012)
$Trpm7^{\Delta 17}$	Deletion of exon 17 in $Trpm7^{fl}$	Constitutive loss-of-function due to a frame shift	Jin et al. (2008)
$Trpm7^{\Delta kinase}$	Deletion of exons 32–36	Constitutive deletion of the kinase domain	Ryazanova et al. (2010)

et al. 2010; Jin et al. 2008). The reasons for this remain unclear. As briefly discussed above, mice heterozygotic for the TRPM7 kinase ablation ($Trpm7^{\Delta kinase/+}$) have reduced Mg levels in the blood, bone, and urine (Ryazanova et al. 2010). In contrast to wild-type control mice, a substantial fraction of heterozygotic animals die shortly after placing them on a Mg-deficient diet. In addition, $Trpm7^{\Delta kinase/+}$ mice exhibit behavioral alterations indicative of Mg deficiency (clasping, tremors, and seizures). Embryonic stem (ES) cells isolated from these animals show reduced TRPM7 currents due to an increased sensitivity to intracellular Mg. Thus, experiments with $Trpm7^{\Delta kinase/+}$ mice indicate that a key aspect of TRPM7 function is a regulation of systemic Mg homeostasis.

Conditional mutagenesis of the *Trpm7fl* allele (Table 1) using Cre/loxP-recombination technologies has been employed to elucidate a spatiotemporal requirement for *Trpm7* during embryonic development. Here, an epiblast-restricted inactivation of *Trpm7* leads to lethality indicating that TRPM7 is required within the embryo proper (Jin et al. 2008). Furthermore, global disruption of *Trpm7* at different embryonic stages using a tamoxifen (TM)-inducible Cre-ER transgene uncovers embryonic lethality during e7–e9. In contrast, TM-induced mutagenesis of *Trpm7* at e14.5 is compatible with prenatal development since healthy *Trpm7* null pups are born with expected Mendelian inheritance. Surprisingly, the TM-induced inactivation of *Trpm7* in adults causes no obvious phenotype, suggesting that *Trpm7* is indispensible only before and during organogenesis (Jin et al. 2008). However, one caveat to keep in mind is the difficulty to accurately assess whether the incomplete deletion of *Trpm7* observed in the tissue of this mouse model is sufficient to induce a true *Trpm7* null phenotype.

Several Cre transgenic lines with tissue-specific recombination activity were used to elucidate the organ-restricted requirements of *Trpm7*. First, deletion of *Trpm7* in the T cell lineage disrupts thymopoiesis and leads to a developmental block of thymocytes and a progressive depletion of thymic medullary cells (Jin et al. 2008). Second, disruption of *Trpm7* in the embryonic ureteric bud causes ablation of the protein in collecting ducts of the postnatal kidney without obvious morphological alterations (Jin et al. 2012). In contrast, deletion of *Trpm7* in the embryonic metanephric mesenchyme leads to inactivation of the gene in renal

tubules of the kidney. The latter mutants show a reduction in glomeruli number, renal tubular dilation, and formation of cysts in the proximal tubules, indicating that *Trpm7* is essential for nephrogenesis. Third, disruption of *Trpm7* in neural crest (NC) cells at e10.5 results in loss of dorsal root ganglion sensory neurons and skin pigment cells (Jin et al. 2012). However, disruption of *Trpm7* in the embryonic neural stem (NS) cells at e10.5 does not influence normal brain development. Studies with NS cells in vitro reveal that *Trpm7* is not essential for their self-renewal and differentiation. In contrast, during in vitro differentiation of induced pluripotent stem cells to NS cells, *Trpm7* disruption prevents the formation of the NS cell monolayer. Thus, *Trpm7* seems essential for NC progenitors but dispensable once the progenitors are committed.

The role of *Trpm7* in cardiogenesis has been studied by heart-restricted mutagenesis of the conditional *Trpm7* allele (Sah et al. 2013). Cardiac deletion of *Trpm7* at e9.0 results in congestive heart failure and death. In contrast, inactivation of Trpm7 at e13.0 produces viable mice with normal ventricular function. Deletion of Trpm7 at an intermediate time point reduces viability of the mutants to 50 %. The surviving mutant mice develop cardiomyopathy associated with heart block, impaired repolarization, and ventricular arrhythmias.

7.2 Zebrafish and Frog

Several loss-of-function mutations in zebrafish *Trpm7* (*zTrpm7*) have been described. *zTrpm7*-deficient animals undergo normal early morphogenesis. However, mutant larvae exhibit multiple defects including loss of touch responsiveness, defective melanin synthesis and apoptotic death of melanophores, defective proliferation of epithelial cells in the exocrine pancreas, and lethality in late larval life (Yee et al. 2011; McNeill et al. 2007; Low et al. 2011; Elizondo et al. 2005, 2010). *zTrpm7* mutant larvae have reduced total levels of Mg and Ca, and addition of supplemental Mg, but not Ca, partially rescues melanophore survival and proliferation of cells in the exocrine pancreas (Yee et al. 2011; Elizondo et al. 2010). *zTrpm7* mutants develop kidney stones and express higher levels of stanniocalcin 1 (stc1) and anti-hyperphosphatemic factor, fibroblast growth factor 23 (fgf23) (Elizondo et al. 2010). Stc1 modulates total Mg and Ca levels both in mutant and wild-type larvae. The levels of Mg and Ca can be normalized in *zTrpm7* mutants by a block of stc1 activity, whereas the formation of kidney stones can be prevented by knockdown of fgf23.

A role of TRPM7 in early embryonic development has also been studied by genetic manipulation of *X. laevis* embryos. Knockdown of *Xenopus Trpm7* (*xTrpm7*) transcripts using morpholino oligonucleotides reveals that *xTrpm7* in conjunction with noncanonical Wnt signaling regulates cell polarity and migration during gastrulation (Liu et al. 2011). The gastrulation defect can be rescued by exogenous Mg and by overexpression of the Mg transporter SLC41A2 or a dominant negative form of Rac. This suggests that TRPM7-mediated entry of Mg plays an important role in vertebrate gastrulation.

7.3 Fruit Fly and Roundworm

D. melanogaster harbors a single *Trpm* gene (*dTrpm*) encoding a channel subunit that lacks a kinase domain. *dTrpm* is highly expressed in the Malpighian tubules (equivalent of mammalian kidneys). *dTrpm* null mutants develop slowly as larvae and arrest as prepupae with morphological defects in the Malpighian tubules (Hofmann et al. 2010). *dTrpm*-deficient larvae show increased Mg levels in the body when raised on Mg-enriched diets indicating that *dTrpm* regulates removal of Mg from the hemolymph by the Malpighian tubules. *dTrpm* may also regulate Zn homeostasis. It was reported that dTrpm-deficient larvae exhibit low Zn levels, and this phenotype can be rescued by Zn supplementation (Georgiev et al. 2010).

C. elegans has three TRPM channels genetically related to TRPM7: GON-2, GTL-1, and GTL-2. Like dTRPM, these channels do not contain enzyme domains. GON-2 and GTL-1 are expressed in the intestine and regulate Mg uptake, while GTL-2 controls Mg excretion by the excretory cells (Teramoto et al. 2005, 2010). Moreover, *gon-2*/*gtl-1* double mutants show reduced body Mg levels and a growth defect, which can be rescued by dietary Mg, but not Ca (Teramoto et al. 2010).

In summary, the experiments with genetically tractable animal models support the idea that TRPM7 and its genetic relatives are essential for early development, organogenesis, and regulation of Mg homeostasis.

8 Role in Hereditary and Acquired Diseases

TRPM7 is not the only ion channel whose activity is controlled by the availability of intracellular Mg · ATP. Early studies identified ATP-sensitive voltage-dependent chloride (Cl-) channels in the sarcoplasmic reticulum of rabbit skeletal muscle (Kourie 1997) and in the plasma membrane of various tissue, including mouse cortical collecting ducts (Meyer and Korbmacher 1996) and human T cells (Cahalan and Lewis 1988). CFTR ion channel gating is regulated by ATP binding and hydrolysis in synergy with intracellular Mg (Ikuma and Welsh 2000), and activity of the ATP-sensitive K^+ channels in pancreatic β-cells is determined by the intracellular concentration ratio of Mg · ATP over Mg · ADP (Tarasov et al. 2004). As such, these ion channels function as sensors of cell metabolism, and any changes in availability of either glucose or oxygen will affect their channel activity with varying impact on cell (patho)physiology. For TRPM7 this was most dramatically demonstrated in a mouse model of transient global ischemia, where small interfering RNA-induced suppression of TRPM7 in the right hippocampus protected neurons from undergoing cell death compared to control (Sun et al. 2009). Ample in vitro studies corroborate the involvement of TRPM7 in oxygen-glucose deprivation-induced neuronal cell death (Aarts et al. 2003; Zhang et al. 2011), either caused by activation of channels by reactive oxygen species (Aarts et al. 2003; Coombes et al. 2011), by changes in extracellular divalent ions (Wei et al. 2007), or by TRPM7-mediated Zn^{2+} accumulation (Inoue et al. 2010). Epidemiologic studies inspired by these findings have performed comparative gene

expression analyses in mice to link TRPM7 to brain-related diseases such as multiple sclerosis, Alzheimer's disease, and stroke and found TRPM7 to be one of 18 common genes to be regulated in these mouse disease models (Tseveleki et al. 2010). However, a prospective, nested case-control study did not find a connection between incident risk of ischemic stroke and variations in the TRPM7 gene (Romero et al. 2009).

The TPRM7 channel kinase seems to play a role in various cardiovascular diseases. Early reports located TRPM7-like currents in pig, rat, and guinea pig ventricular myocytes (Gwanyanya et al. 2004) and most recently in human atrial myocytes (Macianskiene et al. 2012; Zhang et al. 2012a), although the channel kinase's role in this tissue remains to be determined. More is known about TRPM7's function in human cardiofibroblasts, where increased TRPM7-mediated Ca influx has been linked to increased myofibroblast differentiation and fibrogenesis in patients prone to atrial fibrillation (Du et al. 2010).

TRPM7 is also linked to hypertension. Vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats have lower TRPM7 mRNA levels and significantly reduced intracellular Mg levels compared to VSMC from Wistar control, and this is linked to angiotensin II stimulation (Touyz et al. 2006). Interestingly, chronic angiotensin II application increases intracellular Mg in a TRPM7-dependent way leading to enhanced DNA and protein production, indicating cell growth (He et al. 2005). VSMC isolated from the ascending aorta of mouse respond to angiotensin II stimulation by upregulating TRPM7 expression, which triggers a Ca-dependent switch from contractile cell characteristics to a phenotype supporting cell proliferation (Zhang et al. 2012b). In human aortic VSMC, vascular calcification can be prevented on the cellular level by exposing cells to increasing external Mg concentrations, and the use of pharmacological tools implicates TRPM7 to be involved in this process (Louvet et al. 2013). Renal TRPM7 (and TRPM6) is downregulated in a mouse model of hereditary hypomagnesemiar (Yogi et al. 2011), which is further exacerbated by aldosterone administration to induce hypertension (Sontia et al. 2008). Mg supplementation can alleviate the effects induced by aldosterone, including hypertension, inflammation, and fibrosis.

The central function of TRPM7 in processes driving cell growth, proliferation, differentiation, and migration identifies the protein as a possible target in cancer (Sahni et al. 2010). Indeed, reducing TRPM7 expression inhibits proliferation in human head and neck carcinoma (Jiang et al. 2007) and human gastric adenocarcinoma cells (Kim et al. 2008). In other cancer cell lines, this experimental manipulation affects cell migration and invasiveness, such as in A549 lung cancer (Gao et al. 2011), human nasopharyngeal carcinoma (Chen et al. 2010b), BXPC-3 human pancreas adenocarcinoma (Rybarczyk et al. 2012), or MDA-MB-435 breast cancer cells (Meng et al. 2013). When comparing tumor tissue with normal tissue, TRPM7 is generally upregulated as assessed in human pancreatic adenocarcinoma (Rybarczyk et al. 2012; Yee et al. 2011), human breast cancer (Middelbeek et al. 2012), and rat hepatoma (Lam et al. 2012). This has led to the identification of TRPM7 as an independent predictor of poor outcome in breast cancer patients due to increased metastasis formation (Meng et al. 2013; Middelbeek et al. 2012).

Patient survival is inversely related to TRPM7 expression levels in human pancreatic ductal adenocarcinoma, where TRPM7 levels increase at higher tumor staging (Rybarczyk et al. 2012).

Based on TRPM7's unique permeation profile for both Ca and Mg, epidemiologic studies have started to look at the ratio of Ca:Mg intake and cancer risk. The T1482I polymorphism in the TRPM7 gene, thought to contribute to familial amyotrophic lateral sclerosis and Parkinsonism dementia in Guam (Hermosura et al. 2005) but not in Kii, Japan (Hara et al. 2010), is associated with elevated risk of adenomatous and hyperplastic polyps, both risk indicators of colorectal adenoma. This association is particularly strong when the Ca:Mg intake ratio is high (Dai et al. 2007) and gave reason to initiate a randomized placebo-controlled intervention clinical trial investigating whether a reduction of dietary Ca:Mg ratio lowers the risk of adenoma and hyperplastic polyps in patients who do or do not carry the T1482I allele (clinicaltrials.gov: NCT01105169). A retrospective analysis in age-matched prostate cancer patients shows a parallel increase in the serum Ca:Mg ratio and TRPM7 expression levels (Sun et al. 2013). For postmenopausal breast cancer, the medical hypothesis was brought forth that a higher ratio of Ca:Mg serum levels might parallel increased risk (Sahmoun and Singh 2010). Thus, TRPM7 as a Ca- and Mg-conducting ion channel may represent a novel target to be considered in cancer prevention and control.

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TRPM8

Laura Almaraz, Jan-Albert Manenschijn, Elvira de la Peña, and Félix Viana

Contents

Abstract

Transient receptor potential melastatin 8 (TRPM8) was originally cloned from prostate tissue. Shortly thereafter, the protein was identified as a cold- and menthol-activated ion channel in peripheral sensory neurons, where it plays a critical role in cold temperature detection. In this chapter, we review our current understanding of the molecular and biophysical properties, the pharmacology,

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and the modulation by signaling molecules of this TRP channel. Finally, we examine the physiological role of TRPM8 and its emerging link to various human diseases, including pain, prostate cancer, dry eye disease, and metabolic disorders.

Keywords
Cold • Thermoreceptor • Menthol • Pain • Prostate cancer

1 Introduction

Transient receptor potential melastatin 8 (TRPM8), previously designated Trp-p8, was originally cloned from prostate tissue (Tsavaler et al. 2001). No functional characterization was performed in this initial study. Shortly thereafter, the channel was also found in a subpopulation (10–15 %) of small-diameter, cold-sensitive peripheral sensory neurons (McKemy et al. 2002; Peier et al. 2002). Following studies in transgenic mice lacking functional TRPM8 proved that the channel plays a critical role in the detection of environmental cold temperatures (Dhaka et al. 2007; Colburn et al. 2007; Bautista et al. 2007).

2 Gene Structure and Expression

In humans, the *Trpm*8 gene is located on region 2q37.1 of chromosome 2. The gene spans 102,124 bases and contains 25 exons. *Trpm*8 is transcribed into a messenger RNA (mRNA) coding for a protein of 1,104 amino acids. Several shorter isoforms have been described, but they are poorly characterized.

As already mentioned, the TRPM8 transcript was originally identified in the testis and prostate tissue (Tsavaler et al. 2001). These authors showed that TRPM8 was strongly upregulated in prostate tumors and other malignancies. More recently, this upregulation has been confirmed in several other tumors (reviewed by Yee et al. 2010). In the prostate and in prostate cancer cell lines, TRPM8 gene expression is regulated by androgen receptors (Zhang and Barritt 2004). The precise subcellular localization of TRPM8 in this tissue is somewhat controversial, varying with androgen receptor levels and oncogenic status (Zhang and Barritt 2004; Thebault et al. 2005; Bidaux et al. 2007). Still, it is clear that in prostate cells many channels are confined to the endoplasmic reticulum, and channel agonists can trigger Ca^{2+} release and secondary Ca^{2+} entry following emptying of the Ca^{2+} stores (Bidaux et al. 2005; Valero et al. 2011). Recently, two short isoforms of TRPM8 were identified in the metastatic human prostate cell line LNCaP (Bidaux et al. 2012). These short isoforms represent N-terminal fragments of the protein that appear to interact with the C-terminal region.

The expression profile of TRPM8, inferred from expressed sequence tags (EST), suggests a restricted pattern of expression. In addition to prostate, having the highest expression, other tissues like testis, vascular tissue, lung, uterus, placenta, liver, skin, and eye give positive signals. Using a variety of techniques, TRPM8 has been detected in the bladder urothelium and male urogenital tract (Stein et al. 2004), including mouse and human testis and sperm (De Blas et al. 2009; Gibbs et al. 2011; Martinez-Lopez et al. 2011). The bladder wall contains cold- and menthol-sensitive unmyelinated afferents (Jiang et al. 2002). TRPM8 has been detected in human neuroendocrine tumor (NET) cells (Mergler et al. 2004; Louhivuori et al. 2009). The function of TRPM8 in these cells is also unclear: many respond to cooling, but the correlation with responses to TRPM8 agonists is low (Louhivuori et al. 2009). In NET cells, TRPM8 activation is associated with increased secretion of neurotensin, a gut peptide that stimulates the proliferation of various gastrointestinal tissues and tumors. Functional TRPM8 channels have been reported in human melanocytes and melanoma cells (Yamamura et al. 2008). Furthermore, expression of TRPM8 has been reported in rat myocytes isolated from large arteries (e.g., tail, pulmonary, aorta, femoral) (Yang et al. 2006; Johnson et al. 2009; Liu et al. 2013). Whether TRPM8 plays a role in vasomotor responses to local cooling is presently unclear, and further studies are required. A truncated variant of TRPM8 with altered N-terminus has been found in human bronchial epithelial cells but, lacking electrophysiological data, the functionality of this transcript remains uncertain (Sabnis et al. 2008). In lung epithelium, the channel was found to be localized in the endoplasmic reticulum.

Functional and morphological studies have documented that TRPM8 is strongly expressed in a subpopulation of primary sensory neurons within the dorsal root and trigeminal ganglia (McKemy et al. 2002; Peier et al. 2002). Thermosensitive nerve endings of these sensory neurons innervate the skin and mucosae (e.g., cornea, oral cavity, nasal epithelium) (Takashima et al. 2007; Dhaka et al. 2008; Parra et al. 2010). Originally, this population of afferents was thought to conform to a rather homogeneous group of low-threshold cool receptors, but a more detailed analysis has revealed important differences in the function and biochemical phenotype of TRPM8-expressing neurons (Xing et al. 2006; Madrid et al. 2006; Takashima et al. 2007; Madrid et al. 2009; Lippoldt et al. 2013).

While diverse, the neurochemical phenotype of TRPM8-expressing neurons is distinct from that of other sensory neurons. They encompass both unmyelinated C-fibers and lightly myelinated Aδ fibers and show variable expression of various nociceptive markers, including TRPV1, CGRP, and the artemin receptor GFRα3 (Abe et al. 2005; Takashima et al. 2007; Lippoldt et al. 2013). Furthermore, the nerve endings of these fibers terminate in peripheral zones mediating various distinct perceptions of cold and pain, suggesting that TRPM8-expressing neurons may be responsible for a wide range of sensory functions.

3 Molecular Structure of the TRPM8 Protein

Structural details about the TRPM8 protein are still very limited. In humans and rodents, the TRPM8 gene encodes for a 1,104 amino acid protein (Tsavaler et al. 2001; McKemy et al. 2002; Peier et al. 2002). Different modules forming the channel protein have been described (Latorre et al. 2007). TRPM8, like the rest of the TRP channels, has an overall topology similar to that of voltage-gated potassium (K_v) channels, with six transmembrane segments (S1–S6) flanked by large amino- and carboxy-terminals located intracellularly. Charge-neutralizing mutations in the S4 segment and the S4–S5 linker region reduced the channel's gating charge, suggesting they are part of the voltage sensor (Voets et al. 2007a). The putative pore loop is formed by the region between transmembrane segments S5 and S6. TRPM8 subunits are thought to tetramerize into functional channels (Dragoni et al. 2006; Phelps and Gaudet 2007; Stewart et al. 2010; Janssens and Voets 2011). Some studies suggest that oligomerization of TRPM8 is directed by a coiled-coil domain at the C-terminal end of the protein (Tsuruda et al. 2006; Erler et al. 2006). In contrast, another study found that mutants missing the entire C-terminus can tetramerize and localize to the plasma membrane, although they cannot function (Phelps and Gaudet 2007). The TRP domain, also located within the C-terminal region (residues 990–1025), is known to be important in PIP_2 regulation and the energetics of channel opening, i.e., translating drug binding into channel opening (Bandell et al. 2006; Valente et al. 2008). Functional studies in chimeric channels of TRPM8 and TRPV1 suggest that the C-terminus contains structural elements involved in temperature-dependent gating (Brauchi et al. 2006). In addition, the C-terminus accommodates critical sites for the activation of TRPM8 by phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rohacs et al. 2005; Brauchi et al. 2007).

Relatively, little is known about the function of the long intracytoplasmic N-terminus of TRPM8. It contains four conserved regions, TRPM family homology sequences (MHR) (Fleig and Penner 2004; Phelps and Gaudet 2007). Removal of the first 39 amino acids of the protein does not affect channel activity, while larger deletions give rise to non-functional channels (Phelps and Gaudet 2007). A segment encompassing residues 40–86, N-terminal to the first MHR, appears to be involved in plasma membrane localization and in vivo stabilization of the protein (Erler et al. 2006; Phelps and Gaudet 2007). Bioinformatic tools predict the presence of five putative phosphorylation sites on the N-terminal cytosolic tail (Ser9, Thr17, Thr32, Ser121, and Ser367). Some of these residues are confirmed protein kinase A (PKA) phosphorylation sites (Bavencoffe et al. 2010).

A short, leucine-rich conserved sequence among TRPM channels, known as the ICF region (indispensable for channel function), has been characterized in some detail. TRPM8 proteins lacking this region (TRPM8ΔICF), between Leu482 and Asn499, are expressed in significant, but reduced, amount at the cell surface but do not constitute functional channels themselves (Fruhwald et al. 2012). More detailed studies of this variant in TRPM3, including a natural alternative splicing form, suggest that the UCF region is important for proper protein folding.

As detailed below, the main binding sites for chemical agonists and antagonists have been mapped within the six transmembrane domains of TRPM8. In particular, residueTyr745 in the S2 segment is involved in channel activation by menthol and also mediates inhibition by SKF96365 (Bandell et al. 2006; Malkia et al. 2009). In contrast, the agonist effects of icilin require specific residues within the S3 region (Chuang et al. 2004).

TRPM8 channels are also subject to posttranslational modifications affecting their function. The channel is glycosylated at residue Asn934, situated near the putative vestibule of the ion channel pore. This modification is not critical for channel assembly and function but modulates channel activity (Dragoni et al. 2006; Erler et al. 2006; Pertusa et al. 2012). Moreover, glycosylation of TRPM8 affects its association to lipid rafts (Morenilla-Palao et al. 2009). Flanking this glycosylation site, two cysteines (Cys929 and Cys940) forming an intramolecular disulfide bond are essential for channel function (Dragoni et al. 2006). A novel modification of TRPM8, described recently, consists in the covalent linkage of the protein to repeated units of R-3-hydroxybutyrate, giving rise to the attachment of large polymeric chains of poly-(R)-3-hydroxybutyrate (PHB) (Cao et al. 2013). TRPM8 mutants lacking specific PHB-binding sites, and wild-type channels treated with PhaZ7, a PHB depolymerase with serine hydrolase activity, showed strongly reduced response to menthol or cooling. Serine residues in the S3–S4 extracellular linker are critical in supporting PHB interaction and gating of TRPM8.

4 TRPM8 Interacting Proteins

Several studies noted that threshold temperature of recombinant TRPM8 channels (17–25 °C) is consistently lower than that of native channels in menthol-sensitive primary sensory neurons and terminals (Madrid et al. 2006; Malkia et al. 2007; Parra et al. 2010). In fact, temperature sensitivity of recombinant channels is too low to account for the function of cold thermoreceptors in vivo, with firing rates that are temperature modulated at skin temperatures above (33–35 °C) (Reid 2005). Artificial expression of TRPM8 channels in other neurons (e.g., hippocampus) does not restore the thermal sensitivity level observed in primary sensory neurons (de la Peña et al. 2005). These results suggest that temperature sensitivity of TRPM8 must be facilitated by endogenous factors, specific for sensory neurons.

The number of identified proteins interacting directly with TRPM8 is still very limited (http://trpchannel.org/proteins/show?id=TRPM8). Pull-down and co-immunoprecipitation assays have identified Gαq (Zhang et al. 2012), ths μ-opioid receptor OPRM1 (Shapovalov et al. 2013), Pirt (Tang et al. 2013), and the large polymer polyhydroxybutyrate (Cao et al. 2013) as partners of TRPM8. The functional consequence of some of these interactions is described in other sections. Pirt is a membrane protein and is broadly expressed in primary sensory neurons. Expression of Pirt enhanced the responses of TRPM8 channels to agonists (e.g., cold and menthol) (Tang et al. 2013). Moreover, $Pirt^{-/-}$ mice exhibited reduced

responses to cold temperature and chemical cooling agents. The authors ruled out effects of Pirt on cell-surface expression of TRPM8.

An alternative approach to investigate potential partners of TRPM8 is to selectively ablate TRPM8-expressing neurons and analyze the reduction of specific RNA transcripts. This approach will identify functional partners (proteins expressed in the same neurons) but not necessarily biochemical partners. Using this strategy, the laboratory of McKemy reported reductions in KCNK2 (i.e., TREK-1), cadherin 8, PLCδ4, and the artemin receptor GFRα3 (Knowlton et al. 2013; Lippoldt et al. 2013).

5 Biophysical and Pharmacological Characterization of TRPM8

TRPM8 is a non-selective cation channel, with modest calcium permeability. The relative permeability for Ca^{2+} versus Na^{+} (P_{Ca}/P_{Na}) has been estimated at 1–3.3 (McKemy et al. 2002; Peier et al. 2002). At the single-channel level, the current versus voltage relationship of TRPM8 channels is approximated linear with an estimated conductance of ~60–70 pS at 20 °C, increasing to ~95 pS at 30 °C (Zakharian et al. 2010; Fernandez et al. 2011).

A recognized function of TRPM8 in sensory neurons and their terminals is to monitor subtle changes in ambient temperature (Fig. 1) (Dhaka et al. 2007; Bautista et al. 2007; Parra et al. 2010). In agreement with this physiological role, the gating of TRPM8 shows steep temperature dependence, with Q_{10} estimates around 30–40 (McKemy et al. 2002; Voets et al. 2004; Brauchi et al. 2004; Zakharian et al. 2010). Cold activation is accompanied by large changes in enthalpy and entropy and relatively small changes in free energy (Brauchi et al. 2004). The molecular mechanism(s) underlying thermal sensitivity of some TRP channels, including TRPM8, is a major unsolved question. TRPM8 purified from a bacterial expression system and reconstituted into planar lipid bilayers can be gated by temperature (Zakharian et al. 2010). In addition, thermally sensitive modules have been identified in the structure of TRPM8 and other TRP channels (Brauchi et al. 2006; Yao et al. 2011). These results support an intrinsic thermal sensitivity of TRPM8 (reviewed by Latorre et al. 2009).

A functional feature shared by TRPM8 with other TRP channels is the polymodal activation (Jordt et al. 2003), allowing them to act as signal integrators (Tominaga et al. 1998). Apart from physical (i.e., cold temperature) and chemical stimuli (e.g., menthol), TRPM8 is also gated by voltage (Voets et al. 2004; Brauchi et al. 2004). A key question in understanding the function of the channel is to explain how the different sensors on the protein communicate with the pore to gate its opening and closing (reviewed by Voets et al. 2007b).

Both low temperature and menthol increase probability of TRPM8 opening (Po) (McKemy et al. 2002; Voets et al. 2004) (Fig. 2). Moreover, menthol sensitizes the activation of TRPM8 by temperature and vice versa (Fig. 3). TRPM8 is also gated by voltage, increasing Po with depolarization in a sigmoidal fashion (Voets et al. 2004; Brauchi et al. 2004; Fernandez et al. 2011). The voltage dependence of

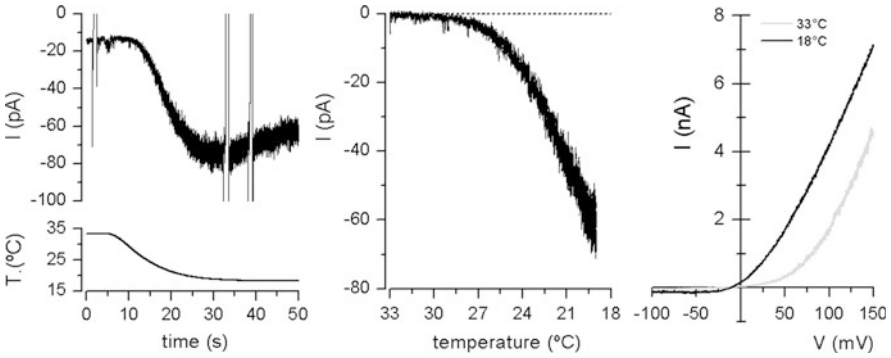

Fig. 1 Effect of cold temperature on whole-cell TRPM8 currents. (*Left*) Current at Vhold = −60 mV in HEK293 cells expressing rat TRPM8 during a temperature ramp from 34 to 19 °C. The sharp transients are due to the application of brief voltage ramps. (*Middle*) Development of inward current as a function of temperature. (*Right*) Current–voltage relationship of the whole-cell current at two different temperatures. Note the marked outward rectification

TRPM8 is characterized by a strong outward rectification at depolarized transmembrane potentials (Fig. 1) and a rapid and potential-dependent closure at negative membrane potentials. The gating charge of TRPM8 is quite small compared to that of classical voltage-gated potassium channels, only around 0.6–0.8 e (Voets et al. 2004; Brauchi et al. 2004; Malkia et al. 2007; Fernandez et al. 2011). As a result, small changes in the Gibbs free energy of the channel can cause large shifts (>100 mV) in the voltage-activation curve (Nilius et al. 2005). At the same time, the shallow voltage dependence curve implies a wide gating window, enabling a precise fine-tuning of the extent of channel activation rather than an all/none type of response. Charge-neutralizing mutations in the S4 segment 4 and the S4–S5 linker region of human TRPM8, specifically Arg842 and Lys856, reduce the channel's gating charge (Voets et al. 2007a), suggesting that this region is part of the voltage-sensing domain, in analogy with voltage-dependent potassium (K_v) channels (Aggarwal and MacKinnon 1996).

A detailed characterization of the voltage- and temperature-dependent gating of TRPM8 channels at the single-channel level revealed a complex gating mechanisms (Fernandez et al. 2011). The increased channel open probability produced by membrane depolarization and cooling was mainly due to a decrease in the duration of closed intervals, compared with a more modest increase in mean open times. The results were best fitted to a multistate gating model, associated with at least two open and five closed states. Moreover, expression of short isoforms of TRPM8 identified in human prostate exerted a negative regulation on TRPM8 activated by cold or menthol, but not by icilin (Bidaux et al. 2012). At the single-channel level, the effect of short isoforms co-expression mimicked the effects of high temperature (i.e., long closures).

In native TRPM8 channels, the apparent temperature activation curve of TRPM8 is shifted to warmer temperatures, compared to recombinant TRPM8 channels

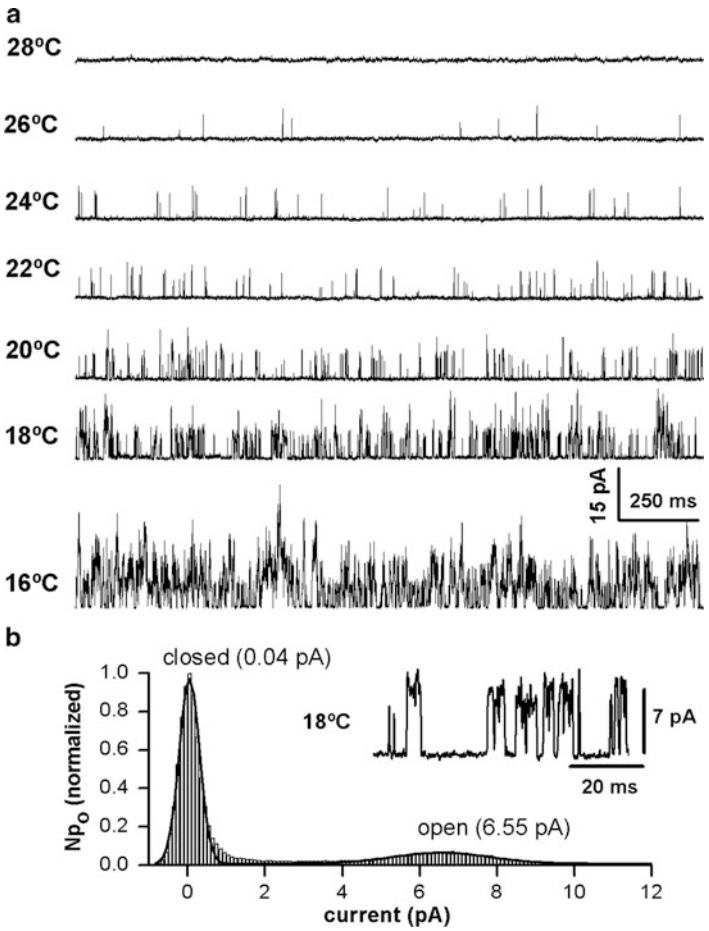

Fig. 2 Cold activation of TRPM8 channels. (**a**) Single-channel current recordings in the cell-attached configuration at different temperatures in a HEK293 cell stably expressing rat TRPM8. Recordings were obtained at a potential of +100 mV in a high potassium solution and filtered at 2 kHz. The patch contained a minimum of four channels. (**b**) Amplitude histogram (0.1 pA bin) showing both the closed state fitted with a Gaussian around zero current and a single open-channel current amplitude with a mean around 6.5 pA, in a cell attach patch at 18 °C over a period of 2 s. The inset shows a 70 ms sample of the trace

expressed in HEK293 cells or hippocampal neurons (de la Peña et al. 2005). This shift is accompanied by an apparent shift in the voltage-dependent activation to more negative potentials (Malkia et al. 2007). Altogether, this explains the large difference in thermal threshold observed between cold thermoreceptor fibers expressing TRPM8 and recombinant channels. Moreover, static warm ambient temperatures shift the temperature threshold of TRPM8 to warmer values (Fujita et al. 2013).

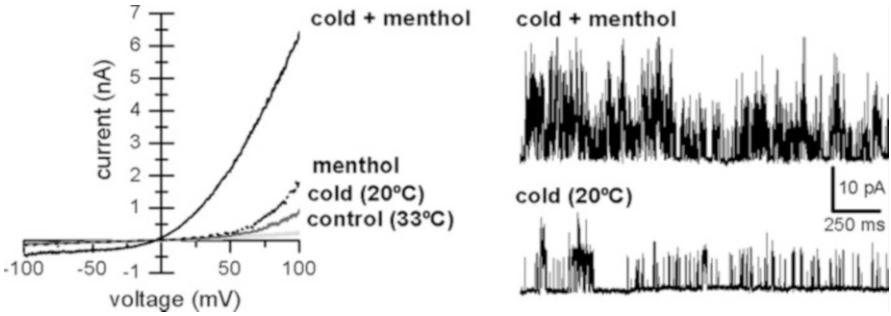

Fig. 3 Potentiation of temperature-dependent activation of TRPM8 by menthol. (*Left*) Current–voltage relationship of the whole-cell current in a HEK293 cell expressing rat TRPM8 under four different conditions: 33 °C, 20 °C, and 100 μM menthol and menthol at 20 °C. (*Right*) Single-channel current recordings in the cell-attached configuration at +100 mV, at 20 °C, and at 20 °C in the presence of 100 μM menthol

5.1 Gating Mechanisms of TRPM8

The activation of TRPM8 by different stimuli has prompted the development of various models to describe the coupling between agonist binding and channel gating. In 2004, Nilius and colleagues proposed that cooling and menthol activate TRPM8 through a shift in the voltage dependence of activation ($V_{1/2}$): both agonists shift the voltage-activation curve of TRPM8 towards more negative potentials, thus increasing the probability of channel openings and boosting inward currents at physiological membrane potentials (Voets et al. 2004). A two-state gating model, with a direct coupling of temperature and voltage, reproduced the macroscopic *G*/*V* curves correctly and could adequately predict the temperature dependence of TRPM8 whole-cell currents at different potentials. However, the complexities of the gating of single TRPM8 channels indicate that models with more states are needed to explain gating mechanisms (Fernandez et al. 2011). Also, studying the thermodynamics of TRPM8 activation by cooling in the presence of menthol and depolarizing voltages, Yang et al. did not observe any effect of these activators on the enthalpy or entropy changes during temperature activation (Yang et al. 2010).

A different approach for describing the gating of TRPM8 was adopted by the group of Ramón Latorre (Brauchi et al. 2004). The so-called allosteric model put forth by these authors describes TRPM8 gating through a scheme of reaction equilibria where the activation by voltage and temperature increases the open probability of the channel both separately and in concert through an allosteric interaction. In this view, sensors for voltage, chemical agonists, and temperature would be located in different protein domains. Consistent with this idea, Matta and Ahern found that very high agonist concentrations activate TRPM8 in a voltage-independent manner and that voltage alone cannot activate TRPM8 maximally (Matta and Ahern 2007), which would be the case if voltage was the final gate in channel activation: the basis of the two-state model. While the allosteric model seems capable of reproducing experimental data and can in principle be expanded

to include any kind of allosteric modulation, the independence of the many fit parameters is not trivial. As pointed out by Zhu (2007), the key difference between the allosteric and the two-state models, the maximum open probability, can be influenced by experimental factors such as desensitization or voltage clamp errors, which become particularly important in the presence of strong receptor stimulation by chemical agonists and/or voltage.

In a more recent publication, Voets et al. (2007a) generated a Monod–Wyman–Changeux (MWC) model explaining the combined effects of voltage, temperature, and menthol on TRPM8 gating. The model was able to faithfully reproduce experimental data, as well as predict the menthol-induced shifts in the voltage-activation curves and the temperature activation threshold. In the absence of ligand, the MWC model is reduced to the two-state model. Advantages of the MWC model over the allosteric model are its simplicity and its ability to predict voltage- and time-dependent kinetics. Nevertheless, this model is not able to accommodate data which point to a menthol-induced increase in the maximum open probability (Matta and Ahern 2007; Malkia et al. 2009).

Finally, the above-cited work by Yang et al. (2010) on the thermodynamics of temperature-induced open probability changes of TRPM8 takes the vision of the allosteric model even further, suggesting that activation of TRPM8 by temperature, voltage, and menthol constitutes three completely separable mechanisms. It remains to be seen how this view can be consolidated with the existing data of agonist-induced effects on TRPM8 voltage dependence.

In a recent publication, Jara-Oseguera and Islas introduced a novel hypothesis in the allosteric mechanism of temperature-dependent activation of TRP channels. In an MWC-type model, they showed that a large reduction in the magnitude of the coupling constant between temperature sensor activation and channel gaiting could reverse the sign of the temperature sensitivity (Jara-Oseguera and Islas 2013). Expressed in simple terms: a heat sensor in a TRPM8 channel that is negatively coupled to the gate would lead to channel opening during cooling.

5.2 Agonists of TRPM8

In addition to cold temperature, TRPM8 can be activated by natural and synthetic cooling mimetic agents like icilin, eucalyptol, and menthol and an abundance of menthol derivatives (Behrendt et al. 2004; Bodding et al. 2007; Beck et al. 2007; Voets et al. 2007b; Viana 2011) (Table 1). Among these agonists, some like WS-12 or compounds identified by Dendreon Corporation are active in the nanomolar range and trigger the apoptotic death of prostate cancer cells. Other natural agonists include volatile chemicals such as hydroxycitronellal, heliotropyl acetone, helional, geraniol, and linalool (Behrendt et al. 2004). It should be noted that many of these agonists are effective only at high concentrations and activate other TRP channels. For example, camphor, an activator of TRPV1 and TRPV3, was recently reported to activate TRPM8 transiently with an EC_{50} of ~4.5 mM (Selescu et al. 2013).

Table 1 TRPM8 agonists

Compound	EC_{50} (μM)	Method
Icilin	0.2 ± 0.1[a]	FL
	1.4[b]	CI
	0.50[c]	EP
	0.36 ± 0.03[d]	EP
Menthol	10.4[b]	CI
	83.6 ± 0.04[c]	EP
	66.7 ± 3.3[d]	EP
WS-12	0.193[b]	CI
	0.680[b]	EP
	0.039[c]	EP
WS-3	3.7 ± 1.7[a]	FL
WS-148	4.1[b]	CI
WS-30	5.6[b]	CI
WS-11	6.25[c]	EP
WS-14	21.19[c]	EP
WS-23	44 ± 7.3[a]	FL
CPS-113	1.2[b]	CI
CPS-369	3.6[b]	CI
Frescolat ML	3.3 ± 1.5[a]	FL
Frescolat MGA	4.8 ± 1.1[a]	FL
Cooling agent 10	6 ± 2.2[a]	FL
PMD-38	31 ± 1.1[a]	FL
Geraniol	$5{,}900 \pm 1{,}600$[a]	FL
Linalool	$6{,}700 \pm 2{,}000$[a]	FL
Eucalyptol	$7{,}700 \pm 2{,}000$[a]	FL
	$3{,}400 \pm 400$[d]	EP
Hydroxycitronellal	$19{,}600 \pm 2{,}200$[a]	FL
Camphor	4,480[e]	CI

Potency of TRPM8 channel activation of various chemical agonists as measured by calcium imaging microscopy (CI), a fluorometric imaging plate reader assay (FL), or patch-clamp electrophysiology (EP)

Whole-cell patch-clamp data are given at + 80 mV[c], +100 mV[b], or −60 mV[d]. The reader is referred to the original publications for compound structures

Data are from:

[a]Behrendt et al. (2004)

[b]Bodding et al. (2007)

[c]Beck et al. (2007)

[d]McKemy et al. (2002)

[e]Selescu et al. (2013)

Icilin activation of TRPM8 requires the concomitant elevation of intracellular calcium, with TRPM8 acting as a coincidence detector of two different stimuli (Chuang et al. 2004; Zakharian et al. 2010). A comparative analysis of rat TRPM8

and icilin-insensitive avian TRPM8 based on the analysis of chimeras and mutant channels revealed the critical residues for icilin sensitivity to be Asn799, Asp802, and Gly805 in the putative transmembrane domain S3 (Chuang et al. 2004). Similarly, during a massive random mutagenesis screen, Tyr745, located in the middle of transmembrane segment S2, was identified as crucial for the menthol sensitivity of mouse TRPM8 (Bandell et al. 2006). This mutant is unable to bind radioactive menthol (Voets et al. 2007a). Residues Tyr1005 and Leu1009 situated in the TRP box of the intracellular C-terminus were also found to participate in channel activation by menthol, but were postulated to be involved in events downstream of menthol binding (Bandell et al. 2006).

As already mentioned, the mechanism of TRPM8 activation by cold and menthol involves a negative shift in the voltage-dependent activation, facilitating the opening of the channel at negative potentials (Voets et al. 2004; Brauchi et al. 2004; Malkia et al. 2007). A similar shift in the voltage dependence has been reported for WS-12 (Bodding et al. 2007). Using tandem tetramers of mutant TRPM8 channels with low affinity for menthol, Janssens and Voets estimated that each channel can bind up to four menthol molecules (Janssens and Voets 2011).

5.3 Antagonists of TRPM8

Identification of potent TRPM8 antagonists and their evaluation in vitro and in vivo have led to a large number of recent patents (reviewed by Malkia et al. 2010; Ferrer-Montiel et al. 2012) (Table 2). Some of these new compounds are effective in the nanomolar range and reduce cold hypersensitivity in different neuropathic pain models, overactive bladder, and melanoma proliferation. In contrast, the first published studies characterized antagonists that lacked selectivity, acting also on other TRP channels, like TRPV1. The list includes drugs like phenanthroline, capsazepine, BCTC, and the related thio-BCTC and CTPC, and the urea derivative SB-452533 (Behrendt et al. 2004; Tousova et al. 2004; Weil et al. 2005; Madrid et al. 2006; Malkia et al. 2007; Valero et al. 2012). TRPM8 has also been found to be potently inhibited by SKF96365 (Madrid et al. 2006; Malkia et al. 2007), a non-specific blocker of various calcium-permeable channels, including receptor-operated channels (Merritt et al. 1990), as well as by several members of the imidazole family of antimycotics, including clotrimazole and econazole (Meseguer et al. 2008; Malkia et al. 2009). AMTB inhibits icilin-evoked responses in TRPM8-expressing cells with moderate potency, exhibiting selectivity for TRPM8 over TRPV1 and TRPV4 (Lashinger et al. 2008). Recently, Ortar et al. reported on a large series of derivatives of (−)-methylamine with TRPM8 antagonism, showing selectivity over TRPA1 and TRPV1 (Ortar et al. 2010). Another recent study showed that the TRPM8 agonist icilin specifically inhibits other modes of TRPM8 activation independently of its interaction site within the S3 segment (Kuhn et al. 2009). Furthermore, the PLA_2 inhibitor p-amylcinnamoylantranilic acid (ACA) also directly inhibits TRPM8, TRPM2, and TRPC6 (Kraft et al. 2006).

Table 2 TRPM8 antagonists

Compound	IC_{50} cold (μM)	IC_{50} menthol (μM)
BCTC	0.68 ± 0.06^{a} (CI)	0.47 ± 0.01^{b} (CI)
	0.54 ± 0.04^{a} (EP)	0.34 ± 0.04^{b} (EP)
		0.143 ± 0.019^{c} (EP)
CTPC	N/A	0.131 ± 0.014^{c} (EP)
Thio-BCTC	N/A	3.5 ± 1.1^{d} (FL)
SB-452533	N/A	0.571 ± 0.077^{c} (EP)
SKF96365	1.0 ± 0.2^{a} (CI)	3 ± 1^{b} (CI)
	0.8 ± 0.1^{a} (EP)	
Econazole	0.42 ± 0.07^{e} (CI)	N/A
Clotrimazole	8 ± 1^{e} (CI)	1.2^{f} (EP)
ACA	N/A	3.9^{g} (FL)
AMTB	N/A	6.23 ± 0.02^{h} (FL)
Capsazepine	12 ± 2^{e} (CI)	18 ± 1^{d} (FL)
Phenanthroline	100 ± 20^{a} (CI)	N/A
	180 ± 20^{a} (EP)	
MAD1d	N/A	0.02 ± 0.002^{i} (SF)
MAD2e	N/A	0.1 ± 0.02^{i} (SF)
AMG9678	6.2 ± 1.9^{j} (BL)	31.2 ± 8.3^{j} (BL)
AMG2850	7.3^{j} (BL)	156 ± 110^{j} (BL)
Compound 496	12 ± 0.9^{j} (BL)	25.8 ± 6.6^{j} (BL)
PBMC	$<0.025^{k}$ (CI)	0.0006^{k} (EP)

Inhibitory potency of various blockers at the cold- or 100 μM menthol-activated TRPM8-wt channel as measured by calcium imaging microscopy (CI), a bioluminescence assay (BL), a fluorometric imaging plate reader assay (FL), a spectrofluorometer (SF), or patch-clamp electrophysiology (EP)

Whole-cell patch-clamp data are given at +80 $mV^{a,b,l}$, +50 mV^{e}, or −70 mV^{f}. Note: the results for AMTB were obtained with icilin rather than menthol. The results for MAD1d and MAD2e were obtained with 20 μM menthol at 22 °C

Data are from:

[a]Malkia et al. (2007)
[b]modified from (Madrid et al. 2006)
[c]Weil et al. (2005)
[d]Behrendt et al. (2004)
[e]Malkia et al. (2009)
[f]Meseguer et al. (2008)
[g]Kraft et al. (2006)
[h]Lashinger et al. (2008)
[i]Ortar et al. (2010)
[j]Gavva et al. (2012)
[k]Knowlton et al. (2011)

In addition, ethanol inhibits TRPM8 function at concentrations on the order of 0.5–3 % (Weil et al. 2005; Benedikt et al. 2007). The actions of TRPM8 blockers on native thermoreceptors are still relatively unexplored, with only a few studies

addressing the antagonism of TRPM8 in sensory neurons (Reid et al. 2002; Madrid et al. 2006; Malkia et al. 2007; Xing et al. 2007; Meseguer et al. 2008; Knowlton et al. 2011) and sensory nerve terminals (Madrid et al. 2006; Parra et al. 2010; Hirata and Oshinsky 2012).

The study by Malkia and colleagues (2007) showed that several antagonist compounds (e.g., BCTC and SKF96365) act as negative allosteric modulators of channel gating, shifting the voltage activation of TRPM8 towards more positive potentials, suppressing the depolarizing effects of temperature and chemical agonists. They showed that co-application of different concentrations of an agonists and an antagonist could be used to titrate the voltage-activation curve of TRPM8 to any desired value, leading to changes in thermal activation threshold. Tyrosine residue 745 at the menthol binding site (Bandell et al. 2006) is critical for inhibition mediated by the compound SKF96365 (Malkia et al. 2009). In contrast, the inhibition by BCTC is unaffected suggesting that at least one other binding site exists on the TRPM8 channel from where this drug exerts its negative allosteric modulation (Malkia et al. 2009).

5.4 Modulation of TRPM8

In excised membrane patches, activity of native TRPM8 channels results in rapid rundown, shifting the thermal activation towards lower temperatures (Reid and Flonta 2002). Moreover, thermodynamic analysis of TRPM8 gating in cell-free patches results in half activation temperatures below 20 °C (Zakharian et al. 2010). This value is clearly erroneous for more physiological conditions (e.g., intact neurons and nerve terminals), where temperature-dependent TRPM8 activity is evident at temperatures >30 °C (Madrid et al. 2006; Malkia et al. 2007; Parra et al. 2010; Fujita et al. 2013). These results suggest the presence of soluble cytosolic factors modulating channel activity. Indeed, several modulatory pathways have been shown to regulate TRPM8 function, altering its sensitivity to temperature and chemical agonists (reviewed by Yudin and Rohacs 2012).

5.4.1 TRPM8 Modulation by Phosphorylation

Protein phosphorylation constitutes one of the main signaling mechanisms modulating ion channel activity. Chemical activation of protein kinase C (PKC) inhibits TRPM8 activity in both heterologous expression systems and cold-sensitive sensory neurons from dorsal root ganglia (Premkumar et al. 2005; Abe et al. 2006; Linte et al. 2007). However, none of the nine putative PKC-phosphorylation sites identified on the TRPM8 channel were involved in the modulation, and PKC-mediated channel internalization is not implicated in this mechanism either. In agreement with these results, PKC activation does not increase the phosphorylation state of TRPM8 but produces a dephosphorylation of the channel by the phosphatase calcineurin. This dephosphorylation is calcium dependent and can be reversed by phosphatase inhibitors. A fraction of cold- and menthol-sensitive DRG neurons in culture respond to proinflammatory mediators: bradykinin, PGE_2, and histamine (Linte et al. 2007). In the presence of bradykinin,

the responses to cold and menthol are partially desensitized, and this effect is totally abrogated by selective PKC inhibitors (Premkumar et al. 2005; Linte et al. 2007). Still, very little is known about the expression of specific PKC isoforms in TRPM8-positive neurons.

TRPM8 activity is also modulated by the cAMP-dependent protein kinase A (PKA). Forskolin and 8-Br-cAMP, activators of the PKA pathway, reduced the response of TRPM8 channels to menthol and icilin (De Petrocellis et al. 2007). In agreement with this finding, application of 8-Br-cAMP reduced cold-evoked responses in cultured DRG neurons (Linte et al. 2007). Moreover, the inhibitory effects of PGE_2 on cold-sensitive neurons were partially reduced by a specific PKA inhibitor (Linte et al. 2007). In contrast, a recent study showed that basal activity of TRPM8 is dependent on phosphorylation by PKA and identified residues Ser9 and Thr17 in the channel N-terminal cytosolic tail as critical targets of this kinase (Bavencoffe et al. 2010). Moreover, they showed that Gi-coupled α2A-adrenoreceptors (α2A-AR) negatively modulate TRPM8 activity by repressing the signaling pathway ending in PKA activation. Stimulation of α2A-AR was also found to inhibit TRPM8-mediated currents in ~20 % of menthol-sensitive sensory neurons, suggesting that a subpopulation of neurons co-express the two proteins. While there are discrepancies as to the nature of TRPM8 modulation by PKA, these findings open a new field of TRPM8 modulation by signaling cross talk with other membrane receptors and channels. Prolonged activation of μ-opioid receptors (OPRM) with morphine also leads to a slowly developing, modest reduction in TRPM8 activity, due to the progressive internalization of the channel in a complex with OPRM1 (Shapovalov et al. 2013).

5.4.2 Direct TRPM8 Modulation by G Proteins

A recent study described the direct inhibition of TRPM8 by Gαq, explaining the inhibition of cold thermoreceptors produced by several inflammatory mediators, such as bradykinin and histamine (Zhang et al. 2012). A brief exposure to the pruritogen chloroquine inhibits TRPM8 by a direct action of activated Gαq (Than et al. 2013). Interestingly, a different study showed that TRPM8 activation can lead to activation of Gαq and calcium release from stores (Klasen et al. 2012).

5.4.3 TRPM8 Modulation by Lipids

The membrane trace phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) activates TRPM8 in the absence of chemical and thermal stimulation, prevents channel rundown in excised patches, and acts as a positive modulator of cold and menthol stimuli (Liu and Qin 2005; Rohacs et al. 2005; Phelps and Gaudet 2007). In lipid bilayers, exogenous PIP_2 facilitates gating of TRPM8 more effectively than other phosphoinositides (Zakharian et al. 2010). Neutralization of positive charges in the TRP domain of the TRPM8 C-terminus impairs interaction with the negatively charged head group of PIP_2, preventing channel activation, with the point mutant R1008Q resulting in the most dramatic phenotype, a 100-fold decrease in the apparent affinity for PIP_2 (Rohacs et al. 2005). Consequently, PLC-mediated PIP_2 hydrolysis completely abolishes TRPM8 activity (Liu and Qin 2005; Rohacs

et al. 2005). The PIP_2 hydrolysis products, DAG and IP_3, have no effect on channel activity indicating that this inhibition results directly from breakdown of PIP_2 rather than downstream signaling molecules and pathways. More recently, it was reported that PIP_2 depletion, or PLC-mediated PIP_2 hydrolysis, leads to a change in channel gating, shifting the voltage dependence towards more positive potentials, contrary to the effect exerted by channel agonists (Daniels et al. 2009). Surprisingly, these authors found that while reducing cold- and menthol-activated currents, this shift in the voltage dependence did not alter the thermal threshold or the menthol dose–response relationship of TRPM8. In contrast, another study found that warm ambient temperatures (e.g., 40 °C) shift the temperature threshold of TRPM8 to warmer values by an undefined mechanism involving PIP_2 but independent of extracellular calcium (Fujita et al. 2013).

TRPM8-dependent currents desensitize markedly in the presence of external calcium and removal of calcium from the extracellular solution reduces this desensitization to ~10 % (McKemy et al. 2002). Cold- and menthol-sensitive currents also desensitize in a Ca^{2+}-dependent manner in primary sensory neurons (Reid and Flonta 2001b). Since then, several reports have documented TRPM8 desensitization during subsequent stimuli of cold or menthol in the presence of divalent cations (Liu and Qin 2005; Rohacs et al. 2005; Malkia et al. 2007). The calcium-dependent adaptation exhibited by TRPM8 can be explained by Ca^{2+}-mediated activation of PLC and subsequent PIP_2 hydrolysis nearby the TRPM8 channel (Liu and Qin 2005; Rohacs et al. 2005; Daniels et al. 2009). A recent report (Mahieu et al. 2010) describes an additional mechanism for Ca^{2+}-induced adaptation. This work shows that increasing concentrations of extracellular divalent cations or protons displace the voltage dependence of TRPM8 activation towards more positive potentials. The results are rationalized by a Gouy–Chapman–Stern model indicating that the effect is due to the well-known phenomenon of surface charge screening (Hille 2001). A distinction should be made between acute desensitization, occurring during a single agonist application, and the reduction in peak response to paired stimuli, better known as tachyphylaxis. Recent work showed that, for TRPM8, both phenomena are calcium-dependent, but, while the former is triggered by calmodulin, the latter is dependent on PLC, PKC, and protein phosphatases, leading to PIP_2 depletion (Sarria et al. 2011).

5.4.4 TRPM8 Modulation by Growth Factors

Many TRPM8-positive neurons express TrkA, and application of its ligand NGF to neuronal cultures increases cold sensitivity of DRG neurons (Babes et al. 2004). Artemin, the ligand for GFRa3, also produces cold hypersensitivity in vivo, but its effects on TRPM8 are not known (Lippoldt et al. 2013).

5.4.5 Endogenous TRPM8 Modulators

Lysophospholipids (LPL) and polyunsaturated fatty acids (PUFA) constitute a distinct group of lipids that modulate TRPM8 channel activity in vitro (Vanden Abeele et al. 2006; Andersson et al. 2007; Bavencoffe et al. 2011) and in vivo

(Gentry et al. 2010). Both are released downstream of phospholipase A_2 (PLA_2) activation. Whereas application of LPLs activates TRPM8 channels in both intact cells and TRPM8-containing membrane patches, PUFAs such as arachidonic acid (AA) reduce the cold, icilin, and menthol sensitivity of heterologously expressed TRPM8 channels. Despite the antagonistic effect of the products released by PLA_2 on TRPM8 activity, the overall effect of PLA_2 inhibition is the suppresion of TRPM8 function (Vanden Abeele et al. 2006; Andersson et al. 2007).

The endovanilloid anandamide and the endocannabinoid NADA inhibit TRPM8 activity (De Petrocellis et al. 2007). Consistent with this finding, several plant-derived cannabinoids including cannabidiol, cannabidiol acid, cannabigerol, Δ^9-tetrahydrocannabinol, and Δ^9-tetrahydrocannabinol acid also inhibit menthol- and icilin-evoked Ca^{2+} increases in TRPM8-expressing cells with IC_{50} values in the submicromolar range (De Petrocellis et al. 2008).

5.4.6 TRPM8 Modulation by Polyphosphates and Polyhydroxybutyrate

Polyphosphates (PolyP) are negatively charged linear polymers of phosphates present in all organisms where they contributes to multiple cellular roles (Wang et al. 2003; Abramov et al. 2007). PolyP are known to form complexes with other endogenous polymers and associate with many membrane proteins, including ion channels. Cellular PolyP modulate TRPM8 activity (Zakharian et al. 2009; Cao et al. 2013). Enzymatic breakdown of intracellular PolyP significantly inhibited menthol-induced responses of TRPM8 without altering total TRPM8 protein level or PIP_2 levels in the plasma membrane. Biochemical analysis showed that PolyP together with its binding companion R-3-hydroxybutyrate associate with the TRPM8 protein in a stable complex, the disruption of which impairs channel activity. Interestingly, Kim and Cavanaugh (Kim and Cavanaugh 2007) also reported modulation of another thermoTRP, TRPA1, by inorganic polyphosphates. These observations suggest that polyphosphates could be important intracellular modulators of thermoTRP channels. As mentioned previously, the covalent bond between TRPM8 and extracellular polyhydroxybutyrate (PHB) is critical for TRPM8 gating (Cao et al. 2013). Whether this interaction is dynamically modulated is unknown at present.

5.4.7 TRPM8 Modulation by Plasma Membrane Compartmentalization

Lipid rafts are specialized regions within the bulk of fluid plasma membrane, enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-anchored proteins (reviewed by Lingwood and Simons 2010). They can concentrate or exclude specific proteins and lipids and facilitate protein–protein interactions important for modulation and signaling events. TRPM8 has been found to localize into lipid rafts and, TRPM8 activity and intramembrane lateral mobility is modulated by lipid raft association (Morenilla-Palao et al. 2009; Veliz et al. 2010). Depletion of cholesterol to disrupt lipid rafts produces a significant potentiation of TRPM8 responses to menthol and cold in both heterologous

expression systems and cultured sensory neurons (Morenilla-Palao et al. 2009). This potentiation arises from a shift in the voltage activation of the channel towards more negative potentials, whereas the maximum conductance remains unaffected. In turn, the negative shift in the voltage activation displaced the temperature threshold for TRPM8 activation towards warmer values, making the channel more sensitive to temperature below the physiological level (Morenilla-Palao et al. 2009).

6 Physiological Functions of TRPM8

In line with the initial description of TRPM8 expression in a subclass of thermosensitive peripheral sensory neurons, early studies focused on its potential role in thermosensation and pain (reviewed by McCoy et al. 2011; Viana and Fernández-Peña 2013). The physiological role of TRPM8 has been explored with selective pharmacological tools, as they became available, and analysis of the phenotype of transgenic animals with disruptions in the *Trpm8* gene (see below).

Cold temperatures evoke multiple sensations, going from pleasant to painful as temperature decreases. These sensations are thought to derive from activity in sensory fibers with different thermal thresholds, carrying sensory information in specific circuits (i.e., labeled lines) (Reid 2005; reviewed by Belmonte et al. 2009). Mild cold applied to the skin excites a small subset of peripheral somatosensory fibers (Hensel 1981). In the same fibers, menthol sensitizes the response to low temperatures (Schafer et al. 1986), the physiological basis for its cooling mimetic action. The sensory effects of cold and menthol have an obvious cellular correlate in primary sensory neurons. Both cooling and menthol evoke inward depolarizing currents leading to depolarization and firing (Fig. 4) (Viana et al. 2002; McKemy et al. 2002; Peier et al. 2002; Okazawa et al. 2002; Reid et al. 2002). The biophysical and pharmacological properties of the non-selective cation current (I_{cold}) found in cold-sensitive neurons (Reid and Flonta 2001b) are consistent with the properties of TRPM8-dependent currents in transfected cells (McKemy et al. 2002; Peier et al. 2002). Later work demonstrated that these neurons express TRPM8 (Takashima et al. 2007; Dhaka et al. 2008). It is important to emphasize that a fraction of peripheral cold-sensitive neurons does not express TRPM8, indicating the presence of additional cold sensors (Thut et al. 2003; Babes et al. 2006; Fajardo et al. 2008; Ciobanu et al. 2009).

Over the years, the importance of TRPM8 in noxious cold detection has gained strength (reviewed by Viana and Fernández-Peña 2013). The evidence is multiple, including hypersensitivity to cold (i.e., cold allodynia) in different pain models (neuropathic, inflammatory) and its attenuation with TRPM8 blockers (Xing et al. 2007; Knowlton et al. 2011), antisense oligonucleotides (Su et al. 2011), or in $Trpm8^{-/-}$ animals (Colburn et al. 2007). Importantly, the temperature-dependent excitability of individual sensory neurons is strongly modulated by the variable expression of potassium channels, allowing the expansion of the operating range of cold receptors (Viana et al. 2002; Madrid et al. 2009).

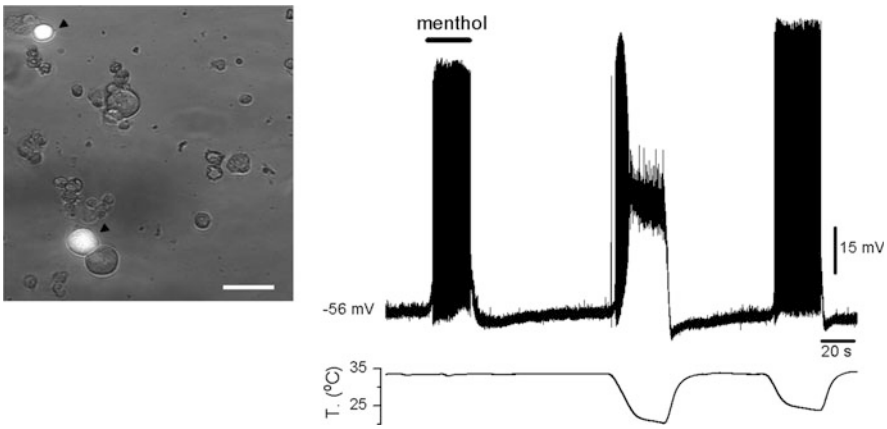

Fig. 4 Activation of TRPM8-expressing neuron by cold and menthol. (*Left*) Fluorescence and transmitted images superimposed, showing two adult mouse trigeminal ganglion sensory neurons expressing YFP (*black arrowhead*) under TRPM8 regulatory sequences. The calibration bar is 50 μm. (*Right*) Response of a TRPM8-YFP trigeminal ganglion neuron, recorded under perforated patch–current clamp, to menthol (100 μM) and to cooling ramps to 19 and 25 °C, respectively

TRPM8 has been detected in high-threshold mechanosensitive colonic afferent fibers expressing TRPV1. Activation of these afferents with icilin cross-desensitizes the response to capsaicin, suggesting a possible antinociceptive effect of TRPM8 in the gut (Harrington et al. 2011).

Exposed to a wide range of environmental temperatures, mammals keep a relatively stable core body temperature (Tb); they are homeotherms. Peripheral cold thermoreceptors are spontaneously active at neutral skin temperatures, sending a steady neural signal to the CNS. The increased activity of cold receptors in a cold environment initiates behavioral and autonomic thermoregulatory responses such as vasoconstriction and increased brown adipose tissue (BAT) thermogenesis, defending the system against heat loss. Similarly, chemical agonists of TRPM8 (menthol and icilin) produce increases in Tb. In contrast, antagonists of TRPM8 lower Tb, approximately 1 °C, in mice and rats (Knowlton et al. 2011; Almeida et al. 2012; Gavva et al. 2012). The reduction is relatively long-lasting. At higher ambient temperatures, the effect of TRPM8 antagonism on Tb is abolished. One study found that adipocytes in BAT express TRPM8. Activation of TRPM8 in BAT causes increased expression of UCP1 and elevated thermogenesis and prevents high-fat-diet-induced obesity (Ma et al. 2012). TRPM8 is also expressed in poikilotherms, animals that do not maintain constant body temperature. In the South African clawed frog *Xenopus laevis*, which lives in ecological niches with temperatures lower than mammalian Tb, activation threshold of TRPM8 is shifted to lower temperatures, suggesting their appropriate tuning to environmental conditions (Myers et al. 2009).

The physiological role of TRPM8 channels detected in the head and flagella of sperm (De Blas et al. 2009) is not clear. They form functional channels sensitive to

BCTC, menthol, icilin, and temperature (Martinez-Lopez et al. 2011). The induction of the acrosome reaction by menthol occurs at very high concentrations and is not fully blocked in *Trpm8*$^{-/-}$ mice (Martinez-Lopez et al. 2011). In a different study, CRISP4, a cysteine-rich protein produced in the proximal regions of the epididymis, was found to inhibit TRPM8 activity (Gibbs et al. 2011).

7 TRPM8: Lessons from Knockouts

Three different mouse transgenic lines null for TRPM8 have been evaluated in the context of temperature sensation, thermoregulation, inflammation, and pain (Dhaka et al. 2007; Colburn et al. 2007; Bautista et al. 2007). The results provide support for multiple physiological roles of TRPM8: cool temperature discrimination, noxious cold sensing, thermoregulation, cold-induced analgesia, and an anti-inflammatory role. The results reported in different studies are generally similar but with some notable differences (e.g., absolute temperature thresholds and reaction times) that may be explained by the intrinsic difficulty in examining aversive responses to cold temperatures in rodents (reviewed by McCoy et al. 2011; Viana and Fernández-Peña 2013).

Mice lacking functional TRPM8 channels exhibit a greatly impaired discrimination of innocuous cold temperatures (Dhaka et al. 2007; Colburn et al. 2007; Bautista et al. 2007) and reduced aversion to noxious cold temperatures (Knowlton et al. 2010). At the same time, these studies clearly show that cold thermosensation is not fully abrogated, neither are cold-evoked responses abolished in sensory neurons, pointing towards additional molecular cold sensors (Reid and Flonta 2001a; Viana et al. 2002; Story et al. 2003; Babes et al. 2006)

Two recent studies investigated the selective ablation of TRPM8-expressing cells using genetically engineered mice that expressed the diphtheria toxin receptor under control of TRPM8 transcriptional promoter. These mice showed profound deficits in the detection of innocuous and noxious cold temperatures (Knowlton et al. 2013; Pogorzala et al. 2013). Interestingly, these deficits were more severe than in *Trpm8*$^{-/-}$ animals, suggesting that these neurons express other cold-sensing mechanisms (e.g., potassium channels). In one study, thermoregulation was severely impaired. In addition, one study found that ablated animals lose the analgesic effect of cold temperature (Knowlton et al. 2013), revealing a dual role of TRPM8-expressing neurons: pronociceptive and antinociceptive.

The precise role of TRPM8 in thermoregulation and energy homeostasis is unclear. Activation of TRPM8 on the skin acts as a thermostat to prevent further cooling (Tajino et al. 2011). Subcutaneous icilin produces a hyperthermic effect, absent in Trpm8$^{-/-}$ mice (Knowlton et al. 2011). A high dose of the TRPM8 antagonist PBMC produced a severe hypothermic response, also absent in *Trpm8*$^{-/-}$ mice (Knowlton et al. 2011). Similarly, the hypothermic effect of M8-B, a TRPM8 antagonist, was abolished in *Trpm8*$^{-/-}$ mice, suggesting an on-target action (Almeida et al. 2012). *Trpm8*$^{-/-}$ mice show only a modest defect in thermoregulation and only under very specific environmental conditions.

A recent study found that *Trpm8*$^{-/-}$ mice, fed ad libitum, had reduced weight compared to wild types. *Trpm8*$^{-/-}$ animals were also very sensitive to damage of pancreatic islets with streptozotocin (STZ), an animal model of type I diabetes (McCoy et al. 2013). Trpm8$^{-/-}$ mice had a prolonged hypoglycaemia after an acute fasting period by a mechanism involving rapid insulin clearance.

Peripheral activation of afferents in the skin by cooling or direct activation of central afferent terminals with menthol resulted in slowing of the central pattern generator for locomotion in the spinal cord of neonatal mice. These effects were abolished in *Trpm8*$^{-/-}$ mice (Mandadi et al. 2009).

In mouse models of colitis, activation of TRPM8 with icilin (but not with menthol) attenuates the inflammatory response. These effects were not observed in *Trpm8*$^{-/-}$ animals (Ramachandran et al. 2013).

Studies in *Trpm8*$^{-/-}$ mice, combined with functional characterization of corneal nerve terminals, showed an important role of TRPM8 in basal and cold-evoked tearing, a mechanism important for proper humidification of the eye surface (Parra et al. 2010). In addition, menthol (100 μM) application fails to increase lacrimation in *Trpm8*$^{-/-}$ mice (Robbins et al. 2012).

8 TRPM8: Role in Hereditary and Acquired Diseases

Altered TRPM8 expression or function has been linked to different disease states. Considering that TRPM8 shows a relatively restricted expression in normal tissues, this anomalous expression offers interesting possibilities as a diagnostic or prognostic marker and for therapeutic intervention (reviewed by Malkia et al. 2010).

8.1 TRPM8 in the Diagnosis and Treatment of Different Types of Cancer

The original study by Tsavaler and colleagues showed that TRPM8 is markedly upregulated in prostate cancer and other malignancies (Tsavaler et al. 2001). This study was followed by several reports confirming TRPM8 expression in prostate cancer and prostate cancer cell lines (Bidaux et al. 2005; Prevarskaya et al. 2007; Valero et al. 2011). The expression of the classical isoform of TRPM8 in prostate is androgen-dependent, increasing with androgen treatment and declining with anti-androgen therapy. TRPM8 mRNA levels could thus be used to predict prostate cancer status. Furthermore, Bai et al. (2010) recently reported that TRPM8 mRNA can be detected in body fluids of men. While the mRNA levels in blood and urine were unchanged between healthy subjects and patients with localized prostate cancer, they increased significantly in patients with metastatic disease, offering a diagnostic tool for distinguishing the two stages of cancer.

However, the specific role(s) of TRPM8 in prostate cancer is unresolved. Zhang and Barritt (2004) found that overstimulation or inhibition of TRPM8 activity reduced the viability of androgen-dependent LNCaP cells. Moreover, in advance

prostate cancer, during the transition to androgen independence, TRPM8 levels are strongly reduced (Bidaux et al. 2005). Recently, it was found that the influence of TRPM8 on proliferation and migration of prostate epithelial cells was restricted to cancer cells (Valero et al. 2012). In contrast, prostate-specific antigen, a kallikrein, activates TRPM8 though bradykinin type 2 receptors (BR2), reducing migration of prostate cancer cells (Gkika et al. 2010). These results are somewhat puzzling, considering that BR2 couple to Gαq, and activation of this G protein exerts powerful inhibition of TRPM8 (Zhang et al. 2012).

Several recent studies have screened TRPM8 expression in other types of tumors. TRPM8 is virtually undetected in normal tissues, becoming highly enriched in the corresponding tumors. In melanoma cells, activation of TRPM8 by menthol leads to sustained calcium influx and reduced cell viability (Yamamura et al. 2008). In breast adenocarcinoma, TRPM8 is mainly overexpressed in small, grade I tumors (Dhennin-Duthille et al. 2011). Thus, contrary to TRPV6, expression of TRPM8 in these tumors is associated with a low proliferative index. In neuroendocrine pancreatic tumor cells, TRPM8 activation increases neurotensin secretion. In human glioblastoma cells, the effects of TRPM8 on cell migration appear to be secondary to changes in the activity of Ca-activated potassium channels (Wondergem and Bartley 2009). This result highlights the need to evaluate the role of TRPM8 channels in the context of multiple ionic mechanisms that are functionally coupled.

In a recent study, D-3263, an orally active TRPM8 agonist developed by Dendreon, underwent phase 1 clinical evaluation for the treatment of advanced solid tumors refractory to standard therapies (http://clinicaltrials.gov/ct2/show/study/NCT00839631). No clinical results have been reported yet. The therapeutic potential of D-3263 is based on the idea that an agonist of TRPM8 will provoke an increased entry of calcium and sodium into the cell, the disruption of calcium and sodium homeostasis, and the specific induction of cell death in TRPM8-expressing tumor cells. The apoptosis observed in prostate and melanoma cells following menthol application is consistent with this notion (Zhang and Barritt 2004; Yamamura et al. 2008; Yang et al. 2009).

8.2 TRPM8 in Diseases of the Urogenital Tract

Besides its relation to prostate cancer, TRPM8 has been linked to other diseases in the urogenital tract. Cold can stimulate contraction of the bladder and trigger the urge to urinate, the bladder cooling reflex. Hypersensitive afferent input from the bladder wall is thought to be involved in the overactive bladder syndrome (OAB) and pain bladder syndrome (PBS). In patients diagnosed with OAB, the density of TRPM8 protein in bladder afferents was increased (Mukerji et al. 2006). AMTB, a specific TRPM8 blocker, reduced the frequency of rat bladder contractions and the visceromotor response to bladder distension (Lashinger et al. 2008). Similarly, cold- or menthol-induced bladder detrusor overactivity in freely moving rats was prevented by the TRPM8 antagonist BCTC (Lei et al. 2013).

8.3 TRPM8 and Cold Pain

In healthy volunteers, a decrease in skin temperature from 34 to 2 °C evokes a monotonic increase in cold pain, with a variable threshold of 14–10 °C (Harrison and Davis 1999). The neural mechanisms of cold pain are complex and involve peripheral activation of nociceptors by low temperature and interactions between different afferent inputs in the CNS, which determine the ultimate sensory experience triggered by a cold stimulus (Fruhstorfer 1984; Yarnitsky and Ochoa 1990; Craig et al. 1996). Several findings indicate the possible involvement of TRPM8 channels in normal and pathological noxious cold sensations (reviewed by Knowlton and McKemy 2011; Viana and Fernández-Peña 2013). Notably, in animal models of neuropathic pain, cold allodynia is significantly attenuated by capsazepine, a TRPM8 blocker (Xing et al. 2007; Zuo et al. 2013), and mice lacking TRPM8 show reduced thermal responses in neuropathic models of cold pain (Dhaka et al. 2007; Colburn et al. 2007). Oxaliplatin is an antitumoral drug used in the treatment of colorectal cancer (Tabernero et al. 2007). A frequent side effect of oxaliplatin is the development of peripheral neuropathies accompanied by cold hypersensitivity. In animal models, oxaliplatin-induced cold pain is accompanied by upregulation of TRPM8 (Gauchan et al. 2009; Kawashiri et al. 2012), and TRPM8 activity plays a role in cold pain to oxaliplatin (Knowlton et al. 2011). Skin fibers immunoreactive for TRPM8 are substantially reduced in individuals suffering Norrbottnian congenital insensitivity to pain (Axelsson et al. 2009). These findings stress the potential use of TRPM8 modulators in the therapeutic management of cold-evoked pain. However, cold is also known to have analgesic effects on pain. TRPM8 agonists can relieve pain in certain neuropathic models (Proudfoot et al. 2006). This goes with the well-known fact that cooling the skin produces a soothing sensation on the background of inflammatory pain. The nervous circuitries involved in these analgesic effects of cold temperature are currently unclear.

8.4 TRPM8 and Migraine

Two genome-wide association studies have identified a robust relationship between genetic markers in or near the TRPM8 gene and susceptibility to migraine without aura, a primary headache of neurovascular origin (Chasman et al. 2011; Freilinger et al. 2012). At present, the role of TRPM8 in the pathogenesis of migraine is unclear.

8.5 TRPM8 and Amyloidotic Polyneuropathy

A recent study showed, using a combination of techniques, that TRPM8 is involved in calcium entry induced by a mutant form of transthyretin in growth cones of cultured DRGs. Because this mutated, insoluble form of transthyretin is involved in familial amyloidotic polyneuropathy, these results suggest a possible role of TRPM8 in the pathogenesis of this peripheral neuropathy (Gasperini et al. 2011).

8.6 TRPM8 and Tearing

Aqueous tears from lacrimal glands play and important protective function of the cornea. Activity in TRPM8-expressing corneal afferents is involved in the regulation of tear secretions (Madrid et al. 2006; Parra et al. 2010; Hirata and Meng 2010; Robbins et al. 2012). Therefore, modulation of TRPM8 function in cold thermoreceptors may be relevant for the pathophysiology and treatment of dry eye syndrome (Parra et al. 2010; Kurose and Meng 2013), a prevalent pathological condition characterized by irritation and pain of the ocular surface. Of note, cold corneal afferents have a relatively high basal firing rate, and this activity is entirely dependent on TRPM8 (Parra et al. 2010).

8.7 TRPM8 in Airway and Vascular Pathologies

During breathing, the mouth and the airways can be exposed to marked changes in temperature, detected by cold-sensitive afferents (Hensel and Zotterman 1951). Many nasal trigeminal sensory afferents express TRPM8, being particularly abundant around blood vessels (Keh et al. 2011). Activation of these afferents with cold temperature or menthol can reduce ventilation and suppress cough reflexes evoked by irritation of the lower airways (Orani et al. 1991; Willis et al. 2011; Plevkova et al. 2013). In addition, TRPM8 has been found in rat pulmonary artery myocytes and expression levels fell dramatically in animal models of pulmonary hypertension (Yang et al. 2006; Liu et al. 2013). It is unclear if TRPM8 downregulation contributes to pulmonary hypertension. Moreover, the vasorelaxing effects of menthol on pulmonary arteries could also be due to its known blocking effect on voltage-gated calcium channels (Swandulla et al. 1987), and studies on *Trpm8*$^{-/-}$ animals are required.

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Part IV

The TRPA1, TRPML and TRPP Subfamilies

TRPA1

Peter M. Zygmunt and Edward D. Högestätt

Contents

Abstract

The transient receptor potential ankyrin subtype 1 protein (TRPA1) is a nonselective cation channel permeable to Ca^{2+}, Na^{+}, and K^{+}. TRPA1 is a promiscuous chemical nocisensor that is also involved in noxious cold and mechanical sensation. It is present in a subpopulation of Aδ- and C-fiber nociceptive sensory

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neurons as well as in other sensory cells including epithelial cells. In primary sensory neurons, Ca^{2+} and Na^{+} flowing through TRPA1 into the cell cause membrane depolarization, action potential discharge, and neurotransmitter release both at peripheral and central neural projections. In addition to being activated by cysteine and lysine reactive electrophiles and oxidants, TRPA1 is indirectly activated by pro-inflammatory agents via the phospholipase C signaling pathway, in which cytosolic Ca^{2+} is an important regulator of channel gating. The finding that non-electrophilic compounds, including menthol and cannabinoids, activate TRPA1 may provide templates for the design of non-tissue damaging activators to fine-tune the activity of TRPA1 and raises the possibility that endogenous ligands sharing binding sites with such non-electrophiles exist and regulate TRPA1 channel activity. TRPA1 is promising as a drug target for novel treatments of pain, itch, and sensory hyperreactivity in visceral organs including the airways, bladder, and gastrointestinal tract.

Keywords

Nociceptor • Sensory transduction • Pain • Hydrogen sulfide • Hyperalgesia • Chemosensitivity • Mechanotransduction • Thermosensitive • Irritants • Allyl isothiocyanate • Menthol • Cannabinoid • Mustard oil • TRPA1 • TRPV1

1 Gene

In 1999, Jaquemar and coworkers described an mRNA transcript encoding a TRP-like protein (~1,100 amino acids) with 18 putative N-terminal ankyrin repeats in human fibroblasts (Jaquemar et al. 1999). The corresponding human gene, denoted *trpa1*, contains 27 exons and is located on chromosome 8 (8q13) (Nilius and Owsianik 2011; Story et al. 2003). Several TRPA1 homologues exist in the animal kingdom, and the ability of TRPA1 to sense potentially harmful electrophilic compounds has been conserved for ~500 millions of years, whereas the thermosensitive properties of TRPA1 have diverged later (Kang et al. 2010; Panzano et al. 2010). The sequence homology of TRPA1 in mammals is only 79 % between primates and rodents, which is important to consider when screening for TRPA1 active drugs for treatment of human diseases (Bianchi et al. 2012; Chen et al. 2013; Chen and Kym 2009).

2 Expression

In sensory neurons from trigeminal, dorsal root, and nodose ganglia, TRPA1 is expressed in both peptidergic and non-peptidergic neurons classified as Aδ- and C-fiber primary afferents (Figs. 1 and 2) (Andrade et al. 2012; Hjerling-Leffler

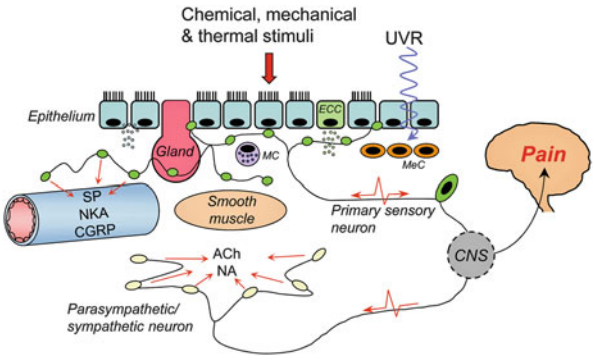

Fig. 1 The human body receives information about the external and internal environment through the somatic senses (thermo-, mechano-, and chemosensation), consisting of different types of primary sensory neurons with myelinated (Aβ), thinly myelinated (Aδ), and unmyelinated (C) nerve fibers. TRPA1 is present on Aδ- and C-fibers and on epithelial cells in the airways, gastrointestinal tract, bladder, and skin. Mucosal enterochromaffin cells (*ECC*), epidermal melanocytes (*MeC*) and mast cells (*MC*) also express TRPA1, which may play an important role in the regulation of gastrointestinal motility, UV radiation (UVR)-induced skin pigmentation and the innate immune system, respectively. In addition to pain signaling, primary sensory neurons participate in visceral reflexes, involving the release of acetylcholine (ACh) and noradrenaline (NA), as well as in local responses to tissue injury. Activation of TRPA1 on primary sensory neurons results in both afferent and efferent signaling. An influx of Na^+ and Ca^{2+} through TRPA1 triggers an action potential and a local release of sensory neuropeptides such as calcitonin gene-related peptide (CGRP), substance P (SP), and neurokinin A (NKA). In blood vessels these neuropeptides cause vasodilation and vascular leakage, signs of inflammation, and TRPA1 activation of the trigeminovascular system, including neurogenic CGRP-mediated vasodilation of cerebral arteries, may contribute to migraine and cluster headache (Benemei et al. 2013; Messlinger et al. 2012; Nassini et al. 2012). In airways, activation of sensory neurons can cause bronchospasm, cough, sneezing, congestion, rhinorrhea and itch (Alenmyr et al. 2009, 2011; Bautista et al. 2013; Taylor-Clark and Undem 2011). In the bladder and gastrointestinal tract, changed properties of sensory signaling are also believed to play an important role in diseases associated with sensory hyperreactivity (Andersson et al. 2010; Bautista et al. 2013; Birder 2013; Holzer 2011)

et al. 2007; Kim et al. 2010). TRPA1 is mostly found in a subpopulation of TRPV1-positive neurons, but non-TRPV1-containing neurons expressing TRPA1 exist, including a small population of myelinated Aβ-fibers, which are activated by innocuous mechanical force (Fig. 2) (Hjerling-Leffler et al. 2007; Kim et al. 2010; La et al. 2011). TRPA1 as well as TRPV1 is also present along the axon of primary afferents, the physiological significance of which is less obvious (Brenneis et al. 2011; Weller et al. 2011). Outside sensory neurons, TRPA1 is found in epithelial cells, melanocytes, mast cells, fibroblasts, odontoblasts, and enterochromaffin cells and β-cells of the Langerhans islets (Fig. 1) (Andrade et al. 2012; Baraldi et al. 2010; Bellono et al. 2013; Bellono and Oancea 2013; Buch et al. 2013; Cao et al. 2012; Earley 2012; Nilius et al. 2012; Oh et al. 2013; Prasad et al. 2008). Many of these cells have sensory properties and communicate with nearby nociceptors (Kwan et al. 2009; Lumpkin and Caterina 2007). TRPA1 may even

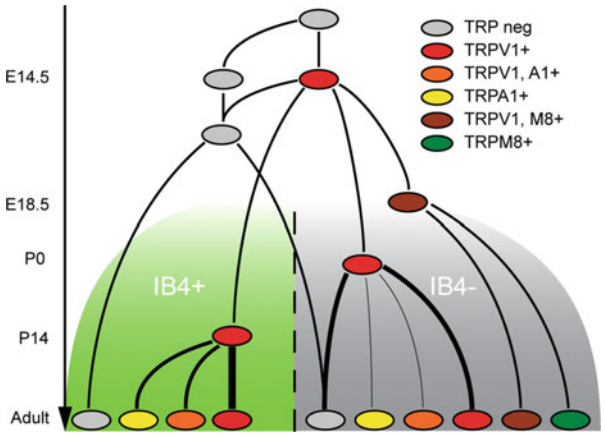

Fig. 2 In adult mouse sensory neurons from dorsal root ganglia, TRPA1 is expressed alone and together with TRPV1 both in peptidergic (IB4−) and non-peptidergic (IB4+) neurons. From a methodological point of view, it is important to consider that before adult age, the TRP expression pattern in sensory neurons is dynamic. From Hjerling-Leffler et al. (2007)

exist in the central nervous system (Vennekens et al. 2012). Importantly, the TRPA1 developmental expression pattern in mouse sensory neurons is highly dynamic before adult age (Fig. 2) and can change dramatically in pathological conditions including inflammatory and neuropathic diseases (Dai et al. 2007; Diogenes et al. 2007; Hjerling-Leffler et al. 2007; Ji et al. 2008; Katsura et al. 2006; Malin et al. 2011; Obata et al. 2005; Oh et al. 2013; Schwartz et al. 2011; Wang et al. 2008a).

3 The Channel Protein Including Structural Aspects

The mammalian TRPA1 belongs to the transient receptor potential (TRP) channel superfamily that consists of 28 different ion channels divided into six subgroups, of which TRPA1 constitutes its own subgroup (Nilius et al. 2012; Nilius and Owsianik 2011; Venkatachalam and Montell 2007). TRPA1 is a nonselective cation channel with six putative transmembrane segments (S1–S6), intracellular N- and C-termini, and a pore loop between S5 and S6 (Fig. 3). The N-terminus (half the size of the protein) contains between 14 and 18 ankyrin repeats that probably are important for protein–protein interactions and insertion of the channel into the plasma membrane (Gaudet 2008; Nilius et al. 2011). Because of the unusually large N-terminal ankyrin repeat domain, it is also possible that TRPA1 is involved in mechanosensation, in which the N-terminal could act as a link between mechanical stimuli and channel gating (Gaudet 2008; Howard and Bechstedt 2004). The N-terminal region contains a large number of cysteines, some of which can form a complex network of protein disulfide bridges within and between monomers (Fig. 4) (Cvetkov et al. 2011;

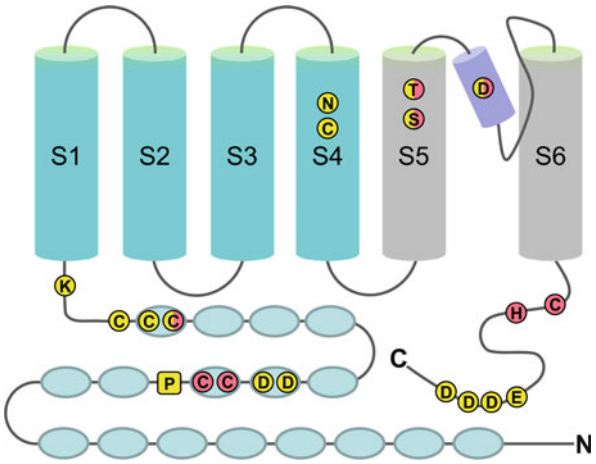

Fig. 3 The functional ion channel TRPA1 is a putative homotetrameric protein complex, where each monomer contains six transmembrane segments (S1–S6) with cytoplasmic N- and C-termini. Between S5 and S6 is the pore region, allowing Ca^{2+} and Na^{+} to enter the cell and K^{+} to flow in the outward direction at physiological membrane potentials. The pore loop Asp918 (D) is supposed to be critical for Ca^{2+} selectivity. The N-terminus with its many ankyrin repeats (*blue ovals*) contains cysteine (C) and lysine (K) residues that are targets for electrophiles and oxidants. Cysteines outside the N-terminus, such as Cys856 (C, S4), as well as other amino acid residues may also contribute to the complex regulation of TRPA1 gating. Hydroxylation of Pro394 (P) in the N-terminus reduces human TRPA1 activity at normoxia. A gain-of-function mutation at Asn855 (N) in S4 of human TRPA1 causes familial episodic pain syndrome. Menthol is a non-electrophilic compound that activates TRPA1 possibly by binding to the amino acid residues Ser873/Ser 877 and Thr874/Thr 878 (S and T) in S5 of the human/mouse TRPA1. Phosphatidylinositol-4,5-bisphosphate (PIP_2), which is cleaved by phospholipase C into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), may regulate TRPA1 activity by interacting with charged residues in the C-terminus, as suggested for TRPV1. Cysteine (C) and histidine (H) residues on the N- and C-termini of mouse TRPA1 are putative binding sites for Zn^{2+}, which potently activates TRPA1. The N- and C-termini contain putative binding sites for Ca^{2+} (D and E), which can sensitize or desensitize human TRPA1. Human and mouse amino acid residues are shown as *yellow* and *red*, respectively, and both colors for common residues

Eberhardt et al. 2012; Gaudet 2008; Wang et al. 2012). N-terminal cysteine and lysine residues are key targets for electrophilic TRPA1 activators, but cysteines outside the N-terminal region may also contribute to channel gating as such cysteines also bind electrophiles (Fig. 4) (Macpherson et al. 2007b; Takahashi et al. 2011; Wang 2012). Furthermore, the potent TRPA1 activator Zn^{2+} may bind to cysteine and histidine residues in the C-terminus (Andersson et al. 2009; Hu et al. 2009). The N- and C-termini have been suggested to contain binding sites for Ca^{2+} that can both sensitize and desensitize TRPA1 (Doerner et al. 2007; Jordt et al. 2004; Sura et al. 2012; Wang et al. 2008b; Zurborg et al. 2007).

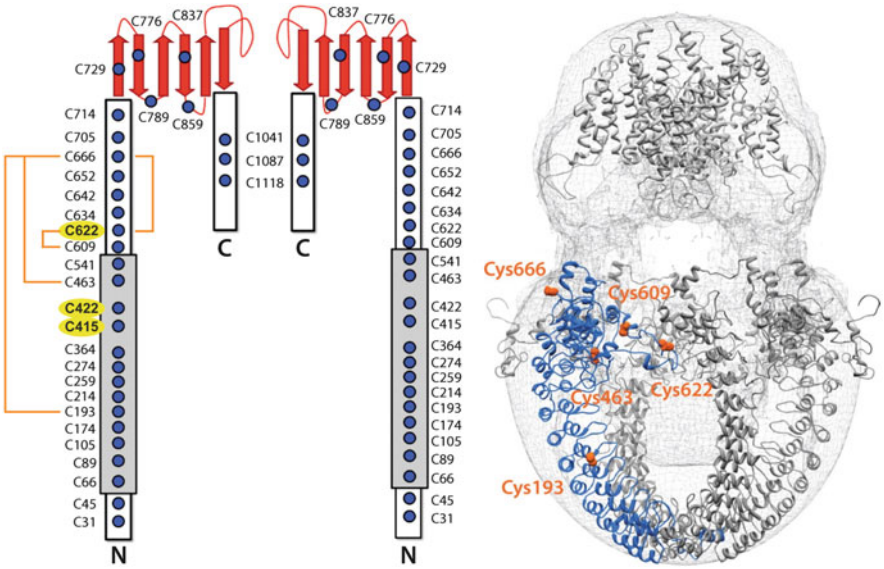

Fig. 4 As shown for the mouse TRPA1, N-terminal cysteines can form a dynamic network of disulfide bonds (*orange lines*), having a substantial impact on the global protein configuration and function. *Blue circles* show cysteine residues, and those identified as critical for activation by electrophilic compounds are highlighted in *yellow*. Notably, the mutation of C622 will affect several disulfide bonds and most likely have drastic effects on the overall protein function also affecting pharmacological interventions not targeting cysteine residues. Modified from Wang et al. (2012)

4 Interacting Proteins

Noxious heat sensitization evoked by bradykinin, which like other endogenous proalgesic or pro-inflammatory agents stimulates various Gq protein-coupled phospholipase C signaling pathways, was shown to be dependent on both TRPA1 and TRPV1, demonstrating a mutual relationship between these two ion channels (Bautista et al. 2006). It was suggested that a release of intracellular Ca^{2+} and an influx of Ca^{2+} via TRPV1 could lead to activation of TRPA1 (Fig. 5). Acute noxious heat responses with an activation threshold around 42 °C were initially expected to be entirely mediated by TRPV1, but studies of TRPV1 knockout mice indicated a contribution of other mechanisms within the temperature range of TRPV1 activation (Caterina et al. 1997, 2000; Davis et al. 2000). Indeed, a recent study provided evidence that TRPA1 contributes to the control of this heat threshold for acute nociceptive thermosensation (Hoffmann et al. 2013). Thus, because of its Ca^{2+} sensitivity, TRPA1 may act as an amplifier to TRPV1 under both normal and pathophysiological conditions (Bautista et al. 2006; Hoffmann et al. 2013). The opposite may also occur as TRPA1 can sensitize TRPV1 in a Ca^{2+} and protein kinase A-dependent manner (Anand et al. 2008; Spahn et al. 2013). In addition to

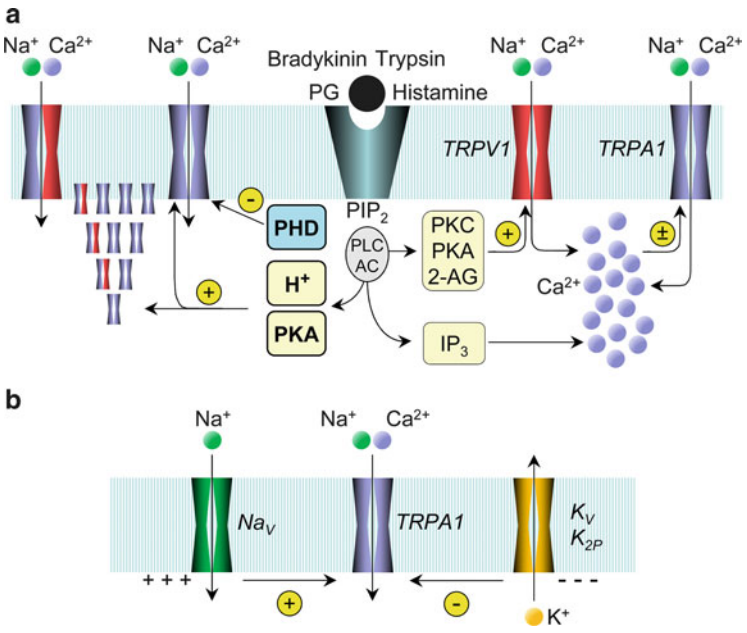

Fig. 5 TRPA1 is part of a complex protein signaling network. (**a**) Pro-inflammatory and pain-producing agents, such as bradykinin, histamine, prostaglandins (PG), and trypsin, acting on G protein-coupled receptors, stimulate via phospholipase C (PLC) or adenylate cyclase (AC) the production of intracellular mediators that directly or indirectly activate TRPV1 and TRPA1. TRPV1 is activated by protein kinase C (PKC), protein kinase A (PKA), and monoacylglycerols, such as 2-arachidonoyl glycerol (2-AG). An increased intracellular Ca^{2+} concentration via release from intracellular Ca^{2+} stores by the action of 1,4,5-triphosphate (IP_3) and influx through TRPV1 can stimulate the Ca^{2+}-sensitive TRPA1, which is also desensitized by Ca^{2+}. PLC stimulation also generates H^+ that can activate TRPA1. PKA may phosphorylate TRPA1 and increase its cell surface expression. The hydroxylation of Pro394 in the N-terminus by the oxygen-dependent prolyl hydroxylase (PHD) suppresses TRPA1 activity at normoxia. Importantly, this break is removed by hypoxia. There is also evidence that TRPA1 and TRPV1 physically interact and create unique hybrid channels. (**b**) Sodium and potassium channels are key in setting the resting membrane potential and the threshold of excitation in neurons. The increased activity in voltage-gated sodium channels (Na_V) by, e.g., ciguatoxins increases the excitability, so that also mild cold temperatures via activation of TRPA1 can evoke action potential discharge, causing cold allodynia, which is a major pain symptom in human chronic pain. Also, potassium channels, such as voltage-sensitive delayed rectifiers (K_V) and two-pore domain channels (K_{2P}), act as excitatory breaks in sensory neurons, and any change in their activity can dramatically affect the membrane excitability and TRPA1 responses

Ca^{2+}, other potential mediators or mechanisms of TRPA1 activation recruited by the phospholipase C signaling pathways include direct channel phosphorylation and the recruitment of TRPA1 to the cytoplasmic membrane by protein kinase A, possibly by interacting with the A-kinase anchor protein 5 (AKAP-5) and other proteins (e.g., CYLD) controlling cytoplasmic levels, trafficking, and turnover of TRPA1 (Fig. 5) (Dai et al. 2007; Schmidt et al. 2009; Stokes et al. 2006; Takahashi et al. 2012; Wang et al. 2008a; Zhang et al. 2008a). Notably, activation of TRPA1

or TRPV1 increased cytoplasmic surface expression of TRPA1, but not TRPV1 (Schmidt et al. 2009). The formation of functional TRPA1 and TRPV1 heterotetramers is another interesting interaction between these proteins (Fig. 5) (Akopian et al. 2007; Salas et al. 2009; Staruschenko et al. 2010).

Voltage-gated sodium and potassium channels are key players controlling cell excitability and intracellular Ca^{2+} levels (Fig. 5). Of particular importance is the expression of certain voltage-gated sodium channels ($Na_V1.7$, $Na_V1.8$, and $Na_V1.9$), and voltage-gated (K_V) and two-pore domain (K_{2P}) potassium channels in sensory neurons associated with perception of thermal, mechanical, and chemical stimuli (Belmonte et al. 2009; Momin and Wood 2008; Noel et al. 2011). As shown by the use of ciguatoxins, activation of Na_V and closure of K_V in sensory neurons induced TRPA1-dependent cold allodynia (Vetter et al. 2012). Also, the chemotherapeutic agent oxaliplatin downregulates the "excitability breaks" K_{2P} and K_V, and upregulates the pro-excitatory channels $Na_V1.8$, hyperpolarization-activated cyclic nucleotide-gated subtype 1 channel (HCN1), and TRPA1 in mouse dorsal root ganglia, whereas $Na_V1.9$ and TRPA1 are upregulated in rat dorsal root ganglia in diabetic animals explaining the lowered thresholds for cold and mechanical responses (Barriere et al. 2012; Descoeur et al. 2011).

TRPA1 activity may be controlled by secretogranin III, a protein present in various endocrine and neuroendocrine cells, as shown in mast cells and HEK293 cells overexpressing these proteins (Prasad et al. 2008). The hydroxylation of a proline residue in the TRPA1 N-terminus by the oxygen-dependent prolyl hydroxylase is believed to depress channel activity at normoxia (Takahashi et al. 2011). This "break" is removed by hypoxia and the prolyl hydroxylase inhibitor dimethyloxalylglycine, providing a potential role for TRPA1 in autonomous tissue adaptation to mild hypoxic conditions by controlling perivascular sensory neurons and tissue blood flow (Zygmunt 2011).

Taken together, a number of proteins that regulate TRPA1 either directly or indirectly have been proposed. The threshold for TRPA1-mediated responses is dependent on the cellular context, including the expression levels of TRPA1 and other ion channels regulating membrane excitability, the phosphorylation and hydroxylation state of TRPA1, and the cytoplasmic Ca^{2+} level (Fig. 5). This is in line with the view that the combinatorial expression of different ion channels, and not a single sensory channel transducer, determines the sensory output (Belmonte and Viana 2008).

5 A Biophysical Description of the Channel Function, Permeation, and Gating

The TRPA1 protein is a nonselective cation channel permeable to both monovalent and divalent ions including Ca^{2+}, Na^+, and K^+. TRPA1 has a high Ca^{2+} permeability compared to most other TRP ion channels and a unitary conductance of ~70 pS and ~110 pS in the inward and outward directions, respectively, under physiological conditions (cell-attached patch configuration and the presence of extracellular Ca^{2+}

and Mg^{2+}) when the channel is constitutively open (Nilius et al. 2011, 2012). The presence of Ca^{2+} and Mg^{2+} in the extracellular solution has a major impact on channel activity, and the single-channel conductance is generally smaller at negative than at positive membrane potentials. However, this voltage dependency is lost in the absence of divalent ions (Nilius et al. 2011). Measurements of the single-channel conductance of wild-type human, rat, and mouse TRPA1 have generated values between 40 and 180 pS depending on the experimental conditions and stimuli used (Table 1). Whether differences in channel conductance related to species exist is yet to be determined.

Interestingly, the TRPA1 pore, the size of which has been estimated as 11.0 Å, can undergo dilation, as shown in the presence of TRPA1 activators, thereby increasing the Ca^{2+} permeability and allowing larger charged molecules to pass through the channel (Banke et al. 2010; Chen et al. 2009; Karashima et al. 2010). This feature of TRPA1 as well as TRPV1 has been explored as an entrance for cell membrane impermeable local anesthetics into sensory neurons to achieve selective nociceptor fiber block (Brenneis et al. 2014). The amino acid residue Asp918 in the putative ion selectivity filter of the pore seems important in determining Ca^{2+} as well as Zn^{2+} permeation through the channel (Fig. 3) (Hu et al. 2009; Karashima et al. 2010; Wang et al. 2008b). This is further supported by the dramatic decrease of TRPA1 single-channel conductance from 111 ± 7 pS to 37 ± 5 pS when Asp918 is replaced by glutamine (D918Q) (Nilius et al. 2011). The pore vestibule is a target not only for the nonselective TRP channel blocker ruthenium red but also for newly developed TRPA1 antagonists such as AZ465, AZ868, and A-967079, whereas HC030031 most likely binds to channel structures outside the pore (Klement et al. 2013; Nyman et al. 2013).

Although there is no apparent voltage sensor in transmembrane segment 4, as shown for voltage-gated potassium channels, TRPA1 displays some voltage dependency, although less pronounced compared to TRPV1 and TRPM8 (Karashima et al. 2009; Samad et al. 2011; Voets et al. 2004). A single point mutation in transmembrane segment 4 of the human TRPA1 causes a shift in channel gating properties with a dramatic increase of inward currents and activation at normal resting potentials (Kremeyer et al. 2010). It has been suggested that a putative voltage-sensing domain of positively charged amino acid residues within the C-terminus could act as a sensor for changes in transmembrane voltage (Samad et al. 2011).

Both inorganic polyphosphates (e.g., PPPi) and Mg-ATP, which stimulates phosphatidylinositol 4-kinase activity and the generation of membrane bound phosphatidylinositol-4,5-bisphosphate (PIP_2), have been shown to preserve TRPA1 single-channel activity in excised inside-out cell membrane patches, suggesting that intracellular modulators mimicking the action of polyphosphates exist and that TRPA1 function is modulated by PIP_2 (Cavanaugh et al. 2008; Karashima et al. 2008; Kim and Cavanaugh 2007). The sites at which polyphosphates and PIP_2 interact with TRPA1 are not resolved, but could include the N-terminal ankyrin repeat domain and the channel pore (polyphosphates) and both the N- and C-termini (PIP_2) (Nilius et al. 2011; Sura et al. 2012). However, the

Table 1 TRPA1 single-channel conductance values

Stimuli	Single-channel conductance	Species	Cell and test condition	References
AITC	98 ± 13 pS (−60 mV)	Mouse	HEK293 (c-a); Ca^{2+} and Mg^{2+}	Nagata et al. (2005)
	101.8 ± 10.9 pS (−60 mV)	Human	HEK293 (c-a); Mg^{2+}	Hinman et al. (2006)
	103 ± 2.9 pS (−100 to +100 mV)	Human	HEK293 (c-a); Mg^{2+}	Hill and Schaefer (2007)
	66 ± 3 pS (−40 mV) 112 ± 4 pS (+40 mV)	Mouse	HeLa (i-o); Mg^{2+}, PPPi	Kim and Cavanaugh (2007)
	64 ± 2 pS (−40 mV) 115 ± 3 pS (+40 mV)	Rat	Trigeminal neuron (i-o); Mg^{2+}, PPPi	Kim and Cavanaugh (2007)
	64.2 ± 5.9 pS (−60 mV)	Rat	HEK293F (c-a); Mg^{2+}	Chen et al. (2008)
	48 pS (−100 mV to 0 mV) 134 pS (0 mV to +140 mV)	Mouse	CHO (i-o); Mg^{2+}, Mg-ATP	Karashima et al. (2010)
Apomorphine	86 pS (−80 to +80 mV)	Human	HEK293 (i-o); Mg^{2+}	Schulze et al. (2013)
Ca^{2+}	119 ± 6.3 pS (−80 mV)	Human	HEK293 (i-o)	Doerner et al. (2007)
CMP1	65.8 ± 6.5 pS (−60 mV)	Rat	HEK293F (c-a); Mg^{2+}	Chen et al. (2008)
Cinnamaldehyde	148 ± 3 pS (− 80 mV)	Rat	HEK293 (c-a); Mg-ATP	Wang et al. (2008b)
Cold	91 ± 4 pS (25 °C, +50 mV) 40 ± 2 pS (10 °C, +50 mV)	Mouse	CHO (c-a); Ca^{2+} and Mg^{2+}	Karashima et al. (2009)
CS gas	120 ± 3 pS (−40 mV)	Human	CHO-K1 (i-o); Mg^{2+}, PPPi	Bessac et al. (2009)
FTS	79 ± 1 pS (−80 to +80 mV)	Human	CHO-TREx (i-o); Mg^{2+}	Maher et al. (2008)
HDI	127 ± 4 pS (−40 mV) 51 ± 4 pS (−40 mV)	Human	CHO-K1 (c-a); Mg^{2+} CHO-K1 (c-a); Ca^{2+} and Mg^{2+}	Bessac et al. (2009)
H_2O_2	94 ± 3 pS (−100 to +100 mV)	Mouse	CHO (c-a); Mg^{2+}	Andersson et al. (2008)
	42 ± 0.5 pS (−50 mV) 73.0 ± 1.0 pS (+50 mV)	Human	CHO (c-a); Ca^{2+} and Mg^{2+}	Bessac et al. (2008)
Hyperoxia	97.3 ± 2.0 pS (−80 to +20 mV) 180.6 ± 10.7 pS (+40 to +80 mV)	Human	HEK293T (i-o); Mg^{2+}, PPPi	Takahashi et al. (2011)
Isovelleral	45 ± 1 pS (−60 mV) 75 ± 1 pS (+60 mV)	Human	CHO (c-a); Ca^{2+} and Mg^{2+}	Escalera et al. (2008)

Methylglyoxal	70–80 pS (−20 to −60 mV) 120 pS (+60 mV) 150 pS (+100 mV)	Mouse	CHO (i-o); MgATP	Andersson et al. (2013)
MIC	124 ± 3 pS (−40 mV)	Human	CHO-K1 (i-o); Mg^{2+}, PPPi	Bessac et al. (2009)
NMM	102.7 ± 12.9 pS (−60 mV)	Human	HEK293 (c-a); Mg^{2+}	Hinman et al. (2006)
Δ^9-THC	63 ± 4 pS (−40 mV) 82 ± 6 pS (+40 mV)	Mouse	HeLa (i-o); Mg^{2+}	Kim et al. (2008)
	80 ± 3 pS (+40 mV)	Rat	Trigeminal neuron (o-o); Mg^{2+}	Cavanaugh et al. (2008)
Trinitrophenol[a]	106 ± 5.5 pS (−100 to +100 mV)	Human	HEK293 (c-a); Mg^{2+}	Hill and Schaefer (2007)
URB597	40 pS (−80 to +80 mV)	Rat	HEK293F (i-o); Mg^{2+}	Niforatos et al. (2007)
Voltage	139 ± 2 pS (−80 to +80 mV)	Rat	HEK293 (c-a); MgATP	Wang et al. (2008b)
	48 pS (−100 mV to 0 mV) 113 pS (0 mV to +140 mV)	Mouse	CHO (i-o); Mg^{2+}, MgATP	Karashima et al. (2010)
	71 pS (−100 mV to 0 mV) 110 pS (0 mV to +80 mV)	Mouse	CHO (c-a); Ca^{2+} and Mg^{2+}	Nilius et al. (2011)
	110 pS (−100 to +100 mV		Divalent-free solution	
Zn pyrithione	83 ± 1 pS (−60 mV)	Mouse	CHO (c-a); Mg^{2+}	Andersson et al. (2009)

AITC, allyl isothiocyanate; CMPI, (4-methyl-*N*-[2,2,2-trichloro-1-(4-nitro-phenylsulfanyl)-ethyl]-benzamide); CS gas, 2-chlorobenzalmalononitrile; FTS, farnesyl thiosalicylic acid; HDI, hexamethylene diisocyanate; MIC, methyl isocyanate; NMM, *N*-methylmaleimide; URB597, 3′-carbamoylbiphenyl-3-yl cyclohexylcarbamate; PPPi, polyphosphates; Δ^9-THC, Δ^9-tetrahydrocannabinol

(c-a) Cell-attached patch configuration; (i-o) excised inside-out patch configuration; (o-o) excised outside-out patch configuration

[a]Trinitrophenol may act indirectly to evoke mechanical activation of TRPA1

role of PIP_2 in TRPA1 channel gating is debated (Dai et al. 2007; Karashima et al. 2008; Kim et al. 2008; Wang et al. 2008b). In contrast, there is no doubt that one of the most important cytosolic regulators of TRPA1 is Ca^{2+}, which has been suggested to gate TRPA1 directly by binding to sites on the N- and C-termini (Doerner et al. 2007; Jordt et al. 2004; Sura et al. 2012; Wang et al. 2008b; Zurborg et al. 2007). The bimodal effect of Ca^{2+} on TRPA1, potentiation or activation at low and inhibition at high intracellular concentrations, most likely reflects independent processes (Wang et al. 2008b). Interestingly, whereas Ca^{2+} and electrophilic compounds require the presence of polyphosphates to activate TRPA1 in excised inside-out cell membrane patches, Δ^9-tetrahydrocannabinol does not, further adding to the intriguing complexity of TRPA1 channel gating (Cavanaugh et al. 2008; Kim and Cavanaugh 2007).

6 Physiological Functions in Native Cells, Organs, and Organ Systems

In addition to its clear function as a chemosensor, there is growing evidence that TRPA1 is involved in thermo- and mechanosensation. Whether TRPA1 is intrinsically thermo- or mechanosensitive remains, however, to be determined.

6.1 TRPA1 and Chemosensation

The proposal of an ionotropic cannabinoid receptor belonging to the TRP ion channel family (Zygmunt et al. 2002), also being sensitive to irritants (Fig. 6), and the frequent use of mustard oil in animal models of acute pain and hyperalgesia prompted a search for Δ^9-tetrahydrocannabinol and mustard oil-sensitive clones upon expression of a rat trigeminal ganglia cDNA library (Jordt et al. 2004). This led us to the identification of the rat and human TRPA1 as ionotropic cannabinoid receptors and targets for mustard oil (Jordt et al. 2004). Another study identified mouse TRPA1 as a target for both electrophilic and non-electrophilic compounds (Bandell et al. 2004), and this also provided a molecular target by which cinnamaldehyde and related spicy compounds induced sensory nerve-mediated vasodilation (Fig. 6). Shortly after, we and others showed that certain thiol-reactive garlic-derived compounds activated TRPA1 (Bautista et al. 2005; Macpherson et al. 2005). Targeted gene mutations identified three cysteines present in the N-terminal region of the human (Cys621, Cys641, and Cys665) and mouse (Cys415, Cys422, and Cys622) TRPA1 as important for electrophilic TRPA1 channel activation (Figs. 3 and 4) (Hinman et al. 2006; Macpherson et al. 2007a). Together, these studies revealed TRPA1 as a detector of thiol-reactive electrophiles and oxidants in addition to non-electrophilic compounds as well as being indirectly regulated by G protein-coupled receptor signaling including the bimodal action of Ca^{2+}.

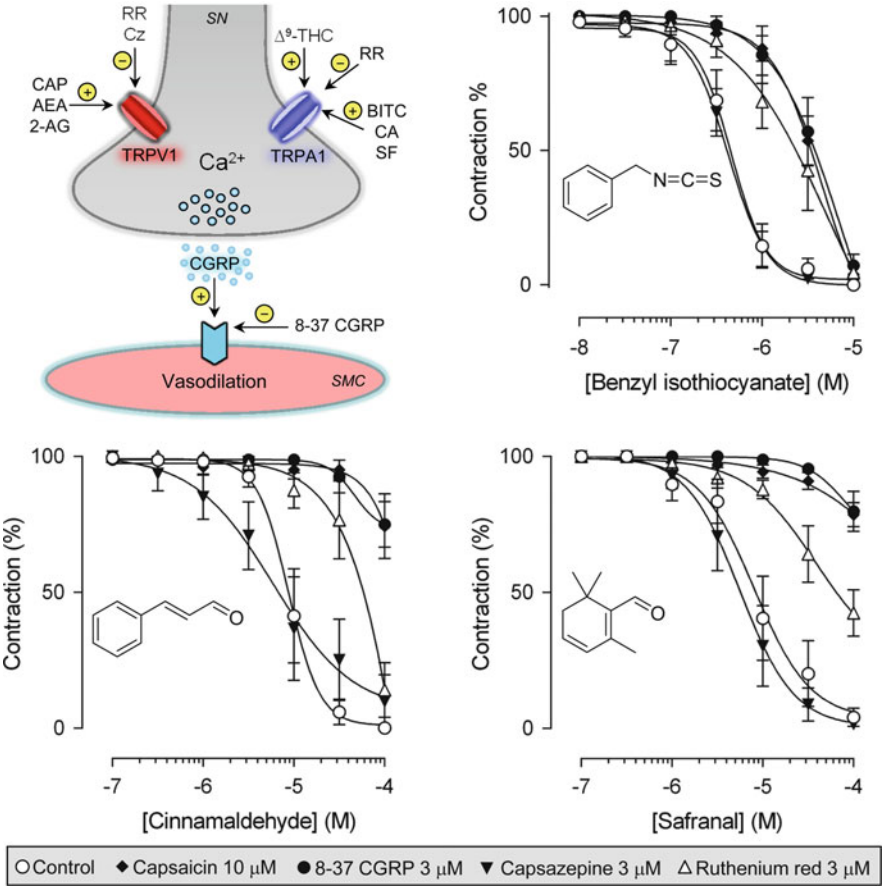

Fig. 6 Early screening of known irritants, using the rat mesenteric artery as a bioassay for sensory neuronal signaling, identified benzyl isothiocyanate (BITC) and similar mustard oil compounds, cinnamaldehyde (CA), and safranal (SF) as activators of a putative TRP channel (TRPA1) on TRPV1-containing primary sensory neurons (authors' unpublished data, 2003 and 2004). Data are expressed as mean ± SEM of five to eight independent experiments (animals) performed as previously described (Zygmunt et al. 2002). This bioassay has also been useful for identifying anandamide (AEA) and similar endogenous *N*-acyl ethanolamines as well as monoacylglycerols, including 2-arachidonoyl glycerol (2-AG), as endovanilloids acting on the capsaicin (CAP) receptor TRPV1 (Movahed et al. 2005; Zygmunt et al. 1999, 2013). Influx of Ca^{2+} through TRPV1 and TRPA1 causes release from sensory neurons (*SN*) of the calcitonin gene-related peptide (CGRP), which through activation of its receptor on the vascular smooth muscle cell (*SMC*) stimulates cyclic adenosine monophosphate (cAMP) production, leading to vasodilation. BITC, CA, and SF displayed a similar pharmacological vasodilator profile as the cannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabinol (Zygmunt et al. 2002); reduced vasodilation after depletion of CGRP-containing perivascular sensory neurons by capsaicin (CAP) or in the presence of the CGRP receptor antagonist (8–37 CGRP) or the nonselective TRP channel blocker ruthenium red (RR), but not in the presence of capsazepine that blocks the vasodilation by TRPV1 agonists (CAP, AEA, and 2-AG) (Zygmunt et al. 1999, 2002, 2013)

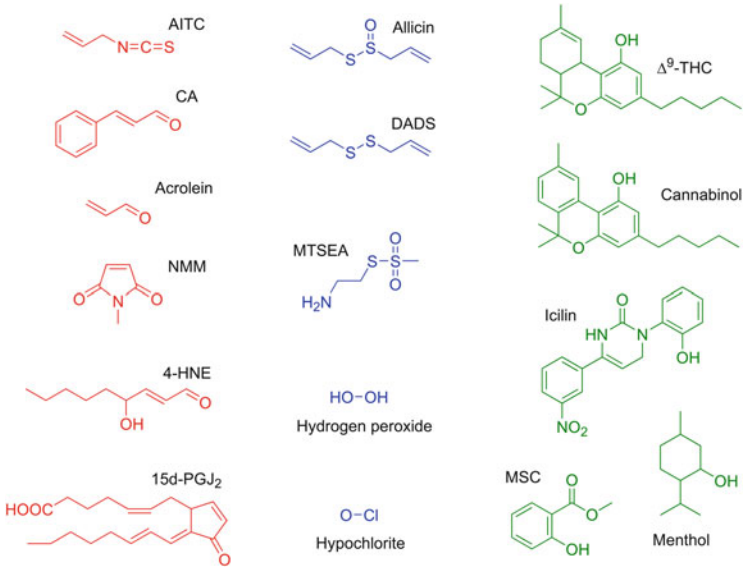

Fig. 7 Chemical structures of key compounds initially used to define TRPA1 as a chemosensor, responding to both exogenous and endogenous cysteine-reactive electrophiles and oxidants as well non-electrophilic compounds. *Red compounds* (*left panel*) bind to cysteines by strong covalent mechanisms, whereas some weaker electrophiles and oxidants, shown as *blue* (*middle panel*), promote disulfide formations within the TRPA1 protein that can be rectified by thiol-reducing agents, such as dithiothreitol. Importantly, the ability of noncovalent compounds (shown as *green*, *right panel*), including the analgesic plant cannabinoids Δ^9-THC and C16 (not shown), to activate TRPA1 may provide opportunities to treat human pain using a TRPA1 agonistic approach (Andersson et al. 2011). This also raises the possibility that chemically similar endogenous compounds may exist to control TRPA1 activity. *AITC* allyl isothiocyanate, *CA* cinnamaldehyde, *NMM* *N*-methylmaleimide, *4-HNE* 4-hydroxy-2-nonenal, *DADS* diallyl disulfide, *MTSEA* 2-aminoethyl methanethiosulfonate, *Δ^9-THC* Δ^9-tetrahydrocannabinol, *MSC* methyl salicylate

Today, a large number of compounds are known to activate mammalian TRPA1 (Fig. 7, Table 2). The ability to activate TRPA1 may explain why the use of apomorphine, disulfiram, glibenclamide, and in the past clioquinol in drug treatment is associated with pain and nausea (Andersson et al. 2009; Babes et al. 2013; Maher et al. 2008; Schulze et al. 2013). Many endogenous and exogenous irritants, including those in spices, mainly bind to cysteine residues in the N-terminus (Andrade et al. 2012; Baraldi et al. 2010; Holzer 2011; Nilius and Appendino 2013; Nilius et al. 2012; Takahashi and Mori 2011). However, other amino acids such as lysine in the N-terminus and cysteines outside the N-terminal region are potential targets for electrophiles and oxidants and may contribute to the regulation of TRPA1 (Eberhardt et al. 2012; Escalera et al. 2008; Ibarra and Blair 2013; Macpherson et al. 2007a; Nilius et al. 2012; Takahashi et al. 2011; Wang et al. 2012). In this context, it is important to remember that several of the N-terminal cysteines may form a complex network of protein disulfide bridges within and between monomers (Fig. 4), and thus, it is not unlikely that mutations of

Table 2 TRPA1 activators

Activator	References
Acetaldehyde and related α,β-unsaturated aldehydes	Bang et al. (2007), Bautista et al. (2006), Macpherson et al. (2007b)
1´-Acetoxychavicol acetate	Narukawa et al. (2010)
N-acetyl-*p*-benzoquinoneimine	Andersson et al. (2011), Nassini (2010)
Acrolein	Bautista et al. (2006)
Allicin	Bautista et al. (2005), Macpherson et al. (2005)
Allyl isothiocyanate	Bandell et al. (2004), Jordt et al. (2004)
2-Aminoethyl methanethiosulfonate	Macpherson et al. (2007a)
Apomorphine	Schulze et al. (2013)
p-Benzoquinone	Andersson et al. (2011)
Benzyl isothiocyanate	Jordt et al. (2004)
Bupivacaine	Brenneis et al. (2014)
Ca^{2+}, Cd^{2+}, Cu^{2+}	Andersson et al. (2009), Jordt et al. (2004)
Caffeine	Nagatomo and Kubo (2008)
Camphor	Alpizar et al. (2013)
Cannabinol	Jordt et al. (2004)
Capsiate	Shintaku et al. (2012)
Carbon dioxide (CO_2)	Wang et al. (2010)
Carvacrol	Xu et al. (2006)
Chlordantoin	Maher et al. (2008)
Chlorpromazine	Hill and Schaefer (2007)
Cinnamaldehyde	Bandell et al. (2004)
Citral	Stotz et al. (2008)
Clioquinol	Andersson et al. (2009)
Clotrimazole	Meseguer et al. (2008)
CMP1 and other thioaminal analogs	Chen et al. (2008)
Croton aldehyde	Andre et al. (2008)
Cyclopentenone prostaglandin metabolites	Andersson et al. (2008), Cruz-Orengo et al. (2008), Maher et al. (2008), Materazzi et al. (2008), Takahashi et al. (2008), Taylor-Clark et al. (2008b)
Diallyl disulfide	Bautista et al. (2005), Macpherson et al. (2005)
Diclofenac	Hu et al. (2010)
1,4-Dihydropyridines	Fajardo et al. (2008b)
Disulfiram	Maher et al. (2008)
Disulfides	Sawada et al. (2008), Takahashi et al. (2011)
Etomidate	Matta et al. (2008)
Eugenol	Bandell et al. (2004)
Farnesyl thiosalicylic acid	Maher et al. (2008)
Flufenamic acid	Hu et al. (2010)
Flurbiprofen	Hu et al. (2010)
Formaldehyde	Macpherson et al. (2007b), McNamara et al. (2007)

(continued)

Table 2 (continued)

Activator	References
Geraniol	Stotz et al. (2008)
Gingerol	Bandell et al. (2004)
GsMTx-4	Hill and Schaefer (2007)
Hexamethylene diisocyanate	Bessac et al. (2009)
Hydrogen sulfide (H_2S from NaHS)	Streng et al. (2008)
Hydrogen peroxide (H_2O_2), superoxide (O_2^-), and other reactive oxygen species	Andersson et al. (2008), Bessac et al. (2008), Sawada et al. (2008)
Hydroxide (OH^-)	Dhaka et al. (2009), Fujita et al. (2008)
4-Hydroxy-2-nonenal, 4-hydroxyhexenal	Andersson et al. (2008), Macpherson et al. (2007b), Taylor-Clark et al. (2008a), Trevisani et al. (2007)
Hypochlorite (OCl^-)	Bessac et al. (2008)
Icilin	Story et al. (2003)
Indomethacin	Hu et al. (2010)
Isoflurane, desflurane	Eilers et al. (2010), Matta et al. (2008)
Isopropyl isothiocyanate	Jordt et al. (2004)
Isovelleral	Escalera et al. (2008)
Ketoprofen	Hu et al. (2010)
Lidocaine	Leffler et al. (2008)
Ligustilide	Zhong et al. (2011b)
Linalool	Riera et al. (2009)
Menthol	Karashima et al. (2007)
Methylglyoxal	Andersson et al. (2013), Cao et al. (2012), Eberhardt et al. (2012), Koivisto et al. (2012), Ohkawara et al. (2012)
Methyl *p*-hydroxybenzoate and analogs	Fujita et al. (2007)
N-methyl maleimide	Hinman et al. (2006)
Methyl isocyanate	Bessac et al. (2009)
Methyl isothiocyanate	Jordt et al. (2004)
Methyl salicylate	Bandell et al. (2004)
Mefenamic acid	Hu et al. (2010)
Morphine	Forster et al. (2009)
Naphthalene metabolites	Lanosa et al. (2010)
Niflumic acid	Hu et al. (2010)
Nicotine	Talavera et al. (2009)
Nitric oxide (NO donors), peroxynitrite ($ONOO^-$ generators), and other reactive nitrogen species	Miyamoto et al. (2009), Sawada et al. (2008), Takahashi et al. (2008)
Nitro-oleic acid	Sculptoreanu et al. (2010), Taylor-Clark et al. (2009a)
5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and analogs	Liu et al. (2010)
Oxygen (O_2)	Takahashi et al. (2011)

(continued)

Table 2 (continued)

Activator	References
4-Oxo-nonenal	Andersson et al. (2008), Taylor-Clark et al. (2008a)
Ozone (O_3)	Taylor-Clark and Undem (2010)
6-Paradol	Riera et al. (2009)
Parthenolide	Materazzi et al. (2013)
Perillaldehyde, perilla ketone	Bassoli et al. (2009)
Phenethyl isothiocyanate	Jordt et al. (2004)
Phenylarsine oxide	Karashima et al. (2008)
Phytocannabinoids	De Petrocellis et al. (2008)
Piperine and other constituents of black pepper	Okumura et al. (2010)
Polygodial	Escalera et al. (2008)
Polyunsaturated fatty acids (PUFAs)	Motter and Ahern (2012)
Probenecid	McClenaghan et al. (2012)
Propofol	Fischer et al. (2010), Lee et al. (2008), Matta et al. (2008)
Proton (H^+)	de la Roche et al. (2013), Dhaka et al. (2009), Takahashi et al. (2008), Wang et al. (2010, 2011)
6-Shogaol	Riera et al. (2009)
Tear gas	Bessac et al. (2009), Brone et al. (2008), Gijsen et al. (2010)
Δ^9-Tetrahydrocannabinol	Jordt et al. (2004)
Δ^9-Tetrahydrocannabiorcol	Andersson et al. (2011)
Thymol	Karashima et al. (2007), Lee et al. (2008)
Toluene diisocyanate	Taylor-Clark et al. (2009b)
Trinitrophenol	Hill and Schaefer (2007)
Umbellulone and derivatives	Nassini et al. (2012), Zhong et al. (2011a)
URB597	Niforatos et al. (2007)
WIN55,212-2	Akopian et al. (2008)
Zn^{2+}	Andersson et al. (2009), Hu et al. (2009)

such amino acids could lead to various conformational changes of the N-terminal region affecting the functional integrity of TRPA1 (Chen et al. 2008; Cvetkov et al. 2011; Eberhardt et al. 2012; Ibarra and Blair 2013; Wang et al. 2012). Indeed, a general defect of TRPA1 channel function caused by cysteine and lysine mutations or the creation of *xenogeneic* (artificial) channels has been reported (Andersson et al. 2013; Chen et al. 2008; Hu et al. 2009; Ibarra and Blair 2013; Miyamoto et al. 2009; Takahashi et al. 2008, 2011; Xiao et al. 2008), and the use of non-electrophilic activators at single supramaximal agonist concentrations may not always be enough to rule out a defect in channel function.

Importantly, the chemical mechanism by which covalent modification of cysteines occurs differs between electrophilic TRPA1 activators also when very close in chemical structure (Bessac et al. 2009; Gijsen et al. 2010; Ibarra and Blair 2013; Sadofsky et al. 2011). The electrophilic paracetamol

(acetaminophen) metabolite *p*-benzoquinone and the noxious fungal sesquiterpenes isovelleral and polygodial, containing α,β-unsaturated dialdehyde moieties, all produced intact TRPA1 responses in the heterologously expressed triple mutant hTRPA1-3C (Escalera et al. 2008; Ibarra and Blair 2013) that was initially used to identify certain N-terminal cysteine residues as key targets for electrophiles (Hinman et al. 2006). The electrophilic ketoaldehyde methylglyoxal displays a dual mechanism of activation of TRPA1 by binding irreversibly to N-terminal lysine 710 and also promoting disulfide formation of N-terminal cysteines (Eberhardt et al. 2012), and the headache-inducing monoterpene ketone umbellulone activates TRPA1 by a non-covalent interaction in addition to covalently reacting with N-terminal cysteines (Zhong et al. 2011a). An interesting example is the cyclopentenone prostaglandin 15-deoxy-δ(12,14)-prostaglandin J2 (Fig. 7), an endogenous electrophilic TRPA1 activator (Andersson et al. 2008; Cruz-Orengo et al. 2008; Maher et al. 2008; Materazzi et al. 2008; Takahashi et al. 2008; Taylor-Clark et al. 2008b), that in contrast to allyl isothiocyanate is antinociceptive when injected into the mouse paw (Weng et al. 2012). Thus, differences in the chemical properties between TRPA1 activators may contribute to unique ligand-specific pharmacology of TRPA1 in terms of sensitization, desensitization, channel trafficking, pungency, and duration of action.

Agents such as complete Freund's adjuvant and carrageenan are widely used to evoke inflammation, hyperalgesia, and allodynia in animals. They all increase the production of numerous inflammatory mediators, some of which act directly (e.g., 4-hydroxynonenal, hydrogen peroxide, 15-deoxy-δ(12,14)-prostaglandin J2) or indirectly (e.g., prostaglandins, bradykinin, histamine, and trypsin) on TRPA1 (Andrade et al. 2012; Mogil 2009; Moilanen et al. 2012). Many of these indirectly acting mediators bind to G protein-coupled receptors that regulate TRPA1 via the phospholipase C signaling pathway including PIP_2 depletion, generation of H^+, Ca^{2+} release, and protein kinase A-mediated phosphorylation (Fig. 5) (Andrade et al. 2012; Bautista et al. 2013; Bessac and Jordt 2008; Cavanaugh et al. 2008; Huang et al. 2010; Nilius et al. 2012). The nociceptive responses triggered by intraplantar injection of complete Freund's adjuvant and carrageenan are sensitive to TRPA1 antagonists (Andrade et al. 2012; Gregus et al. 2012), and TRPA1 plays a key role in the development of carrageenan-induced inflammation (Moilanen et al. 2012). The rat and mouse formalin test is also a widely used pain model for evaluating analgesic compounds on acute pain (first phase) and inflammatory pain (second phase). This biphasic nociceptive response is driven by a direct action of formaldehyde on TRPA1 (Macpherson et al. 2007b; McNamara et al. 2007).

The gasotransmitter H_2S is an endogenous signaling molecule (also produced by bacteria) that can contribute to inflammation and the associated sensitization of nociceptors (Li et al. 2011). This gaseous molecule activates ATP-sensitive potassium channels (K_{ATP}) and T-type voltage-gated calcium channels ($Ca_V3.2$). Our original finding that TRPA1 is activated by H_2S (Streng et al. 2008) together with other studies suggests that H_2S may act in concert with T-type voltage-gated calcium channels to excite nociceptive sensory neurons in the skin, airways, bladder, and colon (Fig. 8) (Andersson et al. 2012; Hsu et al. 2013; Ogawa

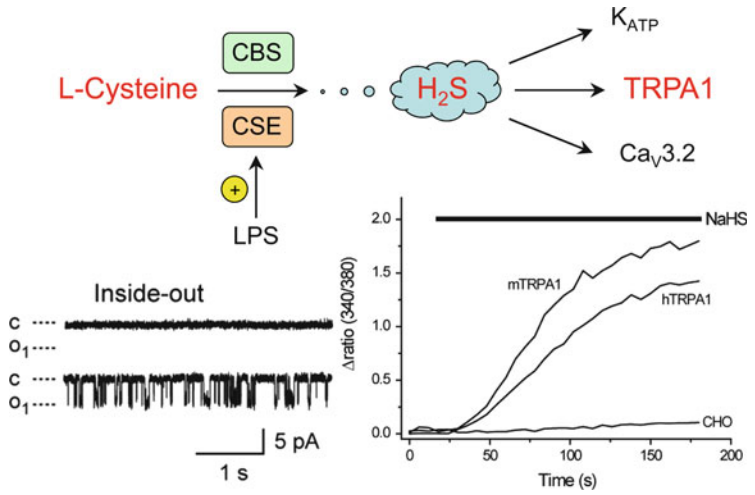

Fig. 8 Hydrogen sulfide (H_2S) is a recently discovered gasotransmitter in mammalians shown to activate the ATP-sensitive potassium channel (K_{ATP}) and the T-type voltage-gated calcium channel ($Ca_V3.2$). H_2S can be produced enzymatically by, e.g., cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), using L-cysteine as a substrate. The production of H_2S is also stimulated by lipopolysaccharide (LPS) derived from bacteria. The H_2S donor NaHS activates both human and mouse TRPA1, as shown by calcium imaging (Streng et al. 2008), and evokes TRPA1 currents in excised inside-out membrane patches, as measured by the patch-clamp technique (Andersson et al. 2012)

et al. 2012; Okubo et al. 2012; Tsubota-Matsunami et al. 2012). In the vasculature, both endothelium and sensory neurons contain powerful vasodilator mediators (Deanfield et al. 2007; Maggi 1995). Stimulation of endothelial cells releases endothelium-derived relaxing factors (EDRFs), such as nitric oxide and the endothelium-derived hyperpolarizing factor (EDHF) (Furchgott and Zawadzki 1980; Petersson et al. 1995, 1997, 1998; Zygmunt and Hogestatt 1996; Zygmunt et al. 1994a, b, 1998), and the activation of TRPV1 and TRPA1 on sensory neurons releases calcitonin gene-related peptide (Figs. 1 and 6) (Bautista et al. 2005; Graepel et al. 2011; Högestätt et al. 2000; Zygmunt et al. 1999, 2000, 2002). With regard to vascular signaling, a multicellular communication seems to exist between capsaicin-sensitive neuronal, endothelial, and smooth muscle cells (Högestätt et al. 2000); H_2S can produce sensory nerve-mediated vasodilation and may share properties with the proposed EDHF (Pozsgai et al. 2012; Tang et al. 2013). However, future studies are needed to explore a possible physiological role of H_2S as an EDHF and TRPA1 activator within the cardiovascular system. The exact mechanism by which H_2S activates TRPA1 is not obvious as H_2S is a mild reducing agent (Zhang et al. 2014). However, it has been suggested that direct or indirect sulfhydration of thiols by H_2S leads to protein activation (Li et al. 2011; Zhang et al. 2014), which is supported by the finding that H_2S can activate TRPA1 in inside-out membrane patches (Fig. 8) (Andersson et al. 2012). Nevertheless,

other indirect mechanisms for H_2S-dependent TRPA1 activation cannot be excluded.

It has been shown that TRPA1 is part of the O_2 homeostatic system, being activated directly by hyperoxia and indirectly by mild hypoxia (Takahashi et al. 2011), of which the latter may indicate an important role for TRPA1 present on perivascular sensory neurons in rectifying hypoxic conditions via release of potent vasodilator peptides.

Extracellular acidification accompanies ischemia, inflammation, and cancer growth, conditions associated with pain and sensitization of nociceptive primary afferents. A recent study disclosed important species differences in the extracellular proton sensitivity of TRPA1 (de la Roche et al. 2013). Interestingly, the human TRPA1 seems to have acquired the ability to sense extracellular acidosis, a property that is absent in rodent and rhesus monkey TRPA1 orthologs (de la Roche et al. 2013). Activation of human TRPA1 is observed at pH levels below 7, and the ensuing Ca^{2+} or current responses are graded, sustained, and reproducible, as shown by calcium imaging and patch-clamp recordings (de la Roche et al. 2013; Takahashi et al. 2008). Amino acids in both the N-terminus (C621) and transmembrane segment 6 (V942 and S943) were identified as crucial for responses to extracellular acidosis (de la Roche et al. 2013; Takahashi et al. 2008). However, the role of C621 in proton sensing is enigmatic, as this cysteine is also present in the rodent and rhesus TRPA1, which are insensitive to extracellular acidosis (de la Roche et al. 2013). These findings also raise concerns about the use of rhesus monkey TRPA1 as a surrogate species in human TRPA1 drug development (Bianchi et al. 2012; Chen et al. 2013).

Rodent TRPA1 is also sensitive to the intracellular acid–base environment, displaying a U-shaped pH response relationship, and mutations of two N-terminal cysteines (C422 and C622) disrupted channel activation evoked by intracellular alkalization (Fujita et al. 2008; Wang et al. 2010, 2011). In this respect, TRPA1 differs from TRPV1, which is inhibited by intracellular acidosis (Chung et al. 2011), although both ion channels may be activated by intracellular alkalosis (Dhaka et al. 2009; Fujita et al. 2008). Notably, the intracellular pH sensitivity of TRPA1 was retained in inside-out patches, ruling out a second messenger role of Ca^{2+} or any other cytosolic components in the pH responses (Dhaka et al. 2009; Fujita et al. 2008; Wang et al. 2010). Its role in both pH and oxygen sensation makes TRPA1 particularly suitable as a mediator of ischemic pain and other ischemia-related protective or adaptive responses, such as hypoxic vasodilation and ischemic preconditioning.

Whereas the sensitivity of TRPA1 to potentially harmful electrophiles and oxidants is in line with its proposed role as a nocisensor, the ability of TRPA1 to respond to many non-electrophilic compounds of diverse chemical structure is intriguing (Fig. 7 and Table 2) (Andrade et al. 2012; Baraldi et al. 2010; Holzer 2011; Nilius et al. 2012). Based on our findings that paracetamol metabolites and non-psychotropic cannabinoids produce spinal antinociception via activation of TRPA1, we have suggested that non-tissue-damaging TRPA1 activators may be developed for treatment of pain (Fig. 9) (Andersson et al. 2011). In this regard, it is

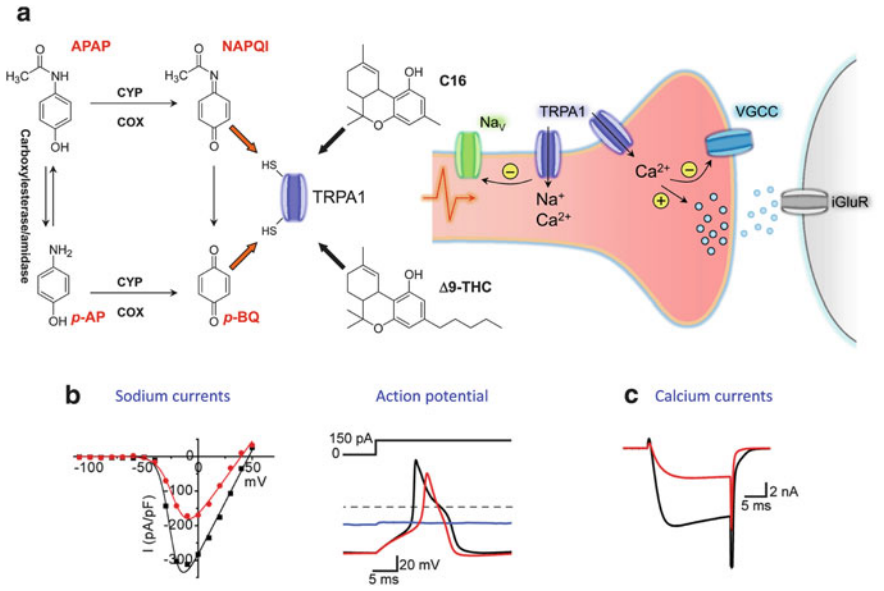

Fig. 9 Activation of TRPA1 by electrophilic paracetamol (acetaminophen) metabolites or non-electrophilic cannabinoids, such as Δ^9-tetrahydrocannabinol and its plant derivative Δ^9-tetrahydrocannabiorcol (C16), in central projections of primary sensory neurons in the spinal cord causes influx of Ca^{2+} and Na^+. This leads to the initial release of the excitatory neurotransmitter glutamate, acting on ionotropic glutamate receptors (iGluR) and the subsequent inhibition of (**a** and **b**) voltage-gated sodium channels (Na_V; *red I–V* trace) and the related action potential (*flat blue line* in the presence and *red line* after washout of TRPA1 activator) as well as (**c**) voltage-gated calcium channels (VGCC, *red trace*). The net result will be the inhibition of incoming pain signals from the periphery. Paracetamol (APAP) is metabolized to *p*-aminophenol (*p*-AP) and electrophilic compounds including *N*-acetyl-*p*-benzoquinoneimine (NAPQI) and *p*-benzoquinone (*p*-BQ) that activate TRPA1 by covalent binding to cysteines (–SH). CYP (cytochrome P450 monooxygenases), COX (cyclooxygenases). Modified from Andersson et al. (2011)

interesting that nonsteroidal anti-inflammatory fenamates also activate TRPA1 (Hu et al. 2010; Peyrot des Gachons et al. 2011). Other non-covalent activators, such as dihydropyridines and clotrimazole, may also serve as templates for the development of analgesic TRPA1 activators (Fajardo et al. 2008b; Meseguer et al. 2008). This approach may not be restricted to a spinal site of action, as the electrophilic TRPA1 activator 15-deoxy-δ(12,14)-prostaglandin J2 produced a peripheral antinociceptive effect in mouse models of pain (Weng et al. 2012).

The electrophilic nature of TRPA1 activators has made it possible to use mass spectrometry to show that such compounds interact covalently with TRPA1 and, hence, TRPA1 is an intrinsically chemosensitive protein (Cvetkov et al. 2011; Macpherson et al. 2007a; Wang et al. 2012). For non-electrophilic activators, mutagenesis and chimeric strategies have also indicated a direct interaction with TRPA1; e.g., menthol was suggested to bind to transmembrane segment 5 of

TRPA1 (Xiao et al. 2008). Patch-clamp recordings of isolated membrane patches suggest that Δ^9-tetrahydrocannabinol also interacts directly with TRPA1 (Cavanaugh et al. 2008; Kim and Cavanaugh 2007). Interestingly, in contrast to Δ^9-tetrahydrocannabinol, several other TRPA1 activators of both electrophilic and non-electrophilic nature (allicin, allyl isothiocyanate, cinnamaldehyde, *N*-ethylmaleimide, 2-aminoethoxydiphenyl borate, methyl salicylate, 2-aminoethyl methanethiosulfonate, and trinitrophenol) as well as Ca^{2+} require a cytosolic component to activate TRPA1, indicating that cannabinoids activate TRPA1 in a unique way (Cavanaugh et al. 2008). The ability of non-electrophilic compounds to activate TRPA1 also raises the interesting possibility that endogenous ligands sharing binding sites with these non-electrophiles exist and regulate TRPA1 activity. However, it still remains to be shown that menthol and other non-covalent activators indeed interact directly with TRPA1.

Some electrophilic and non-electrophilic TRPA1 activators, including apomorphine, camphor, cinnamaldehyde, ligustilide, menthol, nicotine, umbellulone, and thymol, display a bimodal action (activation at low and inactivation at high concentrations) on heterologously expressed TRPA1 that in some cases is species dependent (Alpizar et al. 2013; Karashima et al. 2007; Schulze et al. 2013; Talavera et al. 2009; Zhong et al. 2011a, b). The list of TRPA1 activators is extensive and rapidly growing (Table 2), whereas only a few natural blockers have been described (Bang et al. 2010, 2011; Park et al. 2011; Takaishi et al. 2013). Among these resolvin D2 is the most potent one, inhibiting both TRPA1 and TRPV1 in the nanomolar range as shown in mouse dorsal root ganglion neurons (Park et al. 2011). However, these effects were pertussis toxin-sensitive and therefore most likely not caused by a direct interaction between resolvin D2 and the channel proteins (Park et al. 2011). Although many TRPA1 activators may interfere with other important TRP channels and membrane receptors (e.g., nicotine) that are involved in TRPA1-sensitive sensory signaling (Holzer 2011; Kichko et al. 2013), further studies of the pharmacology of non-covalent activators and the mechanisms behind the bimodal action of certain drugs should provide a deeper understanding of TRPA1 channel function and help to identify novel activators and antagonists with unique pharmacological properties.

6.2 TRPA1 and Noxious Cold Sensation

Ever since TRPA1 was suggested to respond to noxious cold temperatures (Story et al. 2003), its role in cold sensation has been debated. Nevertheless, several studies have shown that acute noxious cold sensation is impaired in TRPA1 knockout mice and, more importantly, that TRPA1 is involved in cold allodynia induced by inflammation, nerve injury, and peripheral neuropathy (Table 3). Furthermore, as shown at a single-channel level, the open probability increased (due to a change in the rate of channel deactivation) by lowering the temperature just below room temperature (Karashima et al. 2009; Sawada et al. 2007). Interestingly, cold responses were independent on Ca^{2+} (Karashima et al. 2009; Sawada et al. 2007),

Table 3 Behavioral assays showing TRPA1-dependent noxious cold sensation

Model	Pain test or stimulus	Inhibition	Species	References
Acute nociception				
Paw	Cold plate (0 °C)	KO	Mouse	Kwan et al. (2006)
Paw	Acetone evaporation	KO	Mouse	Kwan et al. (2006)
Paw	Cold plate (0 °C)	KO	Mouse	Karashima et al. (2009)
Tail	Tail immersion (−10 °C)	KO	Mouse	Karashima et al. (2009)
Paw	Cold plate (10 °C)	KO	Mouse	Gentry et al. (2010)
Inflammation				
CFA (skin)	Cold plate (5 °C)	AntiS	Rat	Obata et al. (2005)
CFA (skin)	Cold plate (5 °C)	AP-18	Rat	Petrus et al. (2007)
CFA (skin)	Cold plate (−5 °C)	HC	Rat	del Camino et al. (2010)
CFA (skin)	Tetrafluoroethane spray (paw)	HC, AntiS	Mouse	da Costa et al. (2010)
Nerve injury				
Spinal nerve ligation	Cold plate (5 °C)	AntiS	Rat	Obata et al. (2005)
Spinal nerve ligation	Cold plate (5 °C)	AntiS	Rat	Katsura et al. (2006)
Spared nerve injury	Cold plate (−5 °C)	HC	Rat	del Camino et al. (2010)
CCI	Acetone spray (paw)	A-967079	Rat	Chen et al. (2011a)
Diabetic neuropathy				
GLOI	Cold plate (10 °C)	KO	Mouse	Andersson et al. (2013)
Iatrogenic				
Oxaliplatin	Acetone evaporation	KO/HC	Mouse	Nassini et al. (2011)
Oxaliplatin	Tail immersion (10 °C)	HC	Rat	Nassini et al. (2011)
Oxaliplatin	Cold plate (5 °C)	KO, HC	Mouse	Zhao et al. (2012)
Oxaliplatin	Acetone evaporation	HC	Mouse	Trevisan et al. (2013)
Oxaliplatin	Cold plate (4 °C)	ADM_09	Rat	Nativi et al. (2013)
Paclitaxel	Paw immersion (4 °C)	HC	Rat[a]	Barriere et al. (2012)
Paclitaxel	Acetone evaporation	KO, HC	Mouse	Materazzi et al. (2012)
Bortezomib	Acetone evaporation	KO, HC	Mouse	Trevisan et al. (2013)
Clioquinol	Cold plate (10 °C)	KO	Mouse	Andersson et al. (2009)

AntiS, antisense oligonucleotides; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; GLOI, glyoxalase-1 inhibition; HC, HC030031; KO, TRPA1 gene knockout

[a]Diabetic animals

but this does not exclude Ca^{2+} as an important regulator of TRPA1-mediated cold responses in vivo. Thus, there is now substantial evidence from both in vitro and in vivo studies that rat and mouse TRPA1 is involved in noxious cold sensation. However, based on in vitro studies of the expressed human TRPA1, the controversy regarding a similar role for human TRPA1 is still ongoing (Bandell et al. 2004; Chen et al. 2013; Cordero-Morales et al. 2011; Jordt et al. 2004; Klionsky et al. 2007; Kremeyer et al. 2010; May et al. 2012; Wang et al. 2013; Zurborg et al. 2007). A recent study concluded that species differences exist and that in contrast to rodent TRPA1, the human variant is incapable to respond to cold (Chen et al. 2013). However, the cold sensitivity of TRPA1-expressing rat sensory neurons seems to depend on the anatomical origin of the neurons (Fajardo et al. 2008a), indicating that the cellular environment including the oxidation state of the cells can dictate cold sensitivity possibly by influencing the conformation of the N-terminal region. This together with other methodological issues may explain why opposite conclusions exist regarding TRPA1 as a cold sensor (Karashima et al. 2009). Whether or not TRPA1 is gated directly by cold remains, however, to be demonstrated and requires studies on the purified ion channel.

6.3 TRPA1 and Noxious Mechanosensation

The interest in mammalian TRPA1 as a mechanosensor was inspired by the large N-terminal ankyrin repeat domain, potentially acting as a gating spring (Gaudet 2008; Howard and Bechstedt 2004), the expression of TRPA1 in hair cells of the inner ear (Corey et al. 2004), and observations in invertebrates that structurally similar TRP channels play key roles in mechanotransduction (Sidi et al. 2003; Walker et al. 2000). Indeed, CHO cells transfected with a *Caenorhabditis elegans* ortholog of the mammalian TRPA1 acquired mechanosensory properties (Kindt et al. 2007). Although a role of the mammalian TRPA1 in hearing could not be demonstrated in subsequent studies of gene knockout mice (Bautista et al. 2006; Kwan et al. 2006), the involvement of TRPA1 in noxious mechanotransduction, including mechanical allodynia or hypersensitivity, has received increasing support over time, as shown in naïve animals and various models of inflammatory and neuropathic pain, using both gene silencing and pharmacological strategies to interfere with TRPA1 expression and function (Table 4). In particular, the introduction of selective TRPA1 inhibitors has made it possible to study the effect of locally inhibiting TRPA1 in the receptive field. Such studies have consolidated TRPA1 as an important component of the mechanotransduction pathway in nociceptive somatosensory neurons (Bonet et al. 2013; da Costa et al. 2010; Fernandes et al. 2011; Kerstein et al. 2009; Kwan et al. 2009). Furthermore, direct mechanical or osmotic stimulation of cultured nociceptor-like sensory neurons induced graded current responses that disappeared or were significantly reduced by pharmacological or genetic inactivation of TRPA1, although the possibility that Ca^{2+} was acting as an upstream mediator of such mechanically induced responses cannot be excluded (Brierley et al. 2011; Vilceanu and Stucky 2010; Zhang et al. 2008b).

Table 4 Behavioral or reflex assays showing TRPA1-dependent noxious mechanosensation

Model	Pain test or stimulus	Inhibition	Species	References
Acute nociception				
Paw	von Frey (paw)	KO	Mouse	Kwan et al. (2006)
Paw	Paw pressure, von Frey	KO	Mouse	Andersson et al. (2009)
Paw	Paw pressure	CHEM	Rat	Wei et al. (2009)
Colon	VMR to distension	KO	Mouse	Brierley et al. (2009)
Stomach	VMR to distension	HC	Rat	Kondo et al. (2009)
Inflammation				
CFA (skin)	von Frey (paw)	AP-18	Mouse	Petrus et al. (2007)
CFA (skin)	Paw pressure	HC	Rat	Eid et al. (2008)
CFA (skin)	von Frey (paw)	AntiS, HC	Mouse	da Costa et al. (2010)
CFA (skin)	von Frey (paw)	HC	Mouse	Lennertz et al. (2012)
LPS (skin)	Paw pressure	KO, AP-18	Mouse	Andersson et al. (2012)
Carrageenan (skin)	Electr. von Frey (paw)	AntiS, HC	Rat	Bonet et al. (2013)
Skin incision	von Frey (paw)	CHEM	Rat	Wei et al. (2012)
TNF-α (skin)	Electr. von Frey (paw)	AP-18	Mouse	Fernandes et al. (2011)
CFA (knee)	Electr. von Frey (paw)[a]	KO, AP-18	Mouse	Fernandes et al. (2011)
CFA (knee)	von Frey (knee)	A-967079	Rat	McGaraughty et al. (2010)
BK (paw)	von Frey (paw)	KO	Mouse	Petrus et al. (2007)
BK (colon)	VMR to distension	AP-18, KO	Mouse	Brierley et al. (2009)
TNBZ (colon)	VMR to distension	KO/AntiS	Mouse/rat	Cattaruzza et al. (2010), Yang et al. (2008)
CPA (bladder)	von Frey (paw)[a]	HU	Rat	Meotti et al. (2013)
Ifosfamide (bladder)	Electr. von Frey (abdomen)[a]	HU	Mouse	Pereira et al. (2013)
Nerve injury				
Spinal nerve ligation	von Frey (paw)	HC	Rat	Eid et al. (2008)
Spared nerve injury	von Frey (paw)	CHEM	Rat	Wei et al. (2013b)
Diabetic neuropathy				
Streptozotocin	von Frey (paw)	CHEM	Rat	Wei et al. (2010a)
Streptozotocin	Paw pressure, von Frey	CHEM	Rat	Wei et al. (2009, 2013b)
GLOI	Paw pressure, von Frey (paw)	KO	Mouse	Andersson et al. (2013)

(continued)

Table 4 (continued)

Model	Pain test or stimulus	Inhibition	Species	References
Iatrogenic				
Oxaliplatin	Paw pressure	KO/HC	Mouse/rat	Nassini et al. (2011)
Cisplatin	von Frey (paw)	KO	Mouse	Nassini et al. (2011)
Paclitaxel	von Frey (paw)	HC	Mouse	Chen et al. (2011b), Materazzi et al. (2012)
Oxaliplatin	Paw pressure	ADM_09	Rat	Nativi et al. (2013)
Oxaliplatin	von Frey (paw)	HC	Mouse	Trevisan et al. (2013)
Bortezomib	von Frey (paw)	HC	Mouse	Trevisan et al. (2013)
Clioquinol	Paw pressure	KO	Mouse	Andersson et al. (2009)

AntiS, antisense oligonucleotides; CFA, complete Freund's adjuvant; CHEM, Chembridge-5861528; CPA, cyclophosphamide; Electr., electronic (anesthesiometer); GLOI, glyoxalase-1 inhibition; HC, HC030031; KO, TRPA1 gene knockout; LPS, lipopolysaccharide; TNBZ, trinitrobenzene sulfonic acid; VMR, visceromotor responses

[a]Secondary hyperalgesia

Except for one study using hypertonic solutions to impose a mechanical force (cell shrinkage) on the cell membrane (Zhang et al. 2008b), heterologous TRPA1 gene expression has so far failed to confer mechanosensitivity to the recipient cells. Functional studies of the purified protein reconstituted in a defined lipid and protein environment will be required to address whether or not TRPA1 is an intrinsically mechanosensitive ion channel.

6.4 TRPA1 and Itch

Itch is a distinct psychophysical sensation associated with a strong urge to scratch. Its sensory and motivational qualities resemble the tickling sensation that may precede cough and sneeze. Itch is mediated by a unique set of primary afferents distinct from those mediating pain (Schmelz et al. 1997). However, painful mechanical stimulation of the skin can inhibit itch at the level of the spinal cord, and this cross-modality interaction may prevent scratch-induced injury (Ross 2011). Itch responses can be evoked by stimulation of two different populations of primary afferent C-fibers: Mechano-insensitive and mechanosensitive fibers selectively activated by histamine and cowhage spicules (containing 5-hydroxytryptamine), respectively (Namer et al. 2008; Roberson et al. 2013). As shown recently, TRPA1 is required for itch-evoked scratching in response to many pruritogens, including cowhage spicules, chloroquine, leukotriene B4, substance P, and reactive oxygen species, acting at least partly through histamine-insensitive primary afferents (Fernandes et al. 2013; Liu et al. 2013; Liu and Ji 2012; Wilson et al. 2011). Intradermal injection of allyl isothiocyanate in the mouse cheek activates pruritogen-responsive second-order neurons in the trigeminal subnucleus caudalis (Akiyama et al. 2010). However, allyl isothiocyanate or cinnamaldehyde produces pain rather than itch sensation when administered to the skin and nasal mucosa in man, possibly reflecting cross-modality inhibition of itch responses (Alenmyr et al. 2009; Namer et al. 2005). A unique pharmacological approach for silencing of capsaicin-sensitive nerve fibers recently disclosed an itch-producing effect of allyl isothiocyanate, providing additional support for a cross-modality interaction between the neural pathways mediating pain and itch (Roberson et al. 2013).

Recent findings have revealed that TRPA1 is an important mediator of non-histaminergic itch and that overactivity in itch-encoding histamine-insensitive primary afferents may be responsible for chronic itch in atopic and allergic contact dermatitis (Cevikbas et al. 2014; Liu et al. 2013; Oh et al. 2013; Wilson et al. 2013). Importantly, the TRPA1 immunoreactivity is dramatically increased in skin biopsies from patients with atopic dermatitis, even in mast cells and keratinocytes that barely express TRPA1 immunostaining in healthy skin (Oh et al. 2013). Thus, TRPA1 is emerging as a promising target for novel anti-pruritic drugs particularly in atopic and allergic contact dermatitis. Pharmacological inhibition of TRPA1 may also break the vicious circle generated by intense scratching in dermatological disease, although disinhibition of itch-encoding neurons may potentially enhance TRPA1-independent itch pathways.

6.5 TRPA1 and the Central Nervous System

While noxious cold, mechanical stress, and environmental irritants can trigger TRPA1 activation in the periphery, the physiological role of TRPA1 on central projections of primary afferents is less obvious, as the central nervous system is rarely exposed to noxious cold or mechanical stress. However, TRPA1 can be activated by several endogenous molecules, including reactive oxygen species and 12-lipoxygenas and cytochrome P450 epoxygenase-derived metabolites, which may be formed in the spinal cord during nociceptive stimulation, nerve injury, and neuroinflammation (Due et al. 2013; Gregus et al. 2012; Lee et al. 2007; Sisignano et al. 2012). The outer layers of the dorsal horn are richly innervated by TRPA1-expressing nerve terminals, as shown by immunohistochemistry (Andersson et al. 2011; Kim et al. 2010). However, transcripts encoding TRPA1 cannot be detected in the rat and mouse spinal cord, and dorsal rhizotomy almost completely eliminates spinal TRPA1 immunostaining in the rat, indicating that most if not all TRPA1-containing nerve terminals originate from primary sensory neurons (Andersson et al. 2011; Kim et al. 2010; Story et al. 2003). However, there are indications that TRPA1 is present in the brain both at the transcriptional and translational levels (Stokes et al. 2006; Vennekens et al. 2012). Using whole-cell patch-clamp recordings, presynaptic TRPA1 channels were demonstrated on glutamatergic neurons in the rat supraoptic nucleus (Yokoyama et al. 2011). Furthermore, expression of TRPA1 in astrocytes was recently identified in rodent trigeminal caudal nucleus and hippocampus, potentially regulating interneuron inhibitory synaptic efficacy, long-term potentiation, and neuronal survival (Koch et al. 2011; Lee et al. 2012; Shigetomi et al. 2012, 2013).

The function of TRPA1 on central projections of primary afferents has not been entirely resolved. In substantia gelatinosa neurons from the spinal dorsal horn and trigeminal sensory nuclei, the TRPA1 activators allyl isothiocyanate and cinnamaldehyde increase the frequency of spontaneous excitatory postsynaptic currents and inhibitory postsynaptic currents, the latter effect being sensitive to tetrodotoxin in spinal cord slices and, hence, attributable to recruitment of inhibitory interneurons (Cho et al. 2012; Kosugi et al. 2007; Uta et al. 2010; Wrigley et al. 2009). Intrathecal administration of TRPA1 activators causes tactile allodynia and occasionally also heat hyperalgesia (Due et al. 2013; Gregus et al. 2012; Proudfoot et al. 2006; Raisinghani et al. 2011; Sisignano et al. 2012; Wei et al. 2013a). However, spinal TRPA1 activation by both electrophilic and non-electrophilic activators can also inhibit acute thermal and mechanical pain, as shown in the modified hot and cold plate tests (using lightly restrained animals) and the paw pressure test, and the formation of electrophilic metabolites in the spinal cord may contribute to the analgesic effect of acetaminophen (Fig. 9) (Andersson et al. 2011). This pharmacological effect of intrathecal administration of TRPA1 activators was attributed to a Ca^{2+}-dependent presynaptic inhibition of voltage-gated calcium and sodium channels (Fig. 9) (Andersson et al. 2011), which is in line with Aδ- and C-fiber-evoked excitatory postsynaptic currents being

inhibited by TRPA1 activators in spinal cord slices, as also shown for capsaicin (Jeffry et al. 2009; Uta et al. 2010; Yue et al. 2013).

Several studies have addressed the role of spinal TRPA1 in models of inflammatory and neuropathic pain using intrathecal administration of TRPA1 blockers. These studies clearly show that spinal TRPA1 participates in mechanical allodynia or hypersensitivity as well as secondary mechanical hyperalgesia following peripheral capsaicin or allyl isothiocyanate administration (da Costa et al. 2010; Fernandes et al. 2011; Wei et al. 2009, 2010a, b, 2011). Thus, while spinal TRPA1 seems to play a key role in central sensitization, pharmacological activation of the same ion channel can also disrupt spinal nociceptive neurotransmission (Andersson et al. 2011). Further studies are required to understand this intriguing dual action of TRPA1 and its implications for the development of TRPA1-based drug therapies.

7 Lessons from Knockouts

Transgenic mice lacking functional TRPA1 channels have been used to understand the physiological and pathophysiological role of this protein and its potential as a pharmacological target for treatment of pain and sensory dysfunction in man (Patapoutian et al. 2009). Initial studies using TRPA1 knockout mice, generated by different genetic strategies, clearly validated TRPA1 as a chemosensor (Bautista et al. 2006; Kwan et al. 2006; Macpherson et al. 2007b; McNamara et al. 2007). Major concerns when using TRPA1 knockout mice are functional compensation by related channels and receptors and significant differences between mouse strains (Patapoutian et al. 2009). This could explain the lack of consensus regarding the role of TRPA1 in noxious cold and mechanical sensation. Nevertheless, many recent studies using TRPA1 knockout mice suggest an involvement of TRPA1 in both noxious cold and mechanical hypersensitivity (Tables 3 and 4), which is also supported by the use of TRPA1 antagonists, such as HC030031 and the structurally similar compound Chembridge-5861528 (Tables 3 and 4), although HC030031 has off-target effects that must be considered when this and chemically similar pharmacological tools are used to understand the physiological role of TRPA1 (Andersson et al. 2013; Fischer et al. 2010). The recent finding that the skin innervation of TRPA1 knockout mice is substantially less compared to wild-type mice is of concern, as it may explain the reduced responsiveness to cold and mechanical stimuli in such mice (Andersson et al. 2013). The type of nerve fiber affected remains, however, to be determined. The rationale of using nonhuman TRPA1 models in vitro and in vivo to develop drugs for treatment of human pain and sensory discomfort has been questioned (Bianchi et al. 2012; Chen et al. 2013). However, as pointed out in a recent review, "approximately 50 % of the molecular targets of the top 100 selling drugs have been knocked out in mouse transgenic models" and "knockout models are likely, in most cases, to provide a productive source of validated targets for future drug development" (Patapoutian et al. 2009).

8 Role in Hereditary and Acquired Diseases

8.1 Hereditary Disease

It is well known that topical skin application of TRPA1 activators can produce burning pain, mechanical and thermal hypersensitivity, and neurogenic inflammation in man (Koltzenburg et al. 1992; Namer et al. 2005; Olausson 1998). The discovery of an autosomal dominant familial episodic pain syndrome, caused by a gain-of-function mutation in TRPA1, has underscored TRPA1 as a key player in pain perception (Kremeyer et al. 2010). Affected individuals display increased hyperalgesia to punctate stimuli after mustard oil application, but are otherwise healthy except for episodes of mainly upper body pain, triggered by physical stress, such as fasting, exposure to cold, and exercise (Kremeyer et al. 2010). The mutation, which is localized to transmembrane segment 4 (N885S), alters the voltage dependence of TRPA1 and renders the channel hyperresponsive to electrophiles and cold at normal resting membrane potentials (Kremeyer et al. 2010). Paradoxical heat sensation was recently linked to a single nucleotide polymorphism in the N-terminus of TRPA1 (E179K), and HEK293T/17 cells transfected with the mutant failed to display cold sensitivity in contrast to the wild-type channel (Binder et al. 2011; May et al. 2012).

8.2 Inflammatory Disease

Animal studies have shown that TRPA1 is involved in thermal and mechanical sensitization induced by inflammation (Tables 3 and 4) (Andrade et al. 2012; Hoffmann et al. 2013). Many endogenous TRPA1 activators, such as reactive oxygen species, α,β-unsaturated aldehydes, and H_2S (Table 2), that are formed during inflammation and bacterial infection may in fact drive or maintain the inflammatory process via activation of TRPA1 on sensory nerve fibers and nonneuronal cells (Andersson et al. 2012; Bautista et al. 2013; Moilanen et al. 2012). Furthermore, inflammatory mediators, such as bradykinin, trypsin, and nerve growth factor, may sensitize TRPA1 or upregulate its expression via different receptor-dependent signaling pathways, leading to hyperalgesia, allodynia, itch, urgency, and other organ-specific symptoms mediated by TRPA1 (Tables 3 and 4) (Dai et al. 2007; Diogenes et al. 2007; Ji et al. 2008; Katsura et al. 2006; Malin et al. 2011; Obata et al. 2005; Schwartz et al. 2011; Wang et al. 2008a). Inflammatory diseases where TRPA1 has been implicated in pain perception, sensory hyperreactivity, or disease progression include arthritis, asthma, dermatitis, inflammatory bowel disease, and pancreatitis (Bautista et al. 2013; Bessac and Jordt 2008; Holzer 2011; Kaneko and Szallasi 2013; Taylor-Clark and Undem 2011).

Cyclophosphamide-induced hemorrhagic cystitis is not only a feared adverse effect, but also a common animal model of interstitial cystitis/bladder pain syndrome, a chronic disease that affects mainly women. The metabolism of both

cyclophosphamide and its congener ifosfamide generates the urotoxic TRPA1 activator acrolein, which produces mucosal injury when accumulating in the urine (Bautista et al. 2006). Interestingly, systemic administration of the TRPA1 blocker HC030031 effectively reduced the bladder overactivity induced by cyclophosphamide pretreatment in rats (Meotti et al. 2013).

8.3 Peripheral Neuropathy

Cold and mechanical allodynia are commonly observed in patients with peripheral neuropathy, and these abnormalities can be mimicked in various animal models of nerve injury. Overexpression of TRPA1 is frequently encountered in spared neurons adjacent to the site of injury, possibly reflecting adaptive responses to neuroinflammatory cues (Ji et al. 2008; Katsura et al. 2006; Obata et al. 2005). Interventional downregulation of TRPA1 expression and function reduces behavioral signs of cold and mechanical allodynia (Tables 3 and 4). Methylglyoxal, a potent TRPA1 activator (Table 2) produced under hyperglycemic conditions, has been implicated as a mediator of diabetic neuropathy, although other endogenous electrophilic compounds may also contribute (Moran et al. 2011). Thus, electrophilic activation of TRPA1 may drive the development of peripheral neuropathy and the associated mechanical sensitization (Tables 3 and 4). The complex neurochemistry of cancer, including trophic cues for neuronal sprouting, may create a chemical microenvironment that promotes TRPA1 expression, sensitization, and activation (Lozano-Ondoua et al. 2013; Ye et al. 2011). Many cancer chemotherapies are neurotoxic and can cause thermal and mechanical allodynia. TRPA1 together with TRPM8 and TRPV1 may contribute to such adverse effects, as demonstrated in animals exposed to different chemotherapeutic agents (Tables 3 and 4).

Altered afferent signaling is believed to play an important role in bladder overactivity, a condition that can be mimicked by intravesical administration of TRPA1 activators (Du et al. 2007; Streng et al. 2008). Bladder overactivity, characterized by urgency with or without incontinence, is commonly associated with infections, outflow obstruction (prostate enlargement), diabetes, and various neurological disorders of the central nervous system, including spinal cord injury and multiple sclerosis (Andersson et al. 2010; Birder 2013; Kanai 2011). A large number of people also suffer from overactive bladders without any obvious cause, a condition referred to as overactive bladder syndrome. In a rat model of bladder overactivity following spinal cord injury, featuring detrusor TRPA1 protein overexpression, antisense oligonucleotide-induced downregulation of TRPA1 in sensory neurons and systemic administration of the TRPA1 blocker HC030031 each reduced non-voiding bladder contractions, as demonstrated by cystometry in anesthetized animals (Andrade et al. 2011). Irritable bowel syndrome is another common illness, characterized by abdominal pain, obstipation, and diarrhea, in which TRPA1-dependent sensory dysfunction may play an important role (Hughes et al. 2013; Ryosuke et al. 2014; Yu et al. 2010).

8.4 Pharmacological Intervention

Although inhibition of TRPA1 seems the most logical treatment strategy to achieve pain relief in inflammatory and neuropathic disease, it is interesting that intraplantar, systemic, or spinal injection of TRPA1 activators can also disrupt nociceptive signaling (Andersson et al. 2011; Materazzi et al. 2013; Ryosuke et al. 2014; Weng et al. 2012). Thus, depending on the site of action and the intrinsic chemical properties of the drug, both antagonists and agonists of TRPA1 could be useful to treat various pain conditions, as shown for TRPV1 (Andersson et al. 2011; Barrière et al. 2013; Kaneko and Szallasi 2013; Mallet et al. 2010; Moran et al. 2011; Patapoutian et al. 2009; Zygmunt et al. 2013). Species differences in function and pharmacology of TRPA1 together with the lack of predictive animal models of pain have complicated the validation of TRPA1 as a drug target in man (Andrade et al. 2012; Baraldi et al. 2010; Bianchi et al. 2012; Chen and Kym 2009; Chen et al. 2008, 2013; de la Roche et al. 2013; Klionsky et al. 2007; Nyman et al. 2013; Patapoutian et al. 2009; Viana and Ferrer-Montiel 2009). A pure antagonist or agonist at TRPA1 in one species may in fact act as a partial agonist or a dual agonist and antagonist in another species (Bianchi et al. 2012; Chen et al. 2008; Klionsky et al. 2007; Xiao et al. 2008). It is, however, encouraging that the TRPA1 antagonist A-967079 is able to attenuate cold allodynia in animals without affecting noxious heat sensation and body temperature, as such unwanted adverse effects have hampered the development of TRPV1 antagonists as analgesics (Moran et al. 2011). The thermoregulatory consequences of intragastric administration of TRPA1 and TRPV1 activators are also not identical (Masamoto et al. 2009). Thus, although TRPA1 is mainly expressed together with TRPV1 on primary afferents, pharmacological interventions with TRPA1 and TRPV1 may produce very different adverse effect profiles. In order to appreciate the full potential of TRPA1 as a safe drug target, more information is needed about the role of TRPA1 in nonneuronal cells and the cardiovascular and metabolic consequences of interfering with this ion channel.

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TRPML1: An Ion Channel in the Lysosome

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Contents

Abstract

The first member of the mammalian mucolipin TRP channel subfamily (TRPML1) is a cation-permeable channel that is predominantly localized on the membranes of late endosomes and lysosomes (LELs) in all mammalian cell types. In response to the regulatory changes of LEL-specific phosphoinositides or other cellular cues, TRPML1 may mediate the release of Ca^{2+} and heavy metal Fe^{2+}/Zn^{2+}ions into the cytosol from the LEL lumen, which in turn may

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regulate membrane trafficking events (fission and fusion), signal transduction, and ionic homeostasis in LELs. Human mutations in *TRPML1* result in type IV mucolipidosis (ML-IV), a childhood neurodegenerative lysosome storage disease. At the cellular level, loss-of-function mutations of mammalian *TRPML1* or its *C. elegans* or *Drosophila* homolog gene results in lysosomal trafficking defects and lysosome storage. In this chapter, we summarize recent advances in our understandings of the cell biological and channel functions of TRPML1. Studies on TRPML1's channel properties and its regulation by cellular activities may provide clues for developing new therapeutic strategies to delay neurodegeneration in ML-IV and other lysosome-related pediatric diseases.

Keywords
Lysosome • Endosome • TRP channel • Membrane trafficking • Ca^{2+} • Phosphoinositide

1 Gene

Human TRPML1 (or mucolipin-1/MCOLN1), the founding member of the TRPML subfamily, is encoded by the *MCOLN1* gene localized on chromosome 19 (19p13.2–13.3; base pair positions 7,587,496–7,598,895) (Bargal et al. 2000; Bassi et al. 2000; Slaugenhaupt et al. 1999; Sun et al. 2000). No splicing variant has been reported for the human *TRPML1* gene. In contrast, the almost identical mouse *Trpml1* gene contains two alternatively spliced isoforms (Slaugenhaupt 2002). Although there are two other *TRPML1*-related genes, i.e., *TRPML2* and *TRPML3*, in human and mouse genomes (Cheng et al. 2010), only one single gene in *C. elegans* and *Drosophila*, *cup*-5 and *trpml* (CG8743), respectively, encodes the TRPML protein, which shares 30–40 % sequence identity with human TRPML1 (Fares and Greenwald 2001). Genetic studies on model organisms suggest that human *TRPML1* plays an evolutionarily conserved role in the cell biology of the lysosome.

2 Expression and Subcellular Localization

TRPML1 is ubiquitously expressed in every mouse tissue, with the highest levels of mRNA expression in the brain, kidney, spleen, liver, and heart (Falardeau et al. 2002; Samie et al. 2009). Consistent with this expression pattern, the loss of TRPML1 results in enlarged late endosomes and lysosomes (LELs) and the accumulation of lysosomal storage materials in most cell types of ML-IV patients and *Trpml1* knockout mice (Slaugenhaupt 2002; Venugopal et al. 2007).

Cellular phenotypes of ML-IV and its mouse model indicate that TRPML1 is predominately localized on the membranes of LELs, but heterologously expressed

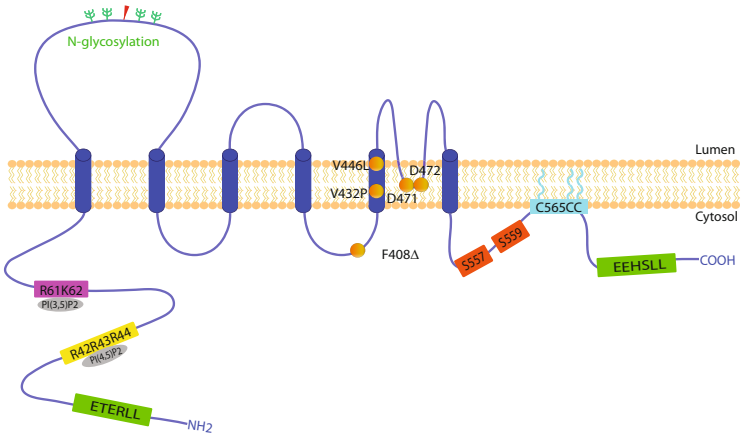

Fig. 1 Structural aspects of TRPML1. TRPML1 consists of six transmembrane (6TM) domains with the amino-terminal (NH_2) and carboxyl-terminal (COOH) tails facing the cytosol. The first luminal loop is uniquely large and contains four N-glycosylation sites and a cleavage site. Two di-leucine motifs ETERL^{15}L and EEHSL^{577}L are located separately at each tail to mediate the localization of TRPML1 to late endosomes and lysosomes (LELs). At the N-terminus of TRPML1, several positively charged amino acid residues are predicted to interact with phosphoinositides with Arg61 and Lys 62 for PI(3,5)P_2 and Arg42/Arg43/Arg44 for PI(4,5)P_2, respectively. At the C-terminus, there are two potential PKA sites (S^{557} and S^{559}) and three potential palmitoylation sites (C$^{565–567}$). Two negatively charged amino acid residues (D^{471} and D^{472}) are potential pore-forming determinants. Gain-of-function mutations are found in the lower part of the S5 (e.g., V^{432}P). Loss-of-function mutations that cause ML-IV patients are throughout the protein (e.g., F408Δ)

GFP or mCherry-tagged TRPML1 proteins are also detected in the early endosomes and plasma membrane (Thompson et al. 2007; Vergarajauregui and Puertollano 2006). Immunostaining and gradient fractionation studies have confirmed the LEL localization of TRPML1 (Kim et al. 2009; Zeevi et al. 2009). GFP fusion proteins of TRPML1 are co-localized nicely (>80 %) with the lysosomal-associated membrane proteins1, 2, and 3 (Lamp1–3) (Manzoni et al. 2004). Moreover, in the gradient fractionation analysis, TRPML1 proteins were found primarily in the Lamp1-positive fractionations (Dong et al. 2010a; Kim et al. 2009).

The LEL localization of TRPML1 is instructed by two di-leucine motifs located separately in the N-terminal and the C-terminal cytosolic tails (see Fig. 1). The N-terminal motif (L^{15}L) interacting with clathrin adaptor protein 1 and 3 (AP1 and AP3) mediates a direct transport of TRPML1 proteins from trans-Golgi network (TGN) to LELs, whereas the C-terminal motif (L^{577}L) directs AP2-dependent internalization from the plasma membrane, which is followed by endocytic trafficking to LELs (Abe and Puertollano 2011; Pryor et al. 2006; Vergarajauregui and Puertollano 2006) (Fig. 1). When both di-leucine motifs are mutated (TRPML1-L^{15}L/AA-L^{577}L/AA), whole-cell TRPML1 currents become detectable (Zhang et al. 2012a).

3 The Channel Structure

Human TRPML1 is a 580-amino acid protein with a molecular mass of 65 kDa (Slaugenhaupt 2002). Due to the lack of a crystal structure for TRPML1, our knowledge about the topology of TRPML1 is mainly gained from bioinformatic analysis and biochemical and structural studies on other TRP channels (Cheng et al. 2010; Dong et al. 2010b). Similar to other TRP channels, TRPML1 consists of six putative transmembrane-spanning domains (TMs, S1–S6) with the amino-terminal (NH_2 or N) and carboxyl-terminal (COOH or C) tails facing the cytosol (Fig. 1). Strikingly, TRPML1 possesses a large highly N-glycosylated luminal loop separating the first two TMs, in which a proteolytic cleavage site with uncharacterized function is located (see Fig. 1) (Kiselyov et al. 2005; Miedel et al. 2006; Puertollano and Kiselyov 2009). The channel pore of TRPML1 is predicted to be formed by the linker or the so-called "pore-loop" region between S5 and S6 (Cheng et al. 2010). Consistently, the pore mutations of TRPML1 are known to affect the conductance and selectivity of TRPML1 channels (Dong et al. 2010a; Pryor et al. 2006; Vergarajauregui and Puertollano 2006). S5 and S6 are presumed to form the channel gate, and the gain-of-function gating mutations are found in the lower part of the S5 domain (Dong et al. 2009; Grimm et al. 2012; Xu et al. 2007) (Fig. 1). The COOH-terminus of TRPML1 contains two potential PKA sites: ($Ser^{557}$and Ser^{559}; see Fig. 1) (Vergarajauregui et al. 2008b) and three cysteine residues ($C^{565}CC$) for potential palmitoylation to ensure association with LEL membranes (Vergarajauregui and Puertollano 2006). In addition, there are multiple positively charged amino acids (Arg and Lys, Fig. 1) in a polybasic domain of the N-terminus of TRPML1(Dong et al. 2010a). Phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$, an LEL-localized phosphoinositide (Zhang et al. 2012b; Zolov et al. 2012), may directly bind to these sites to activate or increase the channel activity of TRPML1 (Dong et al. 2010a). The plasma membrane-localized $PI(4,5)P_2$, however, inhibits the activity of TRPML1 through distinct sites within the same polybasic domain (Zhang et al. 2012a).

4 Interacting Proteins

To gain a better understanding of how TRPML1 regulates multiple lysosomal functions (Cheng et al. 2010; Grimm et al. 2012), it is important to define the molecular context for TRPML1's channel function. One potential effector of TRPML1 channel is penta-EF-hand apoptosis-linked gene 2 protein (ALG-2). ALG-2, a Ca^{2+} binding protein, is found to directly bind to a stretch of amino acid residues (positions 37–49) on the N-terminus of TRPML1 in a Ca^{2+}-dependent manner (Vergarajauregui et al. 2009). Notably, the aberrant accumulation of enlarged endolysosomes induced by TRPML1 overexpression was dramatically attenuated when the potential ALG-2-interacting sites are mutated (Vergarajauregui et al. 2009). Hence ALG-2 may serve as a downstream Ca^{2+} sensor that couples the TRPML1-mediated lysosomal Ca^{2+} release to cellular

functions (Cheng et al. 2010; Vergarajauregui et al. 2009). Alternatively, ALG-2 may directly regulate TRPML1' channel activity (Cheng et al. 2010; Grimm et al. 2012; Vergarajauregui et al. 2009).

Another TRPML1 interaction partner is lysosome-associated protein transmembrane member proteins (LAPTMs), as demonstrated by a yeast two-hybrid screen and co-immunoprecipitation experiments (Vergarajauregui et al. 2011). Interestingly, knockdown of endogenous LAPTMs by specific siRNA induced the accumulation of endolysosomes with electron dense and multi-laminar structures, reminiscent of storage materials in ML-IV cells (Chen et al. 1998; Slaugenhaupt 2002; Zeevi et al. 2009).

Venugopul et al. identified an interaction between TRPML1 and a molecular chaperone complex including heat shock cognate protein of 70 kDa (Hsc70) and heat shock protein of 40 kDa (Hsp40) (Venugopal et al. 2009). The interaction appears to be through the large intraluminal loop between S1 and S2 of TRPML1 (Venugopal et al. 2009). Hsc70 and Hsp40 are required for recognizing the target cytosolic proteins during chaperone-mediated autophagy (CMA), which is defective in ML-IV fibroblasts (Venugopal et al. 2009). Interestingly, an increase in intracellular Ca^{2+} concentration enhances the co-immunoprecipitation and co-localization between Hsc70 and TRPML1 (Venugopal et al. 2009). TRPML1 is also found to interact with two-pore TPC proteins (Yamaguchi et al. 2011), but whether TRPML1 forms heteromeric channels with TPCs is unknown. Finally, a comprehensive and systematic screen for TRPML1 interactors has been recently performed, which resulted in the discovery of a large set of TRPML1-interacting proteins (Spooner et al. 2013).

5 The Channel Biophysical Properties and Function

5.1 Permeation Properties

The LEL localization of TRPML1 has made it difficult to analyze the permeation and gating properties of the channel. However, the recent development of the whole-endolysosome patch-clamp technique has allowed a direct study of TRPML1 on artificially enlarged lysosomes, which are induced by vacuolin-1, a small-molecule chemical compound that selectively enlarges lysosomes (Dong et al. 2010a; Wang et al. 2012). By using the whole-lysosome recordings, it was shown that TRPML1-mediated currents exhibit strong inward rectification (inward indicates cations moving out of the lysosomal lumen). TRPML1 is permeable to Ca^{2+}, Fe^{2+}, Zn^{2+}, Na^{+}, and K^{+} (Table 1) (Dong et al. 2008, 2009; Xu et al. 2007). TRPML1 has been shown to be impermeable to protons, although TRPML1-mediated currents are potentiated by low luminal pH (pH_L) (Dong et al. 2008; Xu et al. 2007) and loss of TRPML1 reportedly affected the lysosomal luminal pH (Miedel et al. 2008; Venkatachalam et al. 2008).

The single-channel conductance (see below) is 76 pS (from −140 mV to −100 mV) and 11 pS (from −80 mV to −40 mV) (Table 1; Xu et al. 2007) for

Table 1 Summary of the channel properties of TRPML1

Properties	TRPML1
Tissue distribution	Ubiquitously expressed with the highest expression levels in the brain, kidney, spleen, liver and heart
Subcellular localization	Late endosomes and lysosomes (LELs)
Ion selectivity	Non-selective: Ca^{2+}, Fe^{2+}, Zn^{2+}, Na^{+}, K^{+}, etc.
I–V plot	Strong inwardly rectifying (inward indicating cations flowing out of the lumen to the cytoplasm)
Single channel conductance (pS)	45 pS (−140 mV to −40 mV)
Activation mechanisms	Voltage; low luminal pH; $PI(3,5)P_2$
Activators	$PI(3,5)P_2$; ML-SA1; SF-22; SF-51
Inhibitors	Sphingomyelins; $PI(4,5)P_2$; Verapamil, Gd^{3+}, and La^{3+}
Interacting proteins	TRPML2, TRPML3; TPC2; ALG-2; LAPTMs; Hsc70; and Hsp40
Cellular functions	Lysosomal membrane trafficking; lysosomal exocytosis; autophagy; lysosomal ion homeostasis
Human diseases	Mucolipidosis IV and Niemann-Pick diseases
Genetic models	Human MLIV patients; Mouse TRPML1 knockout; *Drosophila trpml* mutant; *C elegans* CUP-5 mutant

$TRPML1^{Va}$ and 45 pS (−140 mV to −40 mV) for wild-type TRPML1 (Zhang et al. 2012a). Lysosome transmembrane potentials are presumed to be positive in the luminal side (> + 30 mV), which may provide a driving force for cation efflux from the LEL lumen into the cytosol (Dong et al. 2010b; Morgan et al. 2011).

The putative pore of TRPML1 is formed by the linker region of S5 and S6 (Fig. 1), which constitutes the pore loop and the selectivity filter of the channel (Cheng et al. 2010). Consistent with this prediction, replacing two negatively charged amino acid residues in the pore loop with positively charged ones ($D^{471}D^{472}$-KK) results in a pore-dead non-conducting channel (Dong et al. 2010a; Grimm et al. 2012; Xu et al. 2007).

5.2 Gating

The putative channel gate of TRPML1 is formed by S5 and S6. A proline substitution at Vla^{432} (Vla^{432} P or *Va*, a mutation at the homologous position in TRPML3 causing the varitint-waddler (*Va*) phenotype with pigmentation and vestibular defects in mice (Di Palma et al. 2002; Xu et al. 2007; Fig. 1) in the lower part of S5 in TRPML1 results in gain-of-function (GOF) constitutively active TRPML1 channels at both the plasma membrane and endolysosomal membranes (Xu et al. 2007). The constitutive channel activity caused by Pro substitutions is proposed to be related to locking or facilitating channel conformation at the open state (Dong et al. 2010a; Grimm et al. 2012; Xu et al. 2007). Furthermore, unlike the wild-type TRPML1 channel, $TRPML1^{Va}$ showed a dramatically increased plasma membrane localization, suggesting that the constitutive release of luminal cations

(most likely Ca^{2+}) promotes the delivery of TRPML1 to plasma membrane, likely via lysosome exocytosis (Dong et al. 2009).

Using whole-endolysosome and whole-cell recordings, endogenous activators and inhibitors have been identified for TRPML1. First, phosphoinositides are shown to regulate TRPML1 in a compartment-specific manner. $PI(3,5)P_2$, a phosphoinositide that is mainly localized in the LEL, potently activates TRPML1 with an $EC_{50} = 48$ nM, potentially through a direct binding mechanism (Dong et al. 2010a; Zhang et al. 2012a). Neutralizing the potential $PI(3,5)P_2$ binding sites in the N-terminus ($R^{42}R^{43}R^{44}K^{55}R^{57}R^{61}R^{62}$, 7Q; Fig. 1) completely abolished the activation effect (Zhang et al. 2012a). On the other hand, $PI(4,5)P_2$, a plasma membrane-specific phosphoinositide, inhibits TRPML1 in the inside-out patches (Zhang et al. 2012a). Interestingly, the inhibitory effect of $PI(4,5)P_2$ is also largely abolished in TRPML1-7Q, suggesting that the positively charged amino acid residues in the N-terminus of TRPML1 are required for the isoform-specific regulation of TRPML1's channel activity.

To further investigate the activation mechanisms of TRPML1, several synthetic small-molecule compounds have been recently identified as TRPML agonists (Grimm et al. 2010; Shen et al. 2012). Of them, Mucolipin Synthetic Agonist 1 (ML-SA1) robustly activates TRPML1 at low micromolar concentrations with a response comparable to $PI(3,5)P_2$ (Shen et al. 2012). These agonists may be helpful not only in investigating the gating mechanisms of TRPML1 but also in probing the cell biological functions of the channel (Grimm et al. 2012).

5.3 Channel Function

The permeation and gating properties of TRPML1 suggest that the channel functions of TRPML1 are to release $Ca^{2+}/Fe^{2+}/Zn^{2+}$from the LEL lumen in response to various cellular cues (Cheng et al. 2010; Shen et al. 2011), such as an alteration of lysosomal concentration of $PI(3,5)P_2$. Ca^{2+} efflux from endosomes and lysosomes is thought to be important for signal transduction, organelle homeostasis, and endosomal acidification (Dong et al. 2010b; Luzio et al. 2007a, 2007b; Morgan et al. 2011). The luminal concentration of Ca^{2+} is ~0.5 mM, which is 5,000-fold higher than the cytosolic Ca^{2+} at ~100 nM (Dong et al. 2010b; Morgan et al. 2011). TRPML1 is a natural candidate for lysosomal Ca^{2+} release, and the released Ca^{2+} may drive organelles fusion or fission within the endocytic pathway (Cheng et al. 2010). Consistently, lysosomal trafficking defects are observed in ML-IV and *TRPML1*$^{-/-}$ cells, suggesting that a primary role of TRPML1 is to mediate Ca^{2+} release from LEL upon physiological stimulations.

As TRPML1 is also permeable to Fe^{2+} and Zn^{2+}, TRPML1 may also participate in the regulation of the cellular homeostasis of these heavy metals (Dong et al. 2008). Indeed, cells that lack TRPML1 exhibit a cytosolic Fe^{2+} deficiency and an overload of lysosomal Fe^{2+}, suggesting that TRPML1 contributes to iron transport out of the lysosomes (Dong et al. 2008). Similarly, the permeability of TRPML1 to Zn^{2+} and elevated Zn^{2+} levels in *TRPML1*$^{-/-}$ cells are suggestive of an

essential role of TRPML1-mediated lysosomal Zn^{2+} transport in maintaining Zn^{2+} homeostasis (Eichelsdoerfer et al. 2010; Kukic et al. 2013).

Lysosomes contain a variety of acid hydrolytic enzymes that mediate the breakdown of waste materials and cellular debris (Luzio et al. 2007a). The activities of these enzymes require the luminal pH (pH_L) to be maintained at 4.5 – 5.0, which is mainly established by a vacuolar (V)-type H^+-ATPase (Luzio et al. 2007a). Despite being impermeable to protons, TRPML1 is potentiated by low pH_L (Dong et al. 2008; Xu et al. 2007), and *TRPML1*$^{-/-}$ cells exhibit hyperacidification (Miedel et al. 2008), suggesting that TRPML1 plays a role in regulating pH_L homeostasis. It is possible that elevated juxtaorganellar $[Ca^{2+}]_{cyt}$ caused by lysosomal Ca^{2+} release via TRPML1 induce H^+ efflux through a unidentified Ca^{2+}-H^+ exchanger (Cheng et al. 2010).

6 Physiological Functions

As a lysosomal Ca^{2+} release channel, in addition to regulating lysosome ion homeostasis (see above), TRPML1 has also been proposed to regulate lysosomal membrane trafficking and signal transduction. Lysosomal membrane trafficking refers to all the membrane fusion and fission steps related to the LEL, including endosome maturation, autophagosome–lysosome fusion, LEL-to-TGN retrograde trafficking, and lysosomal exocytosis. Many of these trafficking steps are known to be Ca^{2+} dependent and are defective in *TRPML1*$^{-/-}$cells, suggesting that TRPML1 functions are important in these processes (LaPlante et al. 2002; Shen et al. 2012; Thompson et al. 2007).

6.1 Endosome Maturation

In the endocytic pathway, early endosomes mature into late endosomes and subsequently lysosomes by undergoing lumen acidification, alterations in featured lipids, and dissociation and recruitment of compartment-associated proteins. The process of late endosomal maturation may be regulated by TRPML1 (Fig. 2), as the lysosomal delivery and subsequent degradation of endocytosed proteins, such as BSA and platelet-derived growth factor receptors (PDGF-R), are delayed in ML-IV fibroblasts and macrophage cells stably expressing shRNA against *TRPML1* (Thompson et al. 2007; Vergarajauregui et al. 2008a). In contrast, such a delay is not observed in HeLa cells with transient knockdown of TRPML1 (Miedel et al. 2008), suggesting that the regulatory role of TRPML1 on endosome maturation is cell type specific and subtle. Alternatively, the accumulation of endocytosed proteins caused by TRPML1 deficiency might be secondary to chronic lysosomal storage or other primary defects associated with TRPML1 deficiency (Miedel et al. 2008).

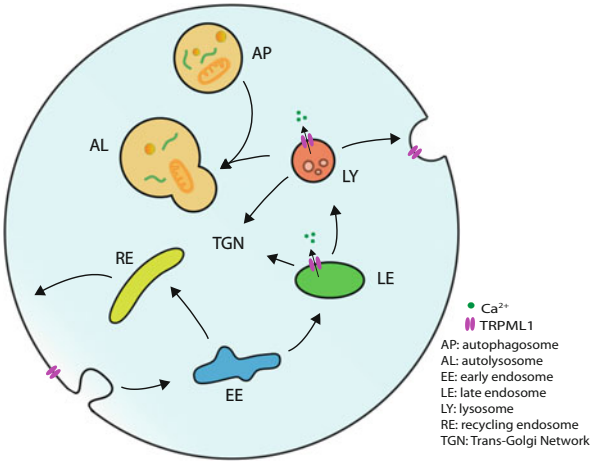

Fig. 2 TRPML1 in lysosomal membrane trafficking. Early endosomes (EEs) are formed upon endocytosis. EEs then undergo endosome maturation into LEs, which become LYs upon further acidification. TRPML1 is predominantly localized in the late endosomes (LEs) and lysosomes (LYs). TRPML1 may mediate Ca^{2+} release from LEs and LYs, which are Ca^{2+} stores with luminal Ca^{2+} concentration approximately 0.5 mM. Transport vesicles derived from LEs and LYs can mediate LEL-to-TGN retrograde trafficking. The autolysosomes (ALs) are formed from fusion of LYs with autophagosomes (APs). LYs can also undergo membrane fusion with plasma membrane (lysosomal exocytosis). Ca^{2+} release from TRPML1 may regulate the above-mentioned trafficking steps

6.2 LEL-to-TGN Retrograde Trafficking

Retrograde trafficking from the LEL to *trans*-Golgi-network (TGN) allows the reutilization of the digested products of lysosomes and the recycling of shuttle proteins that facilitate the transport of lysosomal proteins from TGN to LEL after the biosynthetic processes. For example, the mannose-6-P receptor is required for the lysosomal delivery of hydrolytic enzymes (Luzio et al. 2007b) (Fig. 2). The LEL-to-TGN retrograde trafficking is defective in ML-IV cells, as fluorophore-conjugated lactosylceramide, a lipid that is normally located in the Golgi-like compartment, is accumulated in LEL-like vesicles (Chen et al. 1998). Notably, the delayed or blocked retrograde trafficking in ML-IV and Niemann–Pick C (see below) cells is rescued by expression of TRPML1 or by synthetic TRPML1 agonist ML-SA1 (Shen et al. 2012), suggesting that the channel activity of TRPML1 is required for this specific trafficking step.

6.3 Autophagosome–Lysosome Fusion

The fusion of autophagosomes with lysosomes is essential for the degradation of damaged organelles and aged proteins during autophagy (Fig. 2), thus affecting cell

survival especially under starvation or stress conditions. Loss of TRPML1 function significantly enhances constitutive autophagy but not the starvation-induced autophagy (Vergarajauregui et al. 2008a). In ML-IV fibroblasts, elevated staining of microtubule-associated protein 1A/1B-light chain 3 (LC3, a protein marker for autophagosomes)-positive puncta was observed under complete medium, suggestive of an increase in the basal levels of autophagic flux (Curcio-Morelli et al. 2010; Vergarajauregui et al. 2008a). The constitutive activation of autophagy observed in ML-IV cells and *TRPML1*$^{-/-}$ neurons seems to be caused by both increased autophagosome formation and delayed fusion of autophagosomes with lysosomes (Curcio-Morelli et al. 2010; Vergarajauregui et al. 2008a). Consequently, autophagosomes undergo inefficient digestion and accumulate in TRPML-deficient cells. Interestingly, overexpression of TRPML1 in NRK cells also increases constitutive autophagy, which is also seen in TRPML1-deficient cells. However, because aberrant autophagy is not only found in TRPML1-related diseases but also observed in a wide spectrum of lysosome storage diseases (LSDs) (Lieberman et al. 2012), it remains to be elucidated whether TRPML1 plays a direct role in autophagosome–lysosome fusion.

6.4 Lysosomal Exocytosis

HEK293 cells transfected with TRPML1^{Va} (a gain-of-function mutation) exhibit enhanced lysosomal exocytosis (Dong et al. 2009). On the other hand, lysosome exocytosis induced by lysosome biogenesis transcription factor TFEB requires TRPML1 (Medina et al. 2011), and fibroblasts from ML-IV patients exhibit impaired ionomycin-induced lysosomal exocytosis (LaPlante et al. 2006; Medina et al. 2011). Likewise, shRNA-mediated TRPML1 knockdown in mouse macrophages results in a delay in the plasma membrane transport of the major histocompatibility complex II (MHCII) from LEL compartments in response to the immune stimulation (Thompson et al. 2007). Taken together, these results suggest that activation of TRPML1 may positively regulate lysosomal exocytosis.

6.5 Signal Transduction

Ca^{2+} release from LELs is believed to play an essential role in the transduction of extracellular signals such as glucose-induced insulin secretion in clonal pancreatic beta cells, arterial smooth muscle contraction, T-lymphocyte Ca^{2+} signaling, and neurotransmitter release (Galione et al. 2009). Nicotinic acid adenine dinucleotide phosphate (NAADP) is a Ca^{2+}-mobilizing second messenger produced in response to extracellular stimuli and believed to act on lysosome Ca^{2+} stores (Morgan et al. 2011). TRPML1, as well as two-pore TPC channels, is proposed to be the NAADP receptor (Grimm et al. 2012; Morgan et al. 2011; Zhang and Li 2007). However, while TPCs are shown to be $PI(3,5)P_2$ (but not NAADP)-activated Na^+-selective ion channels in endolysosomes (Wang et al. 2012), NAADP-induced

lysosomal Ca^{2+} release is still intact in TRPML1 KO cells (Yamaguchi et al. 2011). Hence it remains to be established whether TRPML1 plays a role in lysosomal signal transduction.

7 Lessons from Knockouts

Using genetic knockout (KO) approaches, animal models of ML-IV have been well established in mice, *C. elegans*, and *Drosophila*, providing opportunities to better understand the underlying pathogenic mechanisms at the organism and cellular levels and develop potential therapeutic strategies for ML-IV. The first murine model of TRPML1 KO generated by Slaugenhaupt's group (Venugopal et al. 2007) displays neurological, gastric, and opthalmological abnormalities that are reminiscent of the clinic features of ML-IV patients, which include mental retardation, retinal degeneration, constitutive achlorhydria, and iron deficiency. The progressive neurodegeneration phenotypes are prominent in TRPML1 KO mice, which exhibit gait changes at an age of 3 months and gradually develop hind-limb paralysis and typically die at the age of 8–9 months (Venugopal et al. 2007). At the cellular level, ML-IV-like dense granulomembranous storage bodies are observed in $TRPML1^{-/-}$ neurons and glial cells. Evident vacuolization was seen in parietal cells with elevated serum gastrin (Venugopal et al. 2007).

ML-IV-like phenotypes are also observed in the knockout models of *C. elegans* and *Drosophila*. The *cup-5* mutant worms exhibit decreased degradation of endocytosed proteins and accumulation of large vacuoles labeled with LEL markers, indicative of the defective endocytic trafficking (Fares and Greenwald 2001). Studies on the *Drosophila* model of ML-IV demonstrate that progressive neuronal death in *TRPML*-null flies is likely due to impaired autophagy, which results in the accumulation of lysosomal lipofuscin and damaged mitochondria and hence high levels of apoptosis (Venkatachalam et al. 2008). Meanwhile, the inefficient clearance of apoptotic neurons by *trpml*-null glial cells may aggravate cell death in neurons.

8 MI-IV and Other TRPML1 Related Diseases

ML-IV is an autosomal recessive lysosomal storage disorder first described as a new variant of the mucolipidoses characterized by prominent accumulation of lipids and cholesterols inside the cells (Berman et al. 1974). The most common clinical features of ML-IV patients include severe psychomotor retardation, retinal degeneration, and constitutive achlorhydria (Slaugenhaupt 2002). ML-IV-causing *MCOLN1* mutations have been identified predominantly in Ashkenazi Jews (Bargal et al. 2001). To date, there are more than 20 known mutations identified, most of which are severe loss-of-function mutations. Mutations with milder phenotypes, such as ΔF408 (Fig. 1), have only partial loss of the channel function (Dong et al. 2008). No effective treatment has been identified for ML-IV.

TRPML1's role may also be extended to other LSDs including Niemann–Pick A and C diseases (Shen et al. 2012). NPC exhibits lysosomal accumulation of sphingomyelin (SM), cholesterol, and glycolipids and insufficient activity of acid sphingomyelinase (aSMase). TRPML1-mediated lysosomal Ca^{2+} release is dramatically reduced in NPC cells, suggestive of a potential block of channel activity in NPC lysosomes (Shen et al. 2012). Interestingly, TRPML1 channel activity is inhibited by SM, but potentiated by aSMase. In the cellular assays, increasing TRPML1's expression or activity was shown to reduce lysosome storage and cholesterol accumulation in NPC cells (Shen et al. 2012). Collectively, TRPML1 channel deregulation in the lysosome may be a primary pathogenic mechanism that causes secondary lysosome storage, presumably by blocking TRPML1-dependent lysosomal trafficking in NPC.

9 Concluding Remarks

TRPML1 is a lysosomal Ca^{2+} release channel that is important for the regulation of lysosomal membrane trafficking and lysosome ion homeostasis. However, it is still difficult to know whether any of the cellular defects are directly caused by TRPML1 deficiency or indirectly caused by the chronic storage of lysosomal materials. Employing approaches to acutely activate and inhibit TRPML1's channel function may prove helpful in distinguishing these possibilities.

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TRPML2 and Mucolipin Evolution

Jaime García-Añoveros and Teerawat Wiwatpanit

Contents

Abstract

The TRPML2 protein, encoded by the *Mcoln2* gene, is one of the three mucolipins (TRPML1–3), a subset of the TRP superfamily of ion channels. Although there are no thorough studies on the cellular distribution of TRPML2, its mRNA appears to be largely restricted to lymphocytes and other immune cells. This contrasts with the ubiquitous expression of TRPML1 and the limited but diverse expression of TRPML3 and clearly suggests a specialized role for TRPML2 in immunity. Localization studies indicate that TRPML2 is present in lysosomes (including the specialized lysosome-related organelle that B-lymphocytes use for processing of the antigen-bound B-cell receptor), late endosomes, recycling endosomes, and, at a much lower level, the plasma

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membrane. Heterologously expressed TRPML2, like TRPML1 and/or TRPML3, forms ion channels that can be activated by a gain-of-function mutation (alanine to proline in the fifth transmembrane domain, close to the pore) that favors the open state, by a transient reduction of extracellular sodium followed by sodium replenishment, by small chemicals related to sulfonamides, and by PI(3,5)P2, a rare phosphoinositide that naturally accumulates in the membranes of endosomes and lysosomes and thus could act as a physiologically relevant agonist. TRPML2 channels are inwardly rectifying and permeable to Ca^{2+}, Na^{+}, and Fe^{2+}. When heterologously co-expressed, TRPML2 can form heteromultimers with TRPML1 and TRPML3. In B-lymphocytes, TRPML2 and TRPML1 may play redundant roles in the function of their specialized lysosome. Although the specific subcellular function of TRPML2 is unknown, distribution and channel properties suggest roles in calcium release from endolysosomes, perhaps to regulate vesicle fusion and/or subsequent scission or to release calcium from intracellular acidic stores for signaling in the cytosol. Alternatively, TRPML2 could function in the plasma membrane, and its abundance in vesicles of the endocytic pathway could simply be due to regulation by endocytosis and exocytosis. The *Mcoln2* gene is closely downstream from and in the same orientation as *Mcoln3* in the genomes of most jawed vertebrates (from humans to sharks) with the exception of pigs, *Xenopus tropicalis*, and ray-finned fishes. The close homology of TRPML2 and 3 (closer to each other than to TRPML1) suggests that *Mcoln2* and *Mcoln3* arose from unequal crossing over that duplicated a common ancestor and placed both gene copies in tandem. These genes would have come apart subsequently in pigs, Xenopus, and the ancestor to ray-finned fishes. All jawed vertebrates for which we have thorough genomic knowledge have distinct *Mcoln1, 2, and 3* genes (except ray-finned fishes which, probably due to the whole-genome duplication in their common ancestor, have two *Mcoln1*-like genes and two *Mcoln3*-like genes, although only one *Mcoln2* gene). However, the available genomes of invertebrate deuterostomes (a sea urchin, lancelet, and two tunicates) contain a single mucolipin gene that is equally distant from the three vertebrate mucolipins. Hence, vertebrate mucolipins arose through two rounds of gene duplication (the first one likely producing *Mcoln1* and the ancestor to *Mcoln2* and *3*) at some time between the onset of craniates and that of jawed vertebrates. This is also the evolutionary period during which adaptive immunity appeared. Given the restricted expression of TRPML2 in immune cells, this evolutionary history suggests a functional role in the adaptive immunity characteristic of vertebrates.

Keywords

Mucolipin • Mcoln • Mcoln2 • TRPML • TRPML2 • Endosome • Lysosome • Lymphocyte • Ion channel • Channel evolution • Adaptive immunity

This review on TRPML2 updates and revises a previous review article on the same topic from our group (Flores and García-Añoveros 2011) and shall supersede it.

1 Gene Splizing, Location, and Evolution

The TRPML2 protein (also known as mucolipin 2), the product of the *Mcoln2* gene (also known as *Trpml2*), is one of the three vertebrate isoforms (TRPML1–3) of the mucolipin family of transient receptor potential (TRP) cation channels. Their name derives from its paralog mucolipin 1, which is mutated in mucolipidosis type IV, a lysosomal disease. *Mcoln2* was molecularly identified when the *varitint-waddler* mouse mutations, which cause deafness and pigmentation defects, were identified in the adjacent *Mcoln3*. The *Mcoln2* gene maps to human chromosome I (1p22.3) and mouse chromosome 3 (3qH2), very closely downstream (21 and 8 kb, respectively) of *Mcoln3*.

In most tetrapod genomes (35 species) for which there is enough sequence information, as well as in the genome of their closest living relative (the coelacanth *Latimeria chalumnae*), *Mcoln2* is closely downstream of *Mcoln3* and in the same strand (hence "direction"). Two exceptions are an amphibian (*Xenopus tropicalis*) and the pig (*Sus scrofa*). In the frog *Mcoln3* is in the syntenic region (i.e., surrounded by the same genes as in most genomes), whereas its *Mcoln2* has translocated to a new genomic location. However, in the eight available genomes of ray-finned fishes, *Mcoln2* and *Mcoln3* genes are no longer closely linked (in three species—medaka, stickleback, and tetraodon—*Mcoln2* is in the same chromosome as *Mcoln3*, but upstream and at a much greater distance, with many other genes in between). The conservation of the close linkage between *Mcoln2* and *Mcoln3* in most tetrapods plus coelacanth (36/38 genomes) might suggest an evolutionary pressure, such as a shared regulation of expression, to keep both genes together. However, both genes display very different patterns of expression (see below), and our unpublished in situ hybridization (ISH) experiments reveal no alteration of *Mcoln2* expression in *Mcoln3* KO mice. Hence, although possible, there is presently no additional evidence favoring a co-regulatory mechanism for *Mcoln2* and *Mcoln3*. Quite likely, their close linkage has prevented their separation in most evolutionary lineages. Another question to be resolved is how the *Mcoln2* and *Mcoln3* became linked. The one cartilaginous fish genome (that of the elephant shark *Callorhinchus milii*) contains genes for all three mucolipins (Fig. 1), and the partial genomic assembly available as of the writing of this article reveals that in this genome, as in most tetrapods and coelacanth, *Mcoln2* is closely downstream of *Mcoln3* (Byrappa Venkatesh, personal communication). This close linkage, their same orientation, and their high homology (more similar to each other than to *Mcoln1*) suggest that both genes arose from a duplication through ectopic recombination (unequal crossing-over), which generates paralogous genes in tandem, but in that case two genes would have separated in the lineage leading to ray-finned fishes.

While nearly all vertebrate sequences thus far seem to contain genes for all three mucolipins (including mucolipin 2), invertebrates of the deuterostome branch (the

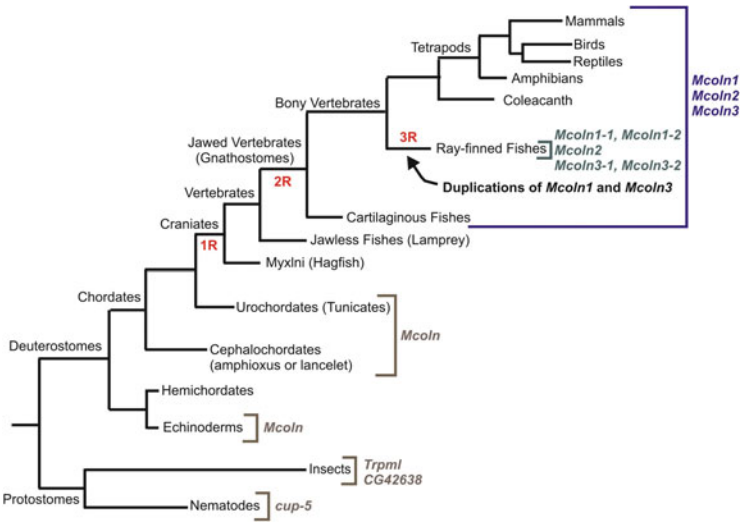

Fig. 1 Cladogram indicating which genes encoding TRPML proteins (also termed mucolipins) are present in the genomes of different animal groups, which reveals the likely evolutionary period when *Mcoln1, 2,* and *3* originated through duplication (sometimes between the onset of craniates and that of jawed vertebrates). *Trpml* and *CG42638* are the two mucolipin genes in *Drosophila melanogaster*, and *cup-5* is the sole mucolipin gene in *Caenorhabditis elegans*. The information regarding mucolipin genes was inferred from a dendrogram assembled in e!Ensembl (http://uswest.ensembl.org/index.html) and from individual searches of *Mcoln* orthologs in the genomes of the sea urchin *Strongylocentrotus purpuratus* (an echinoderm), the lancelet *Branchiostoma floridae* (a cephalochordate), and the elephant shark *Callorhinchus milii* (a cartilaginous fish). The three presumed whole-genome duplications in deuterostomes are indicated as 1R, 2R, and 3R

sea urchin *Strongylocentrotus purpuratus*, which is an echinoderm; the lancelet *Branchiostoma floridae*, which is a cephalochordate; and the tunicates *Ciona intestinalis* and *C. savigni*, which are urochordates) seem to have a single *Mcoln* gene which is equally distant to *Mcoln1, 2,* and *3* (Fig. 1). Hence, at some time between the onset of craniates and that of jawed vertebrates (tetrapods, coelacanth, ray-finned fishes, and cartilaginous fishes, aka Gnathostomes), duplications of an ancestral *Mcoln* gene must have produced all three *Mcoln* paralogs. Completion of the lamprey genome, currently underway, would help further clarify the evolutionary onset of the three vertebrate mucolipins. Based on all the evidence above described, we hypothesized that one of the two presumed whole-genome duplications in the evolutionary line leading to jawed vertebrates (1R or 2R; Fig. 1) gave rise to *Mcoln1* and an *Mcoln2/3* ancestor, which in a subsequent duplication (likely to be local rather than whole genome) generated (also prior to the appearance of jawed vertebrates) the adjacent *Mcoln2* and *Mcoln3* genes. This evolutionary analysis shall override the one we previously published (Flores and García-Añoveros 2011), which was based in incomplete genome data and resulted in some suggestions about mucolipin evolution that are no longer tenable.

A third whole-genome duplication (3R) is believed to have occurred in the common ancestor to ray-finned fishes (Fig. 1). Accordingly, their genomes contain two close paralogs of *Mcoln1* and *Mcoln3*. However, only one *Mcoln2* is present in each of their genomes (Fig. 1). The genomic rearrangements and gene loss that followed this whole-genome duplication may explain the separation of *Mcoln2* and *Mcoln3* genes in this lineage.

The evolutionary history of *Mcoln3* and *Mcoln2* is curiously similar to that of the *Tmem138* and *Tmem216* genes, which are closely linked in tandem in the genomes of the elephant shark, coelacanth, and most tetrapods but have come apart in the branch leading to ray-finned fishes (in which the 3R whole-genome duplication has also similarly resulted in two *Tmem138*-like genes but only one of *Tmem216* gene) as well as in *Xenopus tropicalis* (Venkatesh et al. 2013). Perhaps more significant is the similarity between the evolutionary onset (between the ancestor to craniates and that of jawed vertebrates) and selection of the *Mcoln2* (Fig. 1) and that of adaptive immunity (Flajnik and Kasahara 2010), as it suggests a role of TRPML2 in this cell-based form of immunity characteristic of vertebrates.

The mouse *Mcoln2* gene generates at least two transcripts and polypeptides that result from alternative first exons: TRPML2sv (short, 2,384 bp mRNA and 538 residue protein; NM_001005846) and TRPML2lv (long, 2,418 bp mRNA and 566 residue protein; NM_026656 vs). The first exon of TRPML2sv does not code for protein, whereas the first exon of TRPML2lv encodes the additional 28 amino acids to the amino-terminus of TRPML2lv. Human *Mcoln2* generates only one mRNA and protein of 566 amino acids, the same size as mouse TRPML2lv (Samie et al. 2009).

2 Gene Expression and Protein Localization

By RT-PCR, Trpml2 mRNA was detected in multiple organs of human (lung, trachea, stomach, colon, mammary glands, and peripheral blood lymphocytes with lower levels in the kidney, thymus, testis, and uterus and lowest levels seen in the spleen, adipose tissue, and thyroid) and mouse (the kidney, thymus, trachea, colon, and adipose tissue with lower levels in the lung, liver, pancreas, and testis) (Grimm et al. 2010). However, this technique is overly sensitive and not quantitative, and it may well reveal expression levels that are non-physiological (such as that produced by "leaky" transcription). By quantitative RT-qPCR expression of both alternative mRNAs of mouse, Trpml2 were also detected in multiple organs (all those examined). However, expression of the long Trpml2lv transcript was very low in all organs, and expression of the short Trpml2sv transcript was very low in most. Comparatively high expression levels were only detected in the thymus and spleen (with lower but still above average levels in the kidney and stomach) (Samie et al. 2009). We performed in situ hybridization (ISH) with two non-overlapping probes to separate segments on mouse *Trpml2* mRNA on sections of adult mouse organs as well as sagittal sections of newborn mouse pups and found consistent expression only in the thymus and spleen (Natalie Remis and JG-A, unpublished results). By contrast, ISH with probes to the ubiquitous Trpml1 labeled all organs,

while probes to the more restricted Trpml3 labeled several organs (unpublished results and (Castiglioni et al. 2011)). Hence, while it is possible that Trpml2 mRNA is expressed at very low levels (below detection for ISH) ubiquitously, it is certainly expressed at high levels on cells of the thymus and spleen, which suggest expression in lymphoid cells. This is further supported by the source of most Trpml2 cDNA clones (spleen, bone marrow, thymus, and hematopoietic progenitors) (http://www.informatics.jax.org/searches/estclone_report.cgi?_Marker_key=52259&sort=Tissue at Mouse Genome Informatics) as well as by RT-PCR detection of Trpml2 mRNA in B cells, T cells, primary splenocytes, and mastocytoma and myeloma cell lines (Lindvall et al. 2005). Interestingly, the levels of Trpml2 (but not Trpml3) mRNA were higher in the thymus, spleen, and kidney of *Mcoln1*1 KO than of wild-type mice (Samie et al. 2009), which suggests that the expression of both genes is coordinated in some way and that TRPML2 might be able to compensate for the lack of TRPML1 in these tissues.

In summary, while TRPML1 is ubiquitous and TRPML3 expressed by discrete but diverse cell types, expression of TRPML2 seems highly restricted to lymphocytes and other cells of the immune system.

Although electrophysiological recordings clearly indicate that, when heterologously expressed, some TRPML2 channel localizes to the plasma membrane (Dong et al. 2008; Grimm et al. 2012; Lev et al. 2010; Samie et al. 2009; Zeevi et al. 2010), protein localization studies reveal that most TRPML2, similarly to TRPML1 and TRPML3, localizes to late endosomes and lysosomes (Karacsonyi et al. 2007; Song et al. 2006; Venkatachalam et al. 2006; Zeevi et al. 2009). Tagged TRPML2 heterologously expressed in HEK cells, B-lymphocytes, and HeLa cells accumulated at vesicular structures that co-labeled with markers of lysosomes (Lysotracker, Lamp 3, and Rab 11) and late endosomes (CD63) but not with markers of ER (ER-YFP), Golgi (GFP-Golgin 160; GGA3-VHSGAT-GFP), early endosomes (Rab5, EEA1), late endosomes (Rab7-HA), or secretory vesicles (Rab8) (Karacsonyi et al. 2007; Song et al. 2006; Venkatachalam et al. 2006). Immunoreactivities with antibodies raised against TRPML2 co-localized with the lysosomal marker Lamp1 in untransfected HEK cells, although the specificity of these immunoreactivities has not been demonstrated and there is no confirmation by other means that HEK cells express TRPML2 endogenously (Zeevi et al. 2009). In addition to lysosomes, heterologously expressed, tagged TRPML2 has also been localized in HeLa cells to plasma membrane and tubular endosomes of the Arf6 (GTPase ADP-ribosylation factor 6) pathway, which are recycling endosomes. TRPML2 co-localizes with CD59 and MHC-I, which are also markers of these endosomes, and inhibition of TRPML2 with shRNAs or with a dominant-negative isoform of TRPML2 inhibited the recycling of CD59 from these sorting endosomes back to the plasma membrane (Karacsonyi et al. 2007). However, the inhibition of clathrin-coated endocytosis by co-expression with full-length AP180 (a protein that binds clathrin and causes it to be distributed into a lattice-like pattern thereby preventing clathrin-coated pit formation and clathrin-mediated endocytosis), or with the dominant-negative isoform of Dynamin (Dyn1aK44A, which is incapable of binding GTP), resulted in the accumulation of TRPML2 at the plasma membrane

instead of lysosomal membranes (Venkatachalam et al. 2006). These studies suggested that TRPML2 may be internalized from the plasma membrane through clathrin-coated endocytosis but that once in endosomes it may participate in endosome recycling back to the plasma membrane through the Arf6 pathway.

A problem with the TRPML2 localization studies is that most of them are performed with heterologous overexpression and in cells that do not endogenously express it or have been severely altered through "immortalization" into cell lines. Ideally, these studies would be performed in cells directly obtained from a living organism and that endogenously express TRPML2 (like lymphocytes) with antibodies that recognize endogenous TRPML2 and a control for their specificity (such as a *Mcoln2* KO).

3 The Channel Protein Including Structural Aspects

Like all members of the TRP family, TRPML2 polypeptides are predicted to have their amino- and carboxy-termini in the cytosol, span the membrane six times (with the S1 to S6 domains), and loop in and out of the membrane from the extracellular space by a pore domain situated between S5 and S6. Like all mucolipins, TRPML2 exhibits a large extracellular or luminal loop (~210 amino acids in mouse and human TRPML2) between S1 and S2 (Puertollano and Kiselyov 2009). Although little mutagenesis has been performed in TRPML2, mutagenesis in the other two mucolipins clearly suggests that this large extracellular loop, though distant from the pore in primary sequence, participates in gating the channel.

4 Interacting Proteins

Co-immunoprecipitation and FRET analysis demonstrated that heterologously co-expressed, tagged TRPMLs can associate with one another in all combinations (Grimm et al. 2010; Venkatachalam et al. 2006). Others found that TRPML2 coprecipitated with TRPML1/3, albeit with low efficiency, since only 5–6% of the available TRPML2 co-immunoprecipitated with TRPML1 or TRPML3 (Curcio-Morelli et al. 2010; Zeevi et al. 2009). Finally, heterologous expression revealed that TRPML2 with a of a dominant-negative mutation (DD458/459KK) interacted with and suppressed membrane currents generated by dominant gain-of-function mutations [equivalent to the A419V mutation that was shown to increase channel open probability in the *varitint-waddler* mouse (Grimm et al. 2007; Nagata et al. 2008; Xu et al. 2007)] in TRPML1, 2, and 3, whereas the equivalent dominant-negative mutation in any of the three mucolipins interacted with and inhibited the dominant gain-of-function Va-like mutation (A369P) in TRPML2 (Zeevi et al. 2010). These interactions seemed to specifically occur among mucolipins, as none of them co-immunoprecipitated with the more distant TRPM8 (Zeevi et al. 2010). Hence, TRPML2 can form homomultimers as well as heteromultimers with TRPML1 and/or TRPML3.

Heterologous co-expression of TRPML2 and TRPML3 results in co-localization in Lamp3-positive lysosomes (Venkatachalam et al. 2006), and co-expression of TRPML2 and TRPML1 in B-lymphocytes results in co-localization in Lysotracker-positive lysosomes (Song et al. 2006). In human skin fibroblasts, immunoreactivities with antibodies raised to TRPML1, 2, and 3 co-localize with one another in Lamp1-containing lysosomes, although again it is unclear whether these immunoreactivities represented endogenous TRPML proteins (Zeevi et al. 2009). The co-localization of TRPML2 with TRPML1 appears to be limited to lysosomes, indicating that TRPML2 may have overlapping function with TRPML1. In support, shRNA-mediated knockdown of TRPML2 leads to the appearance of abnormal lysosome storage bodies reminiscent of lysosomes seen in MLIV patients (Zeevi et al. 2009). In summary, TRPML2 can interact with TRPML1 and TRPML3 in lysosomes or late endosomes, although this interaction may be limited and represent a small fraction of the total TRPML2 protein. Hence, although TRPML2 might heteromultimerize with TRPML1 and 3, it likely also forms channels without these subunits.

The heat shock cognate protein of 70 kDa (Hsc70) interacts with the first, large extracellular (or intraluminal, for the vast majority of channels that are in vesicular rather than plasma membranes) loop of TRPML1. HSC70 also co-immunoprecipitates with TRPML2 and TRPML2, although it is unresolved whether this is due to direct binding or to binding to TRPML1 subunits that are heteromultimerized with TRPML2 and/or 3. In any case, this interaction would physiologically only occur in the lumen of the lysosomes. Hsc70 participates in the translocation of cytosolic proteins into the lysosome for degradation, and for this role its presence seems to be required in both the cytosol and the lysosomal lumen. Hence, it was proposed that an interaction between mucolipin channels and intraluminal Hsc70 could facilitate the importation of cytosolic proteins for lysosomal degradation, a process referred to as chaperone-mediated autophagy (Venugopal et al. 2009).

5 A Biophysical Description of the Channel Function, Permeation, and Gating

Even though most TRPML2 probably localizes to the intracellular membranes of endosomes and lysosomes, heterologously expressed TRPML2 results in whole cell recording, and hence some of it must localize to the plasma membrane. Heterologously expressed TRPML2 at the plasma membrane has been activated with gain-of-function mutations that favor the open state (Grimm et al. 2007; Lev et al. 2010; Samie et al. 2009; Zeevi et al. 2010), alterations in extracellular pH (Dong et al. 2008; Lev et al. 2010), transient reduction of extracellular sodium (Grimm et al. 2012), some synthetic small molecules (Grimm et al. 2012), and the phosphoinositide PI(3,5)P2 (Dong et al. 2010). These recordings demonstrated that TRPML2 mediates inward currents at negative potentials and no outward currents at positive ones, the same inward rectification of TRPML3 and TRPML1 (Grimm

et al. 2007; Nagata et al. 2008; Xu et al. 2007) and hence a characteristic of mucolipins. Ion substitution experiments and reversal potentials revealed that the mutated TRPML2 channel is permeable to Ca^{2+}, Na^{+}, and Fe^{2+} (Dong et al. 2008), which led to the suggestion that TRPML2, like TRPML1, could transport iron from the lumen of endosomes and lysosomes into the cytosol.

The mutation used to generate constitutive activation of TRPML2 generated an A369P substitution in the fifth transmembrane domain (S5). This mutation mimicked the equivalent (A419P) substitution in TRPML3 caused by the *varitint-waddler* mutation, which constitutively activates the channel (Grimm et al. 2007; Nagata et al. 2008; Xu et al. 2007). In HEK293 cells, TRPML2 (A369P), but not wild-type TRPML2, generated currents and calcium overload. However, heterologous expression of the wild-type human TRPML2 in *Drosophila* S2 (Schneider) cells produced currents. The reasons for this constitutive activity are unknown.

Thus far, three small chemical compounds previously found in a screen for agonists of TRPML3 (Grimm et al. 2010) have also been found to activate wild-type TRPML2 heterologously expressed in HEK293 cells (Grimm et al. 2012). These are SF-21 (4-chloro-N-(2-morpholin-4-ylcyclohexyl)benzenesulfonamide), SF-41 (1-(2,4-dimethylphenyl)-4-piperidin-1-ylsulfonylpiperazine), and SF-81 (4,6-dimethyl-3-(2-methylphenyl)sulfonyl-1-propan-2-ylpyridin-2-one). These compounds are synthetic and it is unclear whether similar compounds exist in living organisms that might act as natural agonists of TRPML2 and TRPML3.

Another stimulus shown to trigger currents in cells heterologously expressing wild-type TRPML2 is the transient lowering of extracellular sodium followed by the replenishment of sodium or other permeant cations (Grimm et al. 2012), a maneuver that was previously found to activate TRPML3 (Grimm et al. 2010; Kim et al. 2007). However, it is unclear whether these ionic changes occur in a natural setting and thus whether this form of channel activation bears physiological relevance.

The presence of TRPML2 in endosomes and lysosomes suggests potential physiological evidence to two other channel activators, pH and PI(3,5)P2. The lumen of these organelles is characterized by a low pH, and thus its effect on the constitutively active TRPML2 (A369P) was explored. However, the results obtained were opposite, and while one report found that lowering the pH from 7.2 to 4.6 increased inward currents (Dong et al. 2008), another study reported that lowering the pH from 7.5 to 6.0 reduced the currents by 25 % (Lev et al. 2010). The reasons for this difference are unknown. Activation of wild-type TRPML2 was also achieved by PI(3,5)P2, a rare phosphoinositide that is specifically found in endosome and lysosome membranes (Dong et al. 2010). This led to the suggestion that activation of mucolipins in endosomes and lysosomes would cause a release of calcium from their lumen into the surrounding cytosolic space that could trigger their fusion or, if already fused, their scission.

Single channel recordings of TRPML2 have only been reported once from artificial lipid bilayers that incorporated TRPML2 protein that had been previously translated in mammalian cells or in vitro (Curcio-Morelli et al. 2010). The single channel properties thus obtained were a small conductance (7.44 pS for TRPML2 and 10.2 pS for TRPML3), as well as partial permeability to anions for both

channels. These single channel properties differ from the single channel conductance (50–70 pS) and permeability to cations of TRPML3 expressed in the plasma membrane of mammalian cells (Nagata et al. 2008) and are uncharacteristic of TRP channels. Because artificial lipid bilayers are not a physiological environment, the single channel properties of TRPML2 should be corrected or confirmed by recordings from patches of biological membranes.

6 Physiological Functions in Native Cells, Organs, and Organisms

We do not know the function of TRPML2, which is not surprising given the lack of *Mcoln2* KO mice, of human diseases with mutations on *Mcoln2*, and of the lack of targeted inhibition (by specific antagonists, RNA interference, or cell culture-based gene targeting) in cells (such as lymphocytes) that clearly express TRPML2 endogenously. However, heterologous expression of GFP::TRPML2 or GFP::TRPML1 (in which GFP is tagged at the carboxy-terminus of the mucolipins) caused an enlargement or clumping of the lysosomes in these cells quite reminiscent of the pathological vacuolation of lysosomes in MLIV patients. This pathology was not generated by KO of the *Mcoln1* gene in these cells (*Mcoln2* could not be targeted) nor by heterologous expression of TRPML2::GFP or TRPML1::GFP (in which GFP is tagged at the amino-terminus of the mucolipins). A reasonable (although not exclusive) interpretation of these results was that TRPML1 and TRPML2 function redundantly in these cells and that the C-terminus-tagged mucolipins act as dominant negatives. If these assumptions are correct, expression of these constructs would be revealing the effects of interfering with endogenous TRPML1 and TRPML2 function in the lysosomes of B-lymphocytes, which are specialized in the processing of the internalized, antigen-bound B-cell receptor (Song et al. 2006). This does not reveal the precise role or roles of TRPML2 in lysosomal function, but, as for the other mucolipins, reasonable hypothesis includes calcium release from endosomes and/or lysosomes to regulate vesicle fusion or scission, calcium release from intracellular acidic stores for signaling purposes, and release of iron or other metals from organelles into the cytosol (Cheng et al. 2010). Alternatively, the abundance of TRPML2 in intracellular membranes could be due to the regulation of its levels in the plasma membrane through endocytosis and exocytosis, and hence its physiological role could take place in the plasma membrane.

Regardless of its subcellular function, the restricted expression of TRPML2 in immune cells, its co-localization with CD59 and the major histocompatibility complex class I (MHC-I) (Karacsonyi et al. 2007), and its presence in and effects on the specialized lysosomes of B-lymphocytes (Song et al. 2006) all point to roles in adaptive immunity. This is further suggested by our evolutionary analysis, which shows that both adaptive immunity (Flajnik and Kasahara 2010) and the *Mcoln2* gene (Fig. 1) arose at about the same time in the deuterostome lineage and remain present in the same extant vertebrate species.

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TRPML3

Christian Grimm, Maria Barthmes, and Christian Wahl-Schott

Contents

Abstract

TRPML3 belongs to the MCOLN (TRPML) subfamily of transient receptor potential (TRP) channels comprising three genes in mammals. Since the discovery of the pain sensing, capsaicin- and heat-activated vanilloid receptor (TRPV1), TRP channels have been found to be involved in regulating almost all kinds of our sensory modalities. Thus, TRP channel members are sensitive to heat or cold; they are involved in pain or osmosensation, vision, hearing, or taste sensation. Loss or mutation of TRPML1 can cause retina degeneration and eventually blindness in mice and men (mucolipidosis type IV). Gain-of-function mutations in TRPML3 cause deafness and circling behavior in mice. A special feature of TRPML channels is their intracellular expression. They mostly reside in membranes of organelles of the endolysosomal system such as early and late endosomes, recycling endosomes, lysosomes, or lysosome-related organelles. Although the physiological roles of TRPML channels within the endolysosomal

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system are far from being fully understood, it is speculated that they are involved in the regulation of endolysosomal pH, fusion/fission processes, trafficking, autophagy, and/or (hormone) secretion and exocytosis.

Keywords
TRPML • TRPPML3 • TRP • Lysosome • Endosome • Cation channel • Varitint-waddler

Abbreviations

LRO	lysosome-related organelle
LSD	lysosomal storage disease
ML IV	mucolipidosis type IV
TRP	transient receptor potential

1 Gene

The human *trpml3 (mcoln3)* gene resides on the short arm of chromosome 1 at 1p22.3. The gene is split in 12 exons, which entail an open reading frame of 1,659 nucleotides. Humans also express a splice variant, which will be discussed below (see chapter "Protein"). Murine *trpml3* is located on the distal end of chromosome 3 at 3qH2 and, like its full-length human counterpart, has an open reading frame of 1,659 nucleotides.

2 Expression

TRPML3 (synonym: MCOLN3) mRNA was found in a large variety of tissues and organs (Cuajungco and Samie 2008; Grimm et al. 2010; Table 1) which include in humans the kidney, intestine, lung, and thymus as well as major hormone-producing organs or organs under hormonal control such as the adrenal glands, mammary glands, ovary, pancreas, testis, thyroid, and uterus. The UniGene EST profile of human TRPML3 which is an approximate expression pattern inferred from EST sources (Table 1) also suggests high levels of human TRPML3 in the adrenal glands, mammary glands, ovary, pancreas, and uterus as well as parathyroid, and pituitary gland. Highest levels are suggested for the latter two and the adrenal glands. All three are key organs of the endocrine system. These data are largely in agreement with the data provided by NextBio Research Body Atlas (Table 1). RT-PCR results also suggest the presence of TRPML3 mRNA in the human inner ear (Grimm et al. unpublished data).

Table 1 Human and murine TRPML3 expression

Tissue (human)	RT-PCR	UniGene EST	Nextbio body atlas	Tissue (mouse)	RT-PCR	UniGene EST	Nextbio body atlas
Adipose tissue	x	–	x	Adipose tissue	xxx	–	(x)
Adrenal gland	x	xx	xxx	Adrenal gland	–	–	–
Brain	(x)	x	(x)	Brain	(x)	–	–
Cerebellum	–	n.t.	–	Cerebellum	(x)	n.t.	–
Ear	x	–	n.t.	Ear	xxx	xx	x
Eye	n.t.	–	x	Eye	(x)	x	x
Heart	x	–	x	Heart	–	–	x
Intestine	x	x	x	Intestine	(x)	xx	xxx
Kidney	x	x	x	Kidney	xxx	xx	xxx
Liver	–	x	–	Liver	x	xx	x
Lung	x	x	x	Lung	x	–	x
Mammary gland	x	xx	x	Mammary gland	n.t.	(x)	(x)
Muscle	–	x	x	Muscle	–	–	–
Olfactory bulb	n.t.	n.t.	x	Olfactory bulb	x	n.t.	–
Ovary	x	x	x	Ovary	n.t.	–	–
Pancreas	x	x	x	Pancreas	–	–	–
Parathyroid	n.t.	xxx	n.t.	Parathyroid	n.t.	n.t.	n.t.
Pituitary gland	n.t.	xxx	xx	Pituitary gland	n.t.	–	(x)
Salivary gland	–	–	–	Salivary gland	–	–	–
Skin	–	x	x	Skin	xxx	xxx	x
Spleen	(x)	–	(x)	Spleen	x	x	x
Stomach	x	–	x	Stomach	x	xx	–
Testis	x	–	(x)	Testis	x	–	(x)
Thymus	x	x	x	Thymus	xx	xx	x
Thyroid	x	–	x	Thyroid	–	–	–
Trachea	x	x	–	Trachea	x	n.t.	–
Uterus	x	x	x	Uterus	n.t.	–	–

Summary of experimentally derived TRPML3 RT-PCR data (Cuajungco and Samie 2008; Grimm et al. 2010) compared to UniGene EST profile (http://www.ncbi.nlm.nih.gov/UniGene) and NextBio Research Body Atlas (http://www.nextbio.com) data; (x), very low gene expression level; x, moderate gene expression level; xx, strong gene expression level; xxx, very strong gene expression level; –, no expression detected; n.t., "not tested"

In murine tissue the situation looks slightly different. Murine TRPML3 mRNA is mainly found in the inner ear, skin, kidney, and thymus, but not in the adrenal glands, pancreas, or thyroid (Cuajungco and Samie 2008; Grimm et al. 2010; Table 1). This finding is also largely in agreement with the UniGene EST profile for murine TRPML3. According to NextBio Research Body Atlas (Table 1),

Fig. 1 Amino acid sequence of human TRPML3 and schematic drawing of the presumed TRPML3 structure. (**a**) Human TRPML3 full-length amino acid sequence (NP_060768.8). Shown are the putative TMDs of TRPML3 and different mutations. A419P and I362T are the varitint-waddler mutations causing deafness and coat color dilution in mice (Va, A419P and VaJ, A419P + I362T), TRPML3(H283A) is constitutively active due to loss of pH regulation, and TRPML3(D458K) is inactive. *Asterisks* indicate amino acid deletion in a human TRPML3 splice variant (NP_001240622.1). (**b**) Cartoon of TRPML3 protein displaying putative positions of TMDs (TMD1–6), pore (P), and mutations described in (**a**)

highest levels for murine TRPML3 are found in the small intestine, kidney, lung, retinal pigment epithelium, and the stria vascularis of the cochlea, but again only very low levels are detectable in most endocrine glands (Table 1).

The strongest support for expression of TRPML3 in murine inner ear and skin (melanocytes) is derived from TRPML3 varitint-waddler mice. In these mice TRPML3 is mutated in the pore region of the protein (TMD5; A419P), resulting in circling behavior, deafness, and coat color dilution. There are two variants reported, Va (A419P) and Va^J (A419P + I362T). The latter one shows a slightly less severe phenotype (Di Palma et al. 2002; Grimm et al. 2007; Kim et al. 2007; Nagata et al. 2008; Xu et al. 2007; Grimm et al. 2009; Fig. 1 and Table 2). In the murine inner ear, TRPML3 was detected in the organ of Corti sensory hair cells, the stria vascularis of the cochlea, and the utricle (Di Palma et al. 2002; Nagata et al. 2008; van Aken et al. 2008; Grimm et al. 2010; Takumida et al. 2010; Castiglioni et al. 2011; Noben-Trauth 2011). Castiglioni et al. confirmed the expression of murine TRPML3 in the inner ear by using different TRPML3 antibodies in TRPML3−/− controlled experiments. Thus, it was found that vesicles of hair cells and strial marginal cells but not stereocilia ankle links or pillar cells express TRPML3. In the same study, TRPML3 expression was confirmed for vomeronasal and olfactory sensory receptor cells. Here, TRPML3 localizes to cytoplasmic vesicles and vesicles in dendrites (Castiglioni et al. 2011).

3 Protein

The full-length human TRPML3 protein consists of 553 amino acids with a predicted molecular weight of about 64 kDa. The protein has six putative transmembrane domains (TMDs) with the channel pore between TMD5 and TMD6

Table 2 Human and murine TRPML3 mutations.

Mutation	Human	Mouse	Effect	Literature
Spontaneous		A419P	Const. activity; causes deafness and coat color dilution in mice (varitint-waddler mice)	Di Palma et al. (2002); Kim et al. (2007), Grimm et al. (2007), Nagata et al. (2008), Xu et al. (2007)
Spontaneous		I362T	Ameliorates A419P phenotype in mice (varitint-waddler mice)	Di Palma et al. (2002)
Introduced in cDNA		G419P	Const. activity; similar to A419P but weaker	Kim et al. (2007), Grimm et al. (2007)
Introduced in cDNA	A419P		Const. activity	Grimm et al. (2007)
Introduced in cDNA	H283A		Const. activity; not regulated by extracytosolic (luminal) H^+	Kim et al. (2008)
Introduced in cDNA	H283R		Inactivates channel	Kim et al. (2008)
Introduced in cDNA	E361A		Increases activity in high extracellular Na^+	Grimm et al. (2012b)
Introduced in cDNA	D458K		Inactive, dominant negative	Kim et al. (2010)

Summarized are spontaneous and targeted mutations of human and murine TRPML3 and their consequences for TRPML3 function

(Fig. 1). While proline mutations in TMD5 render the channel constitutively active (e.g., A419P varitint-waddler mutant; Xu et al. 2007; Kim et al. 2007; Grimm et al. 2007), mutation of the negatively charged amino acid D458 in the putative pore loop (D458K) renders the channel inactive. D458K was also shown to act as a dominant-negative mutant (Kim et al. 2009; Table 2).

TRPML channels are predicted, like all other TRP channels, to form tetramers. Between TMD1 and TMD2, an unusually long extracellular loop is found which is roughly 200 amino acids in length, depending on TMD prediction. A human splice variant which contains 497 amino acids lacks part of TMD1 and part of the first extracellular loop up to amino acid 131 (Fig. 1a).

Like the full-length human variant, murine TRPML3 consists of 553 amino acids. No splice variants have been reported. Human and mouse TRPML3 proteins share 91 % sequence identity.

The TRPML3-related protein TRPML1 appears to localize almost exclusively to lysosomes due to the presence of classical lysosomal targeting sequences, i.e., two dileucine motifs (D/EXXXLL/I) in the N and C terminus. These motifs are less conserved in TRPML2 (e.g., they are conserved in rodents but not in primates including human) and absent in TRPML3. In contrast, a motif of the following sequence can be found in the N terminus of most, but not all, TRPML3 species: EXXLL (Fig. 1a). A similar motif, DXXLL, was reported to target, e.g., the protein TMEM192 to late endosomes/lysosomes (Behnke et al. 2011), and the following

N-terminal motif was found to be critical for vacuolar targeting of Arabidopsis two-pore channel 1 (TPC1): EXXLI (EDPLI) (Larisch et al. 2012). In contrast, mammalian isoforms of TPC1 or TPC2 generally contain classical dileucine motif(s) in their N- and/or C-termini. Classical D/EXXXLL/I signals interact with heterotetrameric adaptor protein complexes AP1, AP2, AP3, or AP4 that are capable of recruiting clathrin and thereby initiating the assembly and formation of coated vesicles. DXXLL-type dileucine signals were postulated to recruit GGA proteins (Golgi-localized, γ-ear-containing, Arf-binding proteins), a different class of monomeric clathrin adaptors functioning at the TGN (Bonifacino and Traub 2003; Doray et al. 2012). Currently, it is unclear whether this motif is sufficient to target TRPML3 to endolysosomes or whether other interacting proteins are required to recruit TRPML3 to intracellular vesicles (see chapter "Interaction Partners").

4 Channel Function, Permeation, and Gating

Kim et al. (2008) were the first to demonstrate gating of wild-type TRPML3, transiently overexpressed in HEK293 cells by sodium deprivation followed by rapid re-addition of extracellular sodium. Kim et al. further found that the current/voltage relationship of the monovalent cation current showed strong inward rectification with K^+, Na^+, Cs^+, or $NMDG^+$ as the intracellular cation and with Na^+, K^+, and Cs^+ as the extracellular permeating cation. Measurement of reversal potentials with the different external cations and intracellular K^+ indicated monovalent cation selectivity of $Na^+ > K^+ \gg Cs^+$. TRPML3 showed low permeability to Sr^{2+}, Ba^{2+}, and Mg^{2+}. Besides high sodium, low pH also blocks TRPML3 activity (Kim et al. 2008). Regulation by protons was found to be mediated by a string of three histidines (H252, H273, H283) in the large extracytosolic loop between TMD1 and TMD2. Each of the histidines reportedly has a unique role, whereby H252 and H273 retard access of protons to the inhibitory H283. Interestingly, the histidine mutation H283A has the same phenotype as the varitint-waddler mutant A419P and locks the channel in an open state, whereas the H283R mutation inactivates the channel (Kim et al. 2008; Table 2). Block of TRPML3 by low pH is of particular interest since luminal pH drops from early endosomes (pH 6–7) to late endosomes (pH 5–6) and finally to lysosomes (pH 4–5). TRPML3 appears to be expressed by early endosomes, recycling endosomes, but also late endosomes/lysosomes (Kim et al. 2009). Consequently, its activity in these organelles would be affected by their different luminal pH values.

An urgent question is the nature/identity of endogenous ligands/activators of TRPML including TRPML3 channels. Progress in this direction has been made by Xu and colleagues who have recently identified $PI(3,5)P_2$ (phosphatidylinositol 3,5-bisphosphate) as an activator of TRPML channels (Dong et al. 2010; Shen et al. 2012; Zhang et al. 2012). Generally, $PI(3,5)P_2$ appears to be involved in the regulation of a plethora of cellular functions including endolysosome morphology, trafficking and acidification, autophagy, control of membrane and ion transport, and

signaling in response to stresses and hormonal cues (Ho et al. 2012). $PI(3,5)P_2$ is produced through synthesis catalyzed by the phosphoinositide kinase PIKfyve which colocalizes with early endosomes, late endosomes, and lysosomes (Zhang et al. 2012; Zolov et al. 2012). The principal enzymatic activity of PIKfyve is to phosphorylate PI3P to $PI(3,5)P_2$. PIKfyve activity is required for the production of both $PI(3,5)P_2$ and phosphatidylinositol 5-phosphate (PI5P). Defects in the levels of $PI(3,5)P_2$ and PI5P cause premature lethality and degeneration of multiple organs including neurodegeneration (Zolov et al. 2012). $PI(3,5)P_2$ appears to activate not only TRPML channels but also members of another group of endolysosomal cation channels, the two-pore channels TPC1 and TPC2 (Wang et al. 2012). RyR2 channels have also been found to be activated by $PI(3,5)P_2$ (Touchberry et al. 2010). The physiological significance of the $PI(3,5)P_2$ activation of these different channels will require further attention.

In addition to $PI(3,5)P_2$, a number of synthetic, small molecule activators of TRPML channels have been identified recently which have emerged as useful tools to characterize TRPML channel properties in vitro and in vivo and to explore the potential of a pharmacological rescue of ML IV causing human TRPML1 mutant isoforms (Grimm et al. 2010, 2012a; Shen et al. 2012).

As reported before (Grimm et al. 2007, 2010; Xu et al. 2007; Kim et al. 2008), a substantial amount of overexpressed TRPML3, e.g., in HEK293 cells, is detectable in the plasma membrane. Thus, high-throughput screens for small compound agonists could be performed using standard methods. Stable TRPML3 HEK293 cell lines were generated and screened using Fluorescent-based calcium imaging methods. Screening procedures and results have been discussed extensively in previous publications (Grimm et al. 2010, 2012b; Yamaguchi and Muallem 2010; Saldanha et al. 2011). Briefly, 217,969 compounds were tested and candidate compounds were subsequently validated using Fura-2-based single-cell ratiometric calcium imaging and whole-cell patch-clamp methods (Grimm et al. 2010). Cheminformatics analysis placed the candidate compounds into nine chemical scaffolds (SF) and 20 singletons (i.e., compounds sharing no structural resemblance within all other dosed compounds). The nine chemotypes identified were (pyrazol-5-yl)isoxazole-benzenesulfonamides (SF-1), secondary arylsulfonamides (SF-2), tertiary arylsulfonamides (SF-3), sulfonylarylpiperazines (SF-4), 1-(2,2,4-trimethylquinolinyl)-alkylones (SF-5), spirobenzoannulene-arylpyrazolones (SF-6), *t*-butyl-3-methyl-4-(arylsulfonyl)-pyrazol-5-ols (SF-7), arylsulfonylpyridin-2-ones (SF-8), and *t*-butyl-3methylfuran-2-carboxamides (SF-9). For further evaluation in alternative assays, at least one representative of each chemical scaffold (except for SF-9) was selected as well as two singletons, SN-1 and SN-2 (Grimm et al. 2010; Fig. 2). Criteria for this selection were low EC_{50} values for TRPML3 activation, low activation of TRPN1 (used as a negative control), and compound availability (Grimm et al. 2010; Fig. 2). The candidate compounds were inactive against other TRP channels tested, including members of the TRPC, TRPV, TRPM, TRPA, and TRPN subfamilies (Grimm et al. 2010). Candidate compounds were found to be inactive against a plethora of other targets (generally more than 500 each; for further information see http://pubchem.ncbi.nlm.nih.gov; AID: 1448, 1525, 1526, 1562, 2719, 1809, and 2694).

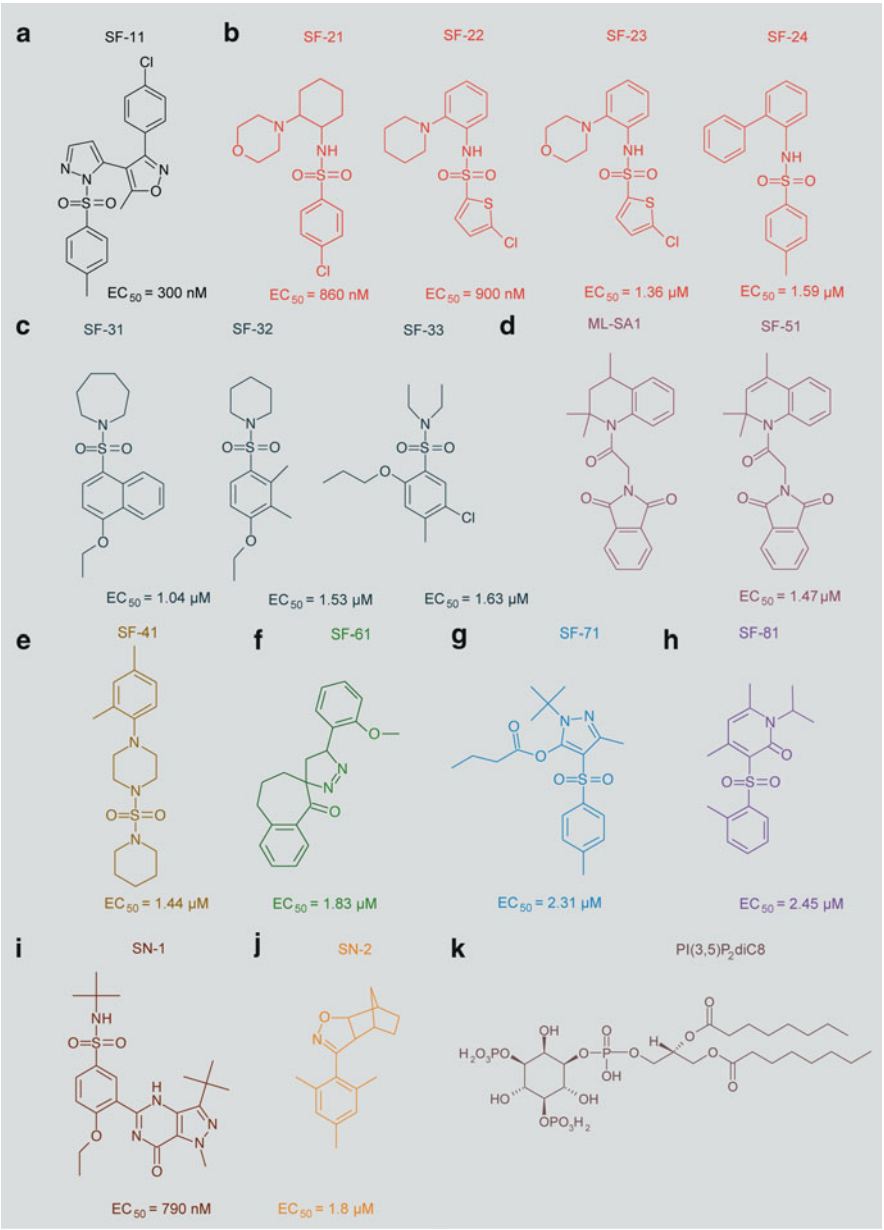

Fig. 2 TRPML3 activators. (**a**–**j**) Shown are the chemical structures of known small compound TRPML3 activators, identified in high-throughput screenings. (**k**) Chemical structure of the putative endogenous TRPML channel activator ($PI(3,5)P_2$). Depicted here is a $PI(3,5)P_2$ isoform with two C8 rests

In addition to TRPML3, some compounds also activated TRPML1, in particular compound SF-22. Since TRPML1 exclusively resides within subcellular organelles, lipophilicity of a drug for therapeutic intervention, e.g., the treatment of ML IV, would be indispensable. To determine lipophilicity, the octanol–water partition coefficient (logP), indicating whether a neutral molecule will prefer an aqueous or organic phase, or the octanol–water distribution coefficient (logD), which produces an apparent partition coefficient for any pH value (combination of pK_a and logP), can be calculated. We calculated the following logD value for SF-22: 3.45 at pH 7.4. Compounds with logD values of >3 are considered lipophilic (Leo et al. 1971; Viswanadhan et al. 1989; Moriguchi et al. 1992). The respective logD values (pH 7.4) for other representative TRPML activators are SF-11 (4.07), SF-51 (3.35), and SF-23 (2.27).

To confirm TRPML3 activation in endolysosomes, we isolated endolysosomes as described previously and performed whole-endolysosomal planar patch-clamp experiments using Nanion's Port-a-Patch system (Schieder et al. 2010a, b). HEK293 cells stably expressing human TRPML3-YFP were used for the isolation of endolysosomes. Inwardly rectifying currents were elicited by applying compound SF-21 (Grimm et al. 2010). These currents were potentiated upon removal of high luminal sodium as described before (Kim et al. 2008; Grimm et al. 2010). These data together with previously published data demonstrate that in overexpression systems, TRPML3 is functional both in the plasma membrane (Grimm et al. 2010; Kim et al. 2008) and in the isolated endolysosomal membranes (Fig. 3). Average whole-endolysosome currents were about 10- to 20-fold smaller than average whole-cell currents (Grimm et al. 2010; Fig. 3).

5 Interaction Partners

In overexpression systems, TRPML3 can interact and form heteromers with its relatives, TRPML1 and TRPML2. This has been shown by several groups (Venkatachalam et al. 2006; Vergarajauregui and Puertollano 2006; Karacsonyi et al. 2007; Kim et al. 2009; Martina et al. 2009; Zeevi et al. 2009, 2010; Curcio-Morelli et al. 2010; Grimm et al. 2010). Currently, it remains however unclear to what extent heteromultimerization events occur in vivo and how they may affect physiological processes. Colocalization of TRPML channels in the same intracellular vesicles seems to be limited (Zeevi et al. 2009). Nevertheless, TRPML2 and TRPML3 channels also traffic, in part, to late endosomes/lysosomes. Kim et al. (2009), for example, found the expression of TRPML3 in different subcellular fractions separated on a Percoll gradient and expression of TRPML1 only in the heavy fraction. TRPML3 was found in fractions positive for Lamp1, a marker for late endosomes and lysosomes; positive for EEA1, a marker for early endosomes; and positive for transferrin receptor (recycling endosomes). Additional immunofluorescence studies using different endolysosomal markers confirmed these results. Whether TRPML1 has a direct impact on lysosomal trafficking of TRPML2 and TRPML3 by heteromultimerization events occurring before reaching the lysosome

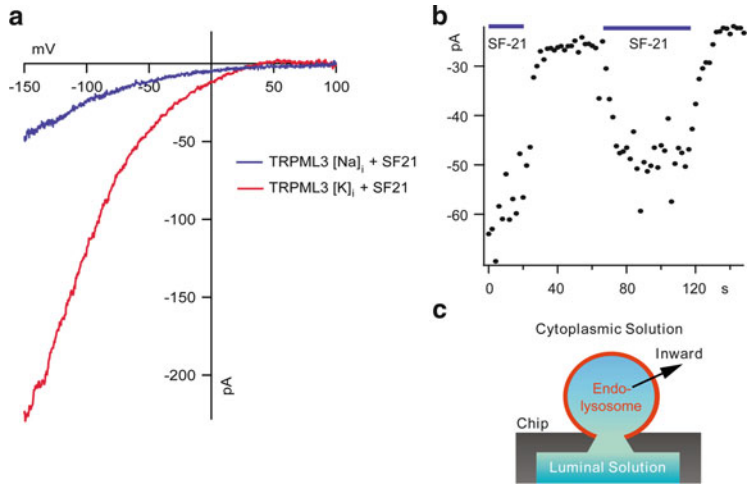

Fig. 3 TRPML3 in endolysosomes. (**a**, **b**) Representative whole-endolysosome patch-clamp measurements of human TRPML3-YFP, overexpressed in endolysosomes, isolated from HEK293 cells, and activated with compound SF-21 (20 μM). Measurements were performed using the planar patch-clamp technique (Port-a-Patch system, Nanion, Munich, Germany), described previously (Schieder et al. 2010a, 2010b). Cytoplasmic solution contained 5 mM Na-MSA, 60 mM KF, 70 mM K-MSA (methanesulfonate), 0.2 mM Ca-MSA, ,and 10 mM HEPES (pH adjusted with KOH to 7.2). Luminal solution was 60 mM Ca-MSA, 70 mM Na-MSA, 5 mM $MgCl_2$, and 10 mM HEPES (pH adjusted with NaOH to 7.2). Alternatively, a luminal solution was used containing 70 mM K-MSA instead of Na-MSA. When using the latter luminal solution, Na-MSA was omitted in the cytoplasmic solution. (**c**) Cartoon depicting the principle of the planar patch-clamp technique

or whether TRPML2 and TRPML3 are directed to lysosomes by other interaction partners is unclear.

Another recent study identified TRPV5/6 as interaction partners of TRPML channels. TRPV5/TRPML3 heteromers showed functional characteristics distinct from the respective homomeric channels (Guo et al. 2013). Most prominently, TRPV5/TRPML3 heteromers showed constitutively active inward rectification at conditions where the respective homomeric channels would be largely closed, i.e., in bath solution containing 140 mM Na^+, 5 mM K^+, 1.5 mM Ca^{2+}, and 1 mM Mg^{2+}. While TRPV5 is active in a bath solution containing 0.1 mM EGTA and no calcium, TRPML3 remains closed and opens only if sodium is replaced by potassium. In contrast, the TRPV5/TRPML3 heteromer is active in both conditions (Guo et al. 2013). Similar results were obtained for TRPV6/TRPML3 heteromers (Guo et al. 2013). FRET results support the interaction between TRPML3 and TRPV5/6. Furthermore, FRET results also indicate interactions between TRPML1 and TRPV5 as well as TRPML1 and TRPV6 with similar FRET efficiencies as shown for TRPML3 and TRPV5 or TRPV6 (Guo et al. 2013; Guo et al. unpublished data). In addition, as mentioned above, a comparison of the amino acid sequences of all TRP channels revealed significant similarities between TRPV5, TRPV6, and

TRPML channels when comparing the pore regions only (TMD5-pore-TMD6). It should also be added here that proline mutations in the TMD5 region of TRPV5/6, in analogy to the varitint-waddler mutation A419P, also had a significant impact on TRPV5/6 channel activity while no effect was found for other TRP channels like TRPM2, TRPV2, or TRPC3 (Grimm et al. 2007; Lee et al. 2010). These data suggest potential structural similarities between TRPMLs and TRPV5/6. Interactions between TRP subfamily members and also across subfamilies have previously been reported, e.g., TRPV5 and TRPV6, TRPC1 and TRPC4, TRPP2 and TRPC1, or TRPP2 and TRPV4 (Tsiokas et al. 1999; Kottgen et al. 2008; Kobori et al. 2009; Stewart et al. 2010; for review see e.g. Gees et al. 2010; Nilius and Owsianik 2011).

TRPV5 cycles between endosomes and the plasma membrane (van de Graaf et al. 2008; deGroot et al. 2008). Thus, surface expression of TRPV5 is tightly regulated allowing well-controlled calcium influx, e.g., in kidney epithelial cells. In fact, endogenously expressed TRPV5 is primarily detectable in intracellular vesicles (deGroot et al. 2008). TRPV5 is internalized in a dynamin- and clathrin-dependent manner. Shortly after endocytosis, calcium and sodium levels in endocytotic vesicles are high and pH is neutral. Under these conditions both TRPV5 and TRPML3 homomers are closed. However, the TRPV5/TRPML3 heteromer would be open under these conditions (Guo et al. 2013) and could thus actively participate in readjusting intraluminal cation levels. Heteromultimeric channels with completely different functional characteristics may thus play important physiological roles within the endolysosomal system. Nevertheless, it remains to be proven if heteromultimerization of TRPMLs with TRPV5/6 is indeed physiologically relevant. In vivo co-immunoprecipitation studies as well as double or triple mouse knockout models may be useful in this context.

6 Physiological Functions in Native Cells, Organs, and Organ Systems

In contrast to TRPML1, no disease-causing human mutations are reported for TRPML3. The only known disease-causing mutations are the murine varitint-waddler mutant isoforms of TRPML3 as described above. These mutations point to expression and physiological roles of murine TRPML3 in the skin and inner ear. Likewise TRPML3 appears to be present in human skin and human inner ear as mentioned above (chapter “Expression”).

According to NextBio Research Disease Atlas (http://www.nextbio.com) which is an application that finds diseases, traits, or conditions associated with, e.g., a gene, sequence region, or SNP whereby the results are grouped by disease and ranked according to statistical significance, a strong downregulation of TRPML3 is associated with viral and bacterial infections, e.g., cytomegalovirus infection (human), influenza (mouse), hemophilus infection (human), or chlamydial infection (human, mouse) but also with adrenal cancer (human), kidney cancer (human), chronic interstitial cystitis (bladder pain syndrome, human), irritable bowel

syndrome (human), skin wounds (human), or acne (human). Strong upregulation is associated with chronic sinusitis (human), lung injury (human, mouse), liver cirrhosis (human), ascites (human), or malignant muscle tumor (human).

In addition, small molecule modulators for TRPML3 have recently been developed and patented for interference with a postulated role of TRPML3 in salty taste perception in primates including humans (patent application number: 20090117563; publication date: 2009-05-07; patent application title: "Identification of TRPML3 as a salty taste receptor and use in assays for identifying taste (salty) modulators and/or therapeutics that modulate sodium transport, absorption or excretion and/or aldosterone and/or vasopressin production or release"). However, TRPML3 expression in taste receptor cells has not been convincingly demonstrated yet (Castiglioni et al. 2011). The invention also relates to the possibility that the TRPML3 gene may modulate sodium metabolism, sodium excretion, blood pressure, fluid retention, cardiac function, or urinary functions such as urine production and excretion. Currently, it seems unclear whether any of these suggested physiological roles indeed relates to TRPML3.

Another study suggested TRPML3 to possibly play a role in pain sensation. Staaf et al. reported that TRPML3 mRNA is present in DRG neurons at very low levels but that, upon nerve damage, these levels rise by an average of 7.5 times in most neurons (Staaf et al. 2009). Castiglioni et al. examined sections of mouse dorsal root and trigeminal ganglia and found no evidence of TRPML3 expression. Using the same antibodies they successfully detected TRPML3 in inner ear, vomeronasal, and olfactory sensory receptor cells as mentioned above. In addition, by RT-qPCR, Castiglioni et al. barely detected an estimated 12 TRPML3 mRNAs per 100 ng total RNA from trigeminal ganglia (700 times less than from the ear) and 4.4 TRPML3 mRNAs per 100 ng total RNA from dorsal root ganglion (2,000 times less than from the ear). The authors therefore concluded that the extremely low concentration of TRPML3 mRNA in dorsal root and trigeminal ganglia compared with inner ear or with TRPA1 levels on DRGs (2,700 times less) would not support a physiological role for TRPML3 in somatosensory neurons.

Other hints for potential physiological role(s) of TRPML3 are derived from cell biological assays using siRNA or other knockdown methods. Thus, for example, it was found that overexpression of TRPML3 produces severe alterations of the endosomal pathway, including enlargement and clustering of endosomes, delayed EGF receptor degradation, and impaired autophagosome maturation (Kim et al. 2009; Martina et al. 2009). Lelouvier and Puertollano (2011) found that inhibition of TRPML3 function caused increased accumulation of luminal Ca^{2+} and that this accumulation had important consequences on endosomal acidification and membrane fusion. Interestingly, endosomal fusion events were increased in the absence of TRPML3 (Lelouvier and Puertollano 2011). Based on these results the authors concluded that TRPML channels may generally play a role in membrane fusion/fission, trafficking, and/or endolysosomal pH regulation.

7 Lessons from Knockout Animals and Role in Hereditary Diseases

Two independently generated TRPML3 knockout mouse models have been published recently (Jörs et al. 2010; Castiglioni et al. 2011). Jörs et al. demonstrated that loss of TRPML3 does not cause deafness or skin abnormalities in mice, quite in contrast to varitint-waddler mice containing gain-of-function mutations as described above. Hearing and balance were evaluated by ABR (auditory brain stem response) measurements and rotarod tests. TRPML3−/− mice showed no circling behavior, head bobbing, waddling, or imbalance when walking. TRPML3−/− mice breed normally and have no acutely visible defects. Clearly, a more detailed and in-depth analysis of TRPML3−/− as well as TRPML double and/or triple knockout mice will be required. In this context it should be kept in mind, as pointed out above, that there appear to be some astonishing differences in expression between humans and rodents which implies that physiological roles of TRPML3 in rodents may not be in all instances directly translatable to humans.

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The TRPP Subfamily and Polycystin-1 Proteins

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Abstract

It has been exciting times since the identification of polycystic kidney disease 1 (PKD1) and PKD2 as the genes mutated in autosomal dominant polycystic kidney disease (ADPKD). Biological roles of the encoded proteins polycystin-1 and TRPP2 have been deduced from phenotypes in ADPKD patients, but recent insights from vertebrate and invertebrate model organisms have significantly expanded our understanding of the physiological functions of these proteins. The identification of additional TRPP (TRPP3 and TRPP5) and polycystin-1-like proteins (PKD1L1, PKD1L2, PKD1L3, and PKDREJ) has added yet another layer of complexity to these fascinating cellular signalling units. TRPP proteins assemble with polycystin-1 family members to form receptor–channel complexes. These protein modules have important biological roles ranging from tubular morphogenesis to determination of left–right asymmetry. The founding members of the polycystin family, TRPP2 and polycystin-1, are a prime example of how studying human disease genes can provide insights into fundamental biological mechanisms using a so-called "reverse translational" approach (from bedside to bench). Here, we discuss the current literature on TRPP ion channels and polycystin-1 family proteins including expression, structure, physical interactions, physiology, and lessons from animal model systems and human disease.

Keywords

TRPP cation channels • Polycystin-1 proteins • Polycystic kidney disease • Cilia • Polycystin physiology

1 Introduction

The founding member of the TRP polycystin ion channel family, TRPP2, was discovered as a gene product mutated in autosomal dominant polycystic kidney disease (ADPKD) (Mochizuki et al. 1996). The TRPP subfamily may be the most ancient among the TRP channels, as members of this subfamily extend from yeast to mammals (Palmer et al. 2005; Venkatachalam and Montell 2007). TRPP proteins assemble with polycystin-1 family members to form receptor–channel complexes. These protein modules have important biological roles ranging from tubular morphogenesis to determination of left–right asymmetry. TRPP channels have evolutionarily conserved functions in primary cilia where they are thought to translate extracellular cues into cellular signalling events.

Table 1 TRPP channel nomenclature

Gene	Protein	TRP
Polycystic kidney disease 2 (PKD2)	Polycystin-2 (PC2)	TRPP2
PKD2-like 1 (PKD2L1)	Polycystin L	TRPP3
PKD2L2	PKD2L2	TRPP5

Table 2 Polycystin-1 family nomenclature

Gene	Protein
Polycystic kidney disease 1 (PKD1)	Polycystin-1 (PC1)
PKD1-like 1 (PKD1L1)	PKD1L1
PKD1L2	PKD1L2
PKD1L3	PKD1L3
PKD and sperm receptor for egg jelly homolog-like (PKDREJ)	PKDREJ

This review summarises the current literature on TRPP channels and polycystin-1 family members. Since not all aspects can be covered in this review, the reader is referred to a number of excellent reviews on TRPP channels and polycystin-1 family proteins (Tables 1 and 2) (Somlo 1999; Somlo and Ehrlich 2001; Delmas et al. 2004a; Anyatonwu and Ehrlich 2004; Delmas 2004, 2005; Cantiello 2004; Nilius et al. 2005; Witzgall 2005; Köttgen and Walz 2005; Giamarchi et al. 2006; Köttgen 2007; Harris and Torres 2009; Zhou 2009; Woudenberg-Vrenken et al. 2009; Tsiokas 2009; Hofherr and Köttgen 2011; Hofherr 2012).

2 TRPP Family Genes

The TRPP (polycystin) subfamily of transient receptor potential (TRP) channels displays a rather limited sequence homology to classical TRP genes (Venkatachalam and Montell 2007). The three human genes encoding for the TRPP protein family are: 1) *polycystic kidney disease 2* (*PKD2*); 2) *PKD2-like 1* (*PKD2L1*); and 3) *PKD2-like 2* (*PKD2L2*). *PKD2* was identified by linkage analysis in ADPKD patients (Mochizuki et al. 1996; Kimberling et al. 1988; Peters and Sandkuijl 1992). Subsequently, the two *PKD2-like* genes were established by homology-based cloning (Nomura et al. 1998; Wu et al. 1998a; Veldhuisen et al. 1999).

Interestingly, TRPP family genes are conserved in vertebrates, invertebrates and yeast, e.g. *pkd2* in *Danio rerio*, *amo* in *Drosophila melanogaster*, *Pkd-2* in *Caenorhabditis elegans*, *suPC2* in sea urchin and *pkd2* in *Schizosaccharomyces pombe* (Palmer et al. 2005; Barr and Sternberg 1999; Watnick et al. 2003; Gao et al. 2003; Neill et al. 2004; Sun et al. 2004). This ancient evolutionary origin has given rise to the hypothesis that TRPP ion channels might comprise the most archetypical TRP subfamily (Venkatachalam and Montell 2007).

PKD2 (GeneID 5,311) is located on chromosome 4q21–4q23 in positive orientation (Mochizuki et al. 1996). The gene is 70,110 base pairs (bp) long and comprises 15 exons (Hayashi et al. 1997). One mRNA transcript has been

established (NM_000297). The TRPP2 protein consists of 968 amino acids (109,561 Da) (NP_000288).

PKD2L1 (GeneID 9,033) is located on chromosome 10q24 in negative orientation (Nomura et al. 1998; Wu et al. 1998a). The gene is 42,341 bp long and comprises 16 exons (Guo et al. 2000a, b). One mRNA transcript has been established (NM_016112), which is translated into TRPP3 (805 amino acids; 91,851 Da) (NP_057196).

PKD2L2 (GeneID 27,039) is located on chromosome 5q31 in positive orientation (Veldhuisen et al. 1999). The gene is 51,032 bp long and comprises 14 exons. One mRNA transcript has been established (NM_014386), which is translated into TRPP5 (613 amino acids; 72,365 Da) (NP_055201).

3 Expression of TRPP Ion Channels

The TRPP family genes are expressed ubiquitously in vertebrates. Large-scale transcriptome analysis detected TRPP mRNA in almost every human and mouse tissue (Su et al. 2004; Wu et al. 2009). The targeted examination of TRPP expression has confirmed and extended these results: *PKD2* and *PKD2L1* transcripts are indeed found in many foetal and adult tissues, including the heart, brain, lung, testis, ovary and kidney (Mochizuki et al. 1996; Nomura et al. 1998; Veldhuisen et al. 1999; Basora et al. 2002; Volk et al. 2003; Murakami et al. 2005; Ishimaru et al. 2006; Huang et al. 2006; Orts-Del'immagine et al. 2012). In contrast, *PKD2L2* transcription appears to be mostly restricted to the testis (Veldhuisen et al. 1999; Guo et al. 2000b; Taft et al. 2002; Chen et al. 2008). Several TRPP splice variants have been described but their functional consequences remain elusive (Mochizuki et al. 1996; Nomura et al. 1998; Veldhuisen et al. 1999; Guo et al. 2000b).

Spatiotemporal control of *PKD2* expression seems to be partially executed on a post-transcriptional level. Micro-RNAs modify gene expression by reducing protein translation through mRNA degradation or translational repression (He and Hannon 2004). The short non-coding microRNA group 17 (miR-17) and the RNA-binding protein bicaudal C (BICC1) apparently antagonise each other to fine-tune TRPP2 translation (Sun et al. 2010; Tran et al. 2010; Patel et al. 2013). miR-17 has been described to repress the expression of *PKD2* post-transcriptionally (Sun et al. 2010; Tran et al. 2010; Patel et al. 2013). Kidney-specific transgenic over-expression of miR-17 produces kidney cysts in mice, while inactivation of miR-17 retards cyst growth in the heterologous mouse PKD model *Kif3a*$^{-/-}$ (Patel et al. 2013). BICC1, on the other hand, promotes *PKD2* mRNA stability and translation efficiency while antagonising the repressive activity of miR-17 (Tran et al. 2010). *Bicc1*$^{-/-}$ animals develop a renal cyst phenotype reminiscent of PKD (Tran et al. 2010). However, future experiments will have to address whether the observed miR-17 and *Bicc1*$^{-/-}$ cyst phenotypes are exclusively linked to decreased TRPP2 protein levels, because miR-17 targets a plethora of genes some of which are known cyst genes (Tran et al. 2010; Cloonan et al. 2008).

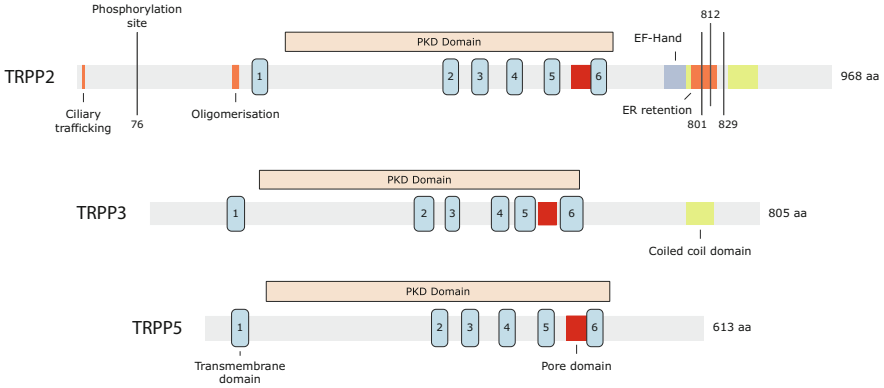

Fig. 1 The TRPP ion channel family

In *C. elegans* and *D. melanogaster*, *PKD2* expression is highly restricted to ciliated cells, predominantly male-specific sensory neurons and spermatozoa, respectively (Barr and Sternberg 1999; Watnick et al. 2003; Gao et al. 2003; Yang et al. 2011; Köttgen et al. 2011). A temporal control of *pkd2* expression has been proposed in the fission yeast, *S. pombe*, where *pkd2* mRNA levels increase following damage to the cell wall (Palmer et al. 2005).

It is intriguing to speculate that the expansion of TRPP gene expression (and therefore most likely TRPP protein function) is evolutionary coupled to the increase in ciliated cell types. There are very few ciliated cells expressing TRPP genes in invertebrates and almost ubiquitous ciliation and TRPP expression in humans.

4 The TRPP Proteins

The nomenclature of TRPP ion channels has been somewhat inconsistent. Initial publications grouped both ADPKD proteins into the polycystin family. The founding member of the TRPP subfamily, polycystin-2, was later classified as TRPP2. The designation TRPP1 has caused some confusion, as it has been used for both polycystin-1 and polycystin-2. We strongly advocate the following TRP nomenclature: 1) *bona fide* TRPP ion channels and 2) receptor-like polycystin-1 family proteins (Tables 1 and 2). Because of ambiguity TRPP1 and TRPP4 should not be used.

TRPP2, TRPP3 and TRPP5 are the three Ca^{2+}-permeable non-selective cation TRPP ion channel proteins (Fig. 1) (Clapham 2003; Nilius and Owsianik 2011). They share a high degree of amino acid sequence identity and similarity. ClustalW sequence alignment of TRPP3 and TRPP2 shows 48 % identity and 70 % sequence similarity (Larkin et al. 2007). TRPP5 is only slightly more distant from TRPP2 with 45 % identity and 68 % similarity (Larkin et al. 2007).

Unfortunately, to date no high-resolution crystal structure is available for any full-length TRP channel (Li et al. 2011). Current TRPP protein models are therefore

based on less accurate experimental methods and predictions. Studies on the architecture of TRP channels in general yielded strong evidence that these channels function as tetramers (Jahnel et al. 2001; Kedei et al. 2001; Moiseenkova-Bell et al. 2008). Monomeric TRP channel subunits are integral membrane proteins with six predicted transmembrane helices (S1–S6), framing a pore-forming loop between S5 and S6 and cytosolic amino- and carboxy-termini of varying sizes (Venkatachalam and Montell 2007; Clapham 2003; Nilius and Owsianik 2011; Li et al. 2011). The prominent feature of TRPP ion channels is a large extracellular loop between S1 and S2, consisting of 245 amino acids in TRPP2, 224 in TRPP3 and 225 in TRPP5. While the transmembrane helices are highly conserved in the TRPP family, major deletions have occurred in the protein termini. Basically, TRPP3 is missing approximately 100 amino acids of the TRPP2 amino-terminus and TRRP5 has lost both sections of the TRPP2 amino- and carboxy-terminus. TRPP sequence divergence in mammals, e.g. amino acid substitution, is mainly observed in the loop regions between the transmembrane helices (Ye et al. 2009).

TRPP2 has six predicted transmembrane domains and their orientation has recently been validated by transient expression of truncated TRPP2 mutants and selective membrane permeabilisation (Hoffmeister et al. 2011a). In line with expectations, both the amino- and carboxy-terminus have been found to protrude into the cytoplasm (Hoffmeister et al. 2011a). The carboxy-terminal region of TRPP2 has attracted particular research interest. It comprises several sequence elements required for proper TRPP2 function, including a Ca^{2+}-binding EF hand ($TRPP2^{754–781}$), two coiled-coil domains (CC1: $TRPP2^{769–796}$/CC2: $TRPP2^{835–873}$), an endoplasmic reticulum retention motif ($TRPP2^{787–820}$) and an acidic amino acid cluster ($TRPP2^{810–821}$) (Fig. 1) (Cai et al. 1999; Hanaoka et al. 2000; Köttgen et al. 2005; Ćelić et al. 2008, 2012; Yu et al. 2009; Petri et al. 2010; Giamarchi et al. 2010). The EF hand has been implicated in ion channel gating and the acidic cluster in protein trafficking, whereas the two coiled-coil domains have been demonstrated to be required for homo- and heteromerisation of TRPP2 subunits (Köttgen et al. 2005; Ćelić et al. 2008, 2012; Yu et al. 2009; Petri et al. 2010; Giamarchi et al. 2010). Structural analysis of recombinant TRPP2 carboxy-terminal fragments and specific amino acid substitutions in full-length TRPP2 have yielded some insight into the molecular mechanisms controlling TRPP2 channel assembly. The formation of functional ion channel homomers or alternatively heteromers seems to be a controlled multistep process which is initiated by the assembly of TRPP2 homomers (Yu et al. 2009; Giamarchi et al. 2010; Feng et al. 2008, 2011). An amino-terminal oligomerisation domain ($TRPP2^{199–207}$), a $cysteine^{632}$-dependent disulphide bond and CC2 apparently stabilise TRPP2 homomers, which are the basis for CC1-mediated interactions to form active ion channels (Yu et al. 2009; Giamarchi et al. 2010; Feng et al. 2008, 2011; Qian et al. 1997; Tsiokas et al. 1997). However, some controversies concerning the sequence of events and the respective TRPP2 multimerisation state remain unsettled (Yu et al. 2009; Giamarchi et al. 2010; Kobori et al. 2009; Molland et al. 2010). Similar data have been reported for TRPP3 (Molland et al. 1824, 2010; Yu et al. 2012). The crystal structure of $TRPP3^{561–805}$ fragments is stabilised by trimerisation (Molland

et al. 1824; Yu et al. 2012). Taken together, these data raise the question whether TRPP proteins form trimers or tetramers, like other TRP ion channels. It is rather difficult to extrapolate from the data provided by the crystal structure of TRPP carboxy-terminal fragments to the full-length ion channel configuration. Further data are required to clarify this issue.

In eukaryotic cells, an additional level of information content is added to protein structures by co- and post-translational modifications. Most frequent are glycosylation and phosphorylation, which are essential for protein processing and function (Tarrant and Cole 2009; Moremen et al. 2012). TRPP2 has been demonstrated to be glycosylated, but a detailed mapping and characterisation of implicated amino acid residues is lacking (Cai et al. 1999; Newby et al. 2002). A large number of phosphorylation sites in TRPP2 have been predicted and identified (Cai et al. 2004; Olsen et al. 2006; Streets et al. 2006, 2010, 2013; Hoffert et al. 2006; Molina et al. 2007; Zanivan et al. 2008; Huttlin et al. 2010; Hsu et al. 2011; Plotnikova et al. 2011). Serine^{76}, serine^{801}, serine^{812} and serine^{829} have been studied in more detail (Köttgen et al. 2005; Cai et al. 2004; Streets et al. 2006, 2010, 2013; Plotnikova et al. 2011; Li et al. 2005a; Fu et al. 2008). Glycogen synthase kinase 3 (GSK3) was shown to phosphorylate serine^{76}, thereby promoting redistribution of plasma membrane TRPP2 to intracellular compartments (Streets et al. 2006). Phosphorylation of serine^{801} appears to be protein kinase D-dependent and increased by epidermal growth factor stimulation (Streets et al. 2010). Phosphorylation of serine^{812} by casein kinase 2 (CK2) contributes to the regulation of TRPP2 trafficking and ion channel activity (Köttgen et al. 2005; Cai et al. 2004; Fu et al. 2008). Serine^{829} is phosphorylated by either aurora A or protein kinase A (PKA) and has been reported to modulate ion channel function (Plotnikova et al. 2011; Streets et al. 2013). It is important to note, however, that the functional importance of these TRPP2 modifications is still difficult to evaluate as overexpressed TRPP2 is trapped in the endoplasmic reticulum, impeding the electrophysiological analysis at the plasma membrane (also see Sect. 6).

5 TRPP Interacting Proteins

TRPP ion channels are embedded in a complex network of protein–protein interactions. After translation, their localisation, function and activity are shaped by a multitude of associated proteins (http://trpchannel.org/families/TRPP) (Tables 3 and 4). Like many TRP proteins, TRPP subunits assemble into functional ion channel homomers, but have also been found to form heteromeric complexes. Their intimate relationship with polycystin-1 family proteins adds yet another layer of complexity to these fascinating proteins. TRPP ion channels and polycystin-1 family proteins constitute versatile receptor–channel modules that use Ca^{2+} as second messenger (Fig. 2). They have been implicated in many signalling cascades, including establishment of body asymmetry and coordination of three-dimensional tissue organisation (Hofherr and Köttgen 2011; Hofherr 2012).

Table 3 TRPP2 protein interactions

Protein	Putative function	References
Adenylyl cyclase 5/6 (AC5/6)	A-kinase anchoring protein complex	Choi et al. (2011)
α-Actinin	Ion channel modulation/actin binding	Li et al. (2005c)
CD2AP	Actin binding	Lehtonen et al. (2000)
Collectrin	Ciliary trafficking	Zhang et al. (2007)
EGFR	Ion channel modulation	Ma et al. (2005)
eIF2α	Regulation of cell proliferation	Liang et al. (2008a)
Filamin A	Ion channel modulation	Sharif-Naeini et al. (2009), Wang et al. (2012)
Filamin B	Ion channel modulation	Wang et al. (2012)
Filamin C	Ion channel modulation	Wang et al. (2012)
FIP2	Protein transport	Li et al. (2008)
FPC (PKHD1)	Ciliogenesis	Kim et al. (2008)
GSK3	Protein transport	Streets et al. (2006)
HAX1	Actin binding	Gallagher et al. (2000)
HERP	Protein degradation	Liang et al. (2008b)
Id2	Regulation of cell proliferation	Li et al. (2005a)
IP3R	Ion channel modulation	Li et al. (2005b)
KIF3A	Tubulin binding	Li et al. (2006)
KIF3B	Tubulin binding/ion channel modulation	Wu et al. (2006)
Kim-1	Development of interstitial fibrosis	Kuehn et al. (2002)
mDia1	Protein transport	Rundle et al. (2004)
NPHP1	Regulation of apoptosis	Wodarczyk et al. (2010)
NPHP9	Protein transport	Sohara et al. (2008)
p97	Protein degradation	Liang et al. (2008b)
PACS-1	Protein transport	Köttgen et al. (2005)
PACS-2	Protein transport	Köttgen et al. (2005)
PATJ	Cell polarity/tight junctions	Duning et al. (2010)
Pericentrin (PCTN)	Assembly of primary cilia	Jurczyk et al. (2004)
PERK	Regulation of cell proliferation	Liang et al. (2008a)
PIGEA-14	Protein transport	Hidaka et al. (2004)
PKD1L1	TRPP signalling module	Field et al. (2011), Kamura et al. (2011)
PKDREJ	TRPP signalling module	Sutton et al. (2006)
PLCγ2	Ion channel modulation	Ma et al. (2005)
Polycystin-1 (PKD1)	TRPP signalling module	Qian et al. (1997), Tsiokas et al. (1997)
PRKCSH	Protein processing	Gao et al. (2010)
RP2	Ion channel modulation	Hurd et al. (2010)
RyR2	Intracellular Ca^{2+} release	Anyatonwu et al. (2007)
SEC10	Ciliary localisation	Fogelgren et al. (2011)
Syntaxin-5 (Stx5)	Ion channel modulation	Geng et al. (2008)

(continued)

Table 3 (continued)

Protein	Putative function	References
TAZ	Protein degradation	Tian et al. (2007)
Troponin-1	Ion channel modulation	Li et al. (2003d)
Tropomyosin-1	Actin binding	Li et al. (2003b)
TRPC1	TRP ion channel	Tsiokas et al. (1999)
TRPC3	TRP ion channel	Miyagi et al. (2009)
TRPC4	TRP ion channel	Du et al. (2008)
TRPC5	TRP ion channel	Sutton et al. (2006)
TRPC7	TRP ion channel	Miyagi et al. (2009)
TRPP2	TRP ion channel	Tsiokas et al. (1997)
TRPV4	TRP ion channel	Köttgen et al. (2008)

Table 4 TRPP3 protein interactions

Protein	Putative function	References
α-Actinin	Ion channel modulation/actin binding	Li et al. (2007)
PKD1L3	TRPP signalling module	Ishimaru et al. (2006), Lopezjimenez et al. (2006)
PKDREJ	TRPP signalling module	Sutton et al. (2006)
Polycystin-1 (PKD1)	TRPP signalling module	Murakami et al. (2005), Bui-Xuan et al. (2006)
RACK1	Ion channel modulation	Yang et al. (2012)
Troponin-1	Ion channel modulation	Li et al. (2003c)
TRPC1	TRP ion channel	Sutton et al. (2006)
TRPC5	TRP ion channel	Sutton et al. (2006)

5.1 The TRPP–Polycystin-1 Signalling Module

PKD1 was the first ADPKD gene to be identified by linkage analysis (Reeders et al. 1985; European Polycystic Kidney Disease Consortium 1994; International Polycystic Kidney Disease Consortium 1995). Mutations in either *PKD1* or *PKD2* cause virtually indistinguishable disease manifestations in humans (Longo and Harrison 2012). *PKD1* is the founding member of the polycystin-1 family, which consists of five large membrane-bound receptor proteins: *PKD1*, *PKD1-like 1 (PKD1L1)*, *PKD1-like 2 (PKD1L2)*, *PKD1-like 3 (PKD1L3)* and *PKD and sperm receptor for egg jelly homolog-like (PKDREJ)* (Reeders et al. 1985; European Polycystic Kidney Disease Consortium 1994; International Polycystic Kidney Disease Consortium 1995; Yuasa et al. 2002; Li et al. 2003a; Sutton et al. 2006). Their signature feature is the tripartite combination of a G-protein-coupled receptor proteolytic site (GPS), the receptor for egg jelly (REJ) domain and the lipoxygenase homology/polycystin, lipoxygenase, atoxin (LH2/PLAT) domain that is separated from the previous two by a transmembrane helix (Qian and Noben-Trauth 2005).

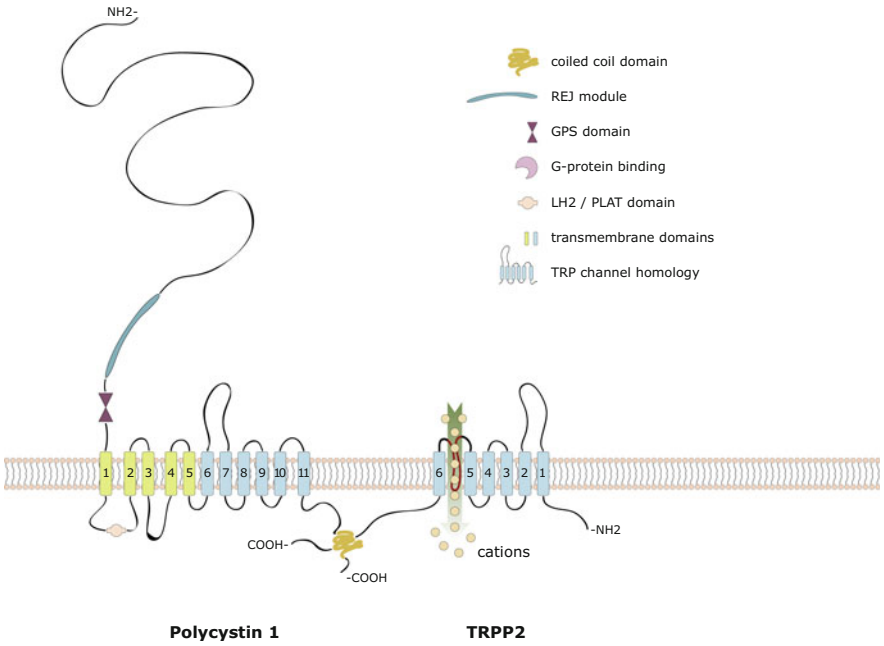

Fig. 2 The TRPP2/polycystin-1 receptor–channel complex

PKD1 (GeneID 5,310) is located on chromosome 16q13.3 in negative orientation. The gene is 47,189 bp long and comprises 92 exons (Reeders et al. 1985; European Polycystic Kidney Disease Consortium 1994; International Polycystic Kidney Disease Consortium 1995). Two mRNA transcripts have been established (NM_001009944, NM_000296), which are translated into polycystin-1 isoform 1 and polycystin-1 isoform 2 (4,303 and 4,292 amino acids; 462,529 and 461,365 Da, respectively) (NP_001009944, NP_000287). In addition, six *PKD1* pseudo-genes *(PKD1P1–PKD1P6)* have been identified in 16q13.11–16q13.13 (GeneIDs: 339,044/283,955/339,039/353,512/348,156/353,511).

PKD1L1 (GeneID 168,507) is located on chromosome 7q12.3 in negative orientation (Yuasa et al. 2002). The gene is 173,748 bp long and comprises 57 exons. One mRNA transcript has been established (NM_138295), which is translated into PKD1L1 (NP_612152), a 315,305 Da protein comprising 2,849 amino acids.

PKD1L2 (GeneID 114,780) is located on chromosome 16q23.2 in negative orientation. The gene is 119,492 bp long and comprises 61 exons (Li et al. 2003a). Two mRNA transcripts have been established (NM_052892, NM_001076780), which are translated into PKD1L2 isoform A and C (2,459 and 991 amino acids; 272,384 and 107,813 Da, respectively) (NP_443124, NP_001070248).

PKD1L3 (GeneID 342,372) is located on chromosome 16q22.3 in negative orientation (Li et al. 2003a). The gene is 70,984 bp long and comprises 30 exons.

One mRNA transcript has been established (XM_001133467), which is translated into PKD1L3 (XP_001133467), a 195,894 Da protein (1,731 amino acids).

PKDREJ (GeneID 10,343) is located on chromosome 22q13.31 in negative orientation. The single exon gene is 7,660 bp long (Sutton et al. 2006). One mRNA transcript has been established (NM_006071). The PKDREJ protein consists of 2,253 amino acids (255,449 Da) (NP_006062).

The interaction of TRPP2 and polycystin-1 has been extensively studied at all levels of complexity (Fig. 2). Loss of either one of these proteins causes severe life-threatening cystic kidney disease (Mochizuki et al. 1996; Reeders et al. 1985; European Polycystic Kidney Disease Consortium 1994; International Polycystic Kidney Disease Consortium 1995). Together they are a prerequisite for the coordination of three-dimensional renal tubular organisation. The TRPP2–PKD1L1 signalling module on the other hand is required for the embryonic establishment of a left–right axis, i.e. the asymmetric arrangement of unpaired organs (also see Sect. 7) (Hofherr 2012; Field et al. 2011; Kamura et al. 2011).

TRPP5 and PKDREJ are abundantly expressed in the male germline, but no physical interaction has been observed (Veldhuisen et al. 1999; Sutton et al. 2006; Hughes et al. 1999).

5.2 TRP–TRPP Heteromers

TRPP2 can interact with several TRP channels, including TRPC1, TRPC3, TRPC4, TRPC5, TRPC7 and TRPV4 (Kobori et al. 2009; Sutton et al. 2006; Tsiokas et al. 1999; Köttgen et al. 2008; Du et al. 2008; Miyagi et al. 2009; Stewart et al. 2010). The respective ion channel function seems to be modulated via the TRP subunit composition, adapting the functional properties of TRPP2 to tissue-specific roles including mechano- and chemosensation (Köttgen et al. 2008).

5.3 TRPP and the Cellular Cytoskeleton

The dynamic coordination of cellular structures is a major function of the cytoskeleton. It is composed of three main components: 1) actin filaments; 2) intermediate filaments; and 3) microtubules (Wickstead and Gull 2011). Actin filaments provide the cell's scaffold. TRPP2 was shown to be attached to actin by direct and indirect means, e.g. by tropomyosin-1 and α-actinin or CD2AP and HAX1, respectively (Li et al. 2003b, c, 2005a; Lehtonen et al. 2000; Gallagher et al. 2000). To guide proper cytoskeletal development, actin filaments can be cross-linked to bundles by filamin proteins (Zhou et al. 2010; Ohta et al. 2006). Filamin A and TRPP2 were reported to regulate pressure sensing in mouse vascular smooth muscle cells, i.e. fine-tuning stretch-activated channels to adapt the vascular myogenic response (Sharif-Naeini et al. 2009). Microtubules, on the other hand, make up the internal structure of cilia and provide platforms for intracellular transport (Rosenbaum and Witman 2002). TRPP2 was shown to bind and co-localise with two motor proteins

required for intra-flagellar transport in primary cilia, KIF3A and KIF3B (Li et al. 2006; Wu et al. 2006). At microtubule-organising centres, TRPP2 interactions with pericentrin and mammalian diaphanous 1 protein (mDia1) apparently modulate the intracellular Ca^{2+} homeostasis (Jurczyk et al. 2004; Rundle et al. 2004).

5.4 TRPP Protein Trafficking

The cellular distribution of TRPP2 is determined by the subsequent interaction with various trafficking proteins. Endoplasmic reticulum, plasma membrane and primary cilia are the most prominent TRPP2 compartments (Witzgall 2005; Cai et al. 1999; Koulen et al. 2002; Pazour et al. 2002; Yoder et al. 2002).

The two proteins implicated in autosomal dominant polycystic liver disease (ADPLD), Sec63p and glucosidase 2 subunit β, function as part of the endoplasmic reticulum translocation, folding and quality control machinery (Drenth et al. 2003; Davila et al. 2004). Their physiological interaction with polycystin-1 and TRPP2 is necessary for module processing. Impairment of either ADPLD gene causes insufficient functional expression of the polycystin-1–TRPP2 receptor–channel complex resulting in cystic disease (Fedeles et al. 2011).

Post-endoplasmic reticulum sorting of TRPP2 seems to be controlled by an intricate interplay of intrinsic TRPP2 amino acid trafficking motifs and their ligands. Multiple motifs have been described including an endoplasmic reticulum retention motif ($TRPP2^{787–820}$), an acidic cluster comprising the $serine^{812}$ phosphorylation site ($TRPP2^{810–821}$), and an amino-terminal ciliary import signal, RVxP (R, arginine; V, valine; x, any amino acid; P, proline) ($TRPP2^{6–9}$) (Cai et al. 1999; Hanaoka et al. 2000; Köttgen et al. 2005; Geng et al. 2006). Phosphorylation of $serine^{812}$ in TRPP2 by casein kinase 2 (CK2) regulates the binding of phosphofurin acidic cluster sorting protein 1 (PACS-1) or PACS-2 and routes TRPP2 to the endoplasmic reticulum, Golgi complex or plasma membrane compartments (Köttgen et al. 2005). In addition, PIGEA-14 promotes the endoplasmic reticulum to *trans*-Golgi complex transport of TRPP2 by way of a carboxy-terminal interaction (Hidaka et al. 2004). Interestingly, germline inactivation of PIGEA-14 causes defective airway kinocilia (Voronina et al. 2009). Ciliary sorting of TRPP2 was proposed to be dependent on Rab8a expression (Hoffmeister et al. 2011b). Interestingly, the latter study showed that TRPP2 takes different routes to the somatic and ciliary plasma membrane. The transport of TRPP2 to the ciliary and to the somatic plasma membrane compartments originates in a COPII-dependent fashion at the endoplasmic reticulum. TRPP2 reaches the *cis*-Golgi complex in either case, but trafficking to the somatic plasma membrane goes through the Golgi complex, whereas transport vesicles to the cilium leave the Golgi complex at the *cis* compartment (Hoffmeister et al. 2011b).

5.5 TRPP and A-Kinase Anchoring Protein (AKAP) Complex

Dysregulation of cyclic adenosine monophosphate (cAMP) signalling has been implicated in the pathogenesis of cystic kidney diseases, i.e. promoting fluid secretion and cell proliferation (Hanaoka and Guggino 2000; Belibi et al. 2004; Torres et al. 2012). The molecular mechanisms leading to increased cAMP production in cyst epithelia are still incompletely understood. Recently, it has been shown that TRPP2 and phosphodiesterase 4C are components of a ciliary A-kinase anchoring protein (AKAP) complex that is disrupted in cystic kidney diseases (Choi et al. 2011). AKAP complexes organise cellular signal transduction by compartmentalising second messenger-regulated enzymes (Wong and Scott 2004; Dessauer 2009). In AKAP complexes, TRPP2 was reported to bind adenylyl cyclase 5/6 (AC5/6), a Ca^{2+}-inhibitable enzyme, in primary cilia (Choi et al. 2011). It has therefore been suggested that loss of TRPP2 inhibitory activity may cause increased AC5/6-mediated cAMP production (Choi et al. 2011).

6 Biophysical Description of the Channel Function, Permeation and Gating

TRPP ion channels have been shown to function as Ca^{2+}-permeable non-selective cation channels.

6.1 TRPP3

TRPP3 was the first member of the TRPP family to be identified as a cation channel (Chen et al. 1999). Expression of TRPP3 in *Xenopus laevis* oocytes gave rise to constitutively active, Ca^{2+}-permeable non-selective cation channels with a high single-channel conductance of 137 pS. High intracellular Ca^{2+} increased channel activity, whereas low extracellular pH decreased conductance (Chen et al. 1999; Shimizu et al. 2009). More recently, TRPP3 activity was found to be voltage dependent and increased upon cell swelling and by alkalinisation (Shimizu et al. 2009, 2011). There have been conflicting results as to whether TRPP3 channel function requires co-expression of PKD1L3 and whether this complex is an acid-sensing channel complex required for sour taste transduction (Yu et al. 2012; Chen et al. 1999; Shimizu et al. 2009; Chaudhari and Roper 2010).

6.2 TRPP5

Limited information is available concerning TRPP5 channel function. No spontaneous channel activity has been observed upon over-expression of TRPP5 in HEK293 cells. In outside-out patches, however, these cells exhibited a channel with a single 25 pS conductance state (Sutton et al. 2006).

6.3 TRPP2

After cloning of *PKD2* in 1996 and description of its channel-like topology (Mochizuki et al. 1996), it took four more years until the first report of the channel function was published (Hanaoka et al. 2000). The reason for the difficulty in measuring TRPP2 channel function at the plasma membrane was explained by the identification of a carboxy-terminal retention motif ($TRPP2^{787–820}$) causing exclusive endoplasmic reticulum localisation of over-expressed TRPP2 (Cai et al. 1999). Carboxy-terminal truncations of TRPP2 deleting the retention motif traffic to the plasma membrane; channel activity has been reported for these truncation mutants (Chen et al. 2001). The cellular localisation and site of action of full-length TRPP2 has been discussed controversially: some groups favour the endoplasmic reticulum as the physiological site of action of TRPP2, others the plasma membrane (Cantiello 2004; Witzgall 2005; Hanaoka et al. 2000; Köttgen et al. 2005; Koulen et al. 2002). The first report on TRPP2 channel function provided evidence that polycystin-1 and TRPP2 interact to form a functional heteromeric complex (Hanaoka et al. 2000). This study reported that TRPP2 alone did not form functional channels, but co-expression of polycystin-1 and TRPP2 promoted the translocation of TRPP2 to the plasma membrane and produced Ca^{2+}-permeable non-selective cation currents (Hanaoka et al. 2000). This finding was later confirmed in neurons where over-expressed polycystin-1 and TRPP2 formed a functional receptor–channel complex (Delmas et al. 2004b).

The functional reconstitution of the polycystin-1/TRPP2 complex in heterologous expression systems could be very useful to screen for ligands and specific inhibitors and to study activation mechanisms as well as regulation of the receptor–channel complex. Considering the fact that functional reconstitution of the polycystin-1/TRPP2 complex in over-expression systems might help to address several fundamental questions in the field, surprisingly little has been published in this area since 2004.

After initial studies reporting TRPP2 whole-cell currents, several single-channel studies characterised the channel properties in more detail (Vassilev et al. 2001; González-Perrett et al. 2001, 2002). The specific biophysical properties and the channel function of TRPP2 are reviewed elsewhere (Delmas et al. 2004a; Cantiello 2004). In brief, TRPP2 has been reported to display a single-channel conductance of 100–200 pS and a spontaneous open probability ranging from 0.2 to 0.4. TRPP2 displays non-selective cation channel activity, with slight preference for divalent cations (Vassilev et al. 2001; González-Perrett et al. 2001; Luo et al. 2003). The properties of endoplasmic reticulum resident TRPP2 were studied at single-channel resolution using reconstitution of microsomes in lipid bilayers (Koulen et al. 2002). These studies confirmed that microsomal TRPP2 functions as a Ca^{2+}-permeable non-selective cation channel, but also showed some biophysical properties that differed from those reported for TRPP2 in other studies (Koulen et al. 2002). One interesting aspect of these studies is the regulation of TRPP2 by Ca^{2+} via a carboxy-terminal EF hand in the channel protein (Koulen et al. 2002). The EF-hand Ca^{2+}-binding motifs of TRPP2 and TRPP3 may be involved in the observed intracellular

Ca^{2+}-dependent activation of the channel (Koulen et al. 2002; Chen et al. 1999). This is supported by the observation that $TRPP2^{742X}$, a naturally occurring pathogenic mutant lacking the EF hand, does not display Ca^{2+} activation (Chen et al. 2001). Furthermore phosphorylation of $serine^{812}$ of TRPP2 regulates the Ca^{2+}-dependent activation of the channel (Cai et al. 2004). Several recent studies investigated the structural and functional properties of the helix-loop-helix structural EF-hand motif in TRPP2 in more detail (Ćelić et al. 2008, 2012; Petri et al. 2010). Structural analysis demonstrated discrete conformational transitions and oligomerisation state changes upon the addition of Ca^{2+} to recombinant TRPP2 fragments ($TRPP2^{720–797}$ [EF hand] and $TRPP2^{704–968}$ [TRPP2 carboxy-terminus]), and the authors propose that the EF hand is required for channel gating (Ćelić et al. 2012).

In summary, the channel function of TRPP channels (in particular TRPP2) has been studied quite extensively but important questions remain. In the case of TRPP2, this is mostly due to the fact that functional reconstitution of this channel at the plasma membrane has proven to be difficult. There has been a report of TRPP2 channel activity at the ciliary membrane, but these recordings are technically challenging (Raychowdhury et al. 2005). The identification of factors that allow the robust reconstitution of the TRPP2 channel complex at the plasma membrane in over-expression systems would undoubtedly help to advance the electrophysiological analysis of this important disease-associated ion channel. The identification of physiological activation mechanisms, regulatory factors and specific inhibitors of TRPP2 will be critical to study channel function under more physiological conditions, i.e. in the native tissue.

7 Physiological Functions of TRPP Channels in Native Cells, Organs, and Organ Systems

The physiological importance of TRPP channels and polycystin-1 family proteins becomes evident in light of severe phenotypes observed upon loss of function in humans and model organisms. The founding members of the polycystin family, TRPP2 and polycystin-1, are a prime example of how studying human disease genes can provide insights into fundamental biological mechanisms using a so-called “reverse translational” approach (from bedside to bench). In fact, studying polycystic kidney disease genes has provided considerable insights into processes like tubular morphogenesis, left–right patterning and signalling through primary cilia.

Homozygous null alleles of *PKD1* or *PKD2* are embryonic lethal in mice and men (also see Sect. 8) (Lu et al. 1997; Wu et al. 1998b, 2000; Paterson et al. 2002; Piontek et al. 2004, 2007; Pennekamp et al. 2002; Garcia-Gonzalez et al. 2010). Loss of function of either gene causes cyst formation in the kidneys, liver and pancreas. Thus, one physiological function of these proteins must be in regulating tubular morphogenesis and maintenance of tubular structures. Both genes have a role in vascular integrity, since loss of function causes cardiovascular phenotypes ranging from cardiac valve defects to aneurysms and abnormal vascular

permeability in knockout mice (Garcia-Gonzalez et al. 2010; Kim et al. 2000; Boulter et al. 2001). The embryonic lethality in PKD knockout mice appears to be caused by vascular defects in the placenta (Garcia-Gonzalez et al. 2010). In addition, TRPP2 is essential for the establishment of left–right asymmetry in vertebrates, as loss of TRPP2 function results in heterotaxy, i.e. the abnormal left–right distribution of visceral organs, in zebrafish and mice (Pennekamp et al. 2002; Bisgrove et al. 2005; Schottenfeld et al. 2007). Extensive efforts have been devoted to investigate the molecular and cellular mechanisms, which are the basis for these physiological functions. It is assumed that polycystins function as signalling proteins, but there is no consensus yet about the precise role of the polycystins in cellular signal transduction (Köttgen 2007; Torres and Harris 2009). Importantly, neither the activation mechanisms nor the downstream components of the enigmatic polycystin signalling pathway are known. One intriguing concept that has emerged from studying polycystin function is the importance of primary cilia in cellular signal transduction and organ development (Hildebrandt and Otto 2005).

7.1 The Ciliary Connection

The primary cilium, once considered an evolutionary relic without apparent function, has recently stepped into the spotlight, as a flood of data indicates that cilia have crucial roles in vertebrate development and human genetic diseases (Hildebrandt et al. 2011; Goetz and Anderson 2010). Research on polycystic kidney disease genes has significantly spurred this Cinderella-like transformation. Cilia are antenna-like structure emanating from the surface of virtually every cell in mammals. Cilia contain an axoneme of nine microtubule doublets surrounded by a ciliary membrane. There is mounting evidence that cilia function as a nexus for signalling pathways, translating extracellular cues into cellular signal transduction events (Goetz and Anderson 2010). Orthologues of polycystin-1 and TRPP2 were first localised on ciliated endings of sensory neurons in *C. elegans* and later in cilia of mammalian cells (Barr and Sternberg 1999; Pazour et al. 2002; Yoder et al. 2002). Meanwhile, a plethora of proteins involved in cystic kidney disease have been detected in primary cilia. Furthermore, genetic ablation of cilia in animal models leads to cyst formation (Hildebrandt et al. 2011; Yoder et al. 1996; Pazour et al. 2000; Lin et al. 2003). It is now generally accepted that cilia play an important role in polycystin function, but the precise mechanisms of polycystins in ciliary signalling remain unresolved. In addition to primary cilia, TRPP2 has been proposed to function in other cellular compartments including the lateral membrane of epithelial cells and the endoplasmic reticulum (Köttgen 2007; Koulen et al. 2002; Scheffers et al. 2002; Li et al. 2005b; Wegierski et al. 2009; Sammels et al. 2010; Mekahli et al. 2012). Despite the apparent importance of primary cilia, other compartment-specific functions of polycystins may also contribute to the diverse roles of these proteins in different organs.

7.2 Activation Mechanisms

The activation mechanism of the polycystin-1/TRPP2 receptor–channel complex is an important open question in the field. Cilia have been shown to function as mechanosensors (Praetorius and Spring 2005). Bending of cilia by tubular fluid triggers cytosolic Ca^{2+} signals (Praetorius and Spring 2001). Polycystin-1 and TRPP2 are required for flow-induced Ca^{2+} signals in renal tubular epithelial cells (Nauli et al. 2003; Boehlke et al. 2010). This finding suggested that the mechanical activation of the polycystin complex could be the critical signal regulating tubular morphogenesis, and it has been proposed that the loss of flow-mediated Ca^{2+} signalling causes cyst formation (Nauli et al. 2003; Nauli and Zhou 2004). While the hypothesis that mechanical signals in the tubule lumen convey spatial information to polarised epithelial cells is intriguing, there is also evidence suggesting that flow-mediated Ca^{2+} signalling may not be required for the regulation of tubular geometry (Köttgen et al. 2008; Kotsis et al. 2013). An alternative hypothesis for the activation mechanism of the polycystin complex may involve yet-to-be identified ligands. To date, there is some indirect evidence supporting such a hypothesis (Barr and Sternberg 1999; Köttgen et al. 2011; Delmas et al. 2004b). Unfortunately, the identification of ligands for orphan receptors is notoriously challenging. But the prospect to deorphanise the polycystin-1/TRPP2 receptor–channel complex holds great promise to gain a better understanding of polycystin signalling and may, in our view, justify concerted efforts to screen for ligands using biochemical, physiological and genetic approaches.

Another interesting hypothesis concerning putative activation mechanisms of polycystins comes from the fact that polycystin-1 bears striking similarities to the so-called adhesion class G-protein-coupled receptors (adhesion GPCRs) (Qian et al. 2002; Wei et al. 2007; Langenhan et al. 2013). The adhesion GPCRs are a unique protein family with more than 30 members, which are involved in multiple signalling pathways. A common feature of these receptors is a GPCR autoproteolysis-inducing (GAIN) domain and cleavage of the extracellular amino-terminus at a G-protein-receptor-coupled proteolytic site (GPS) (Langenhan et al. 2013). It has been shown that the cleaved amino-terminal fragment can bind to the membrane-anchored carboxy-terminal fragment of adhesion GPCRs, where it could act as an endogenous ligand or play a role in binding ligands (Langenhan et al. 2013). Polycystin-1 has a GAIN domain and it was shown that the huge extracellular amino-terminus of polycystin-1 is cleaved autoproteolytically at the GPS motif (Qian et al. 2002; Wei et al. 2007). The cleaved amino-terminus remains tethered to the membrane-bound carboxy-terminal rest of the protein in a covalent manner (Qian et al. 2002). In addition, it was shown that knock-in mice with a non-cleavable GPS site in polycystin-1 are viable but show a rapid onset of cystic kidney disease developing mainly from the distal parts of the nephron (Yu et al. 2007). Further investigation revealed that two endogenous forms of polycystin-1 coexist in wild-type mice: a small amount of uncleaved full-length PC-1 and cleaved polycystin-1, which is more abundant. These studies provide compelling evidence that cleavage is not essential for embryonic development but

required for maintenance of the integrity of the distal nephron segments (Yu et al. 2007). In summary, it is intriguing to speculate that the amino-terminal cleavage product of polycystin-1 could play a ligand-like or a ligand-binding role in the activation and regulation of the polycystin-1/TRPP2 channel complex akin to models proposed for adhesion GPCRs (Langenhan et al. 2013).

7.3 Left–Right Asymmetry

Another interesting connection between cilia and polycystins came from the surprising observation that TRPP2 is required for left–right axis determination in vertebrates (Pennekamp et al. 2002). Loss of TRPP2 has been shown to cause heterotaxy in mice and zebrafish, as well as in three patients with ADPKD (Pennekamp et al. 2002; Bisgrove et al. 2005; Schottenfeld et al. 2007; Bataille et al. 2011). The asymmetric placement of internal organs in vertebrates is established by the action of cilia in a pit-like structure called the embryonic node (McGrath et al. 2003; Hirokawa et al. 2006). Rotating cilia generate unidirectional fluid flow across the node from left to right. TRPP2 is required to sense the fluid flow in immotile cilia of perinodal crown cells (Yoshiba et al. 2012). It is not known whether this process involves chemo- or mechanosensation, or both (Norris and Grimes 2012). Interestingly, loss of *PKD1*, which gives rise to similar phenotypes like *PKD2* in most organs, does not cause heterotaxy. Thus, TRPP2 acts independently of polycystin-1 in the establishment of left–right asymmetry. Recently, it was shown that TRPP2 forms a complex with PKD1L1 in the node to form a sensory protein complex required for left–right patterning (Field et al. 2011; Kamura et al. 2011; Vogel et al. 2010). This finding provides compelling evidence that polycystin-1 and TRPP family members can form distinct signalling modules that are adapted to tissue-specific functions (Hofherr 2012).

7.4 The Modular Concept

There is mounting evidence that TRPP channel subunits can form functional homomeric and heteromeric signalling modules, thereby expanding the diversity of organ-specific functions of these ion channels (also see Sect. 5). As mentioned above, there is strong biochemical and genetic data supporting a role for receptor–channel complexes comprised of different combinations of polycystin-1 family members with TRPP channel subunits. Besides the heavily studied complex of the founding members polycystin-1 and TRPP2 in polycystic kidney disease and the function of the PKD1L1/TRPP2 complex in left–right asymmetry, there is now also data suggesting that a heteromeric complex composed of PKD1L3 and TRPP3 might form an acid-activated complex involved in sour taste (Ishimaru et al. 2006; Huang et al. 2006; Lopezjimenez et al. 2006). Several research groups reported the circumscribed co-expression of PKD1L3 and TRPP3 in a specific subset of mouse taste receptor cells (Ishimaru et al. 2006; Huang et al. 2006; Lopezjimenez

et al. 2006). These cells were found in taste buds and were reported to be distinct from the taste receptor cells mediating sweet, umami and bitter taste. Genetic ablation of the PKD1L3- and TRPP3-expressing cells in mice by targeted expression of attenuated diphtheria toxin leads to a complete loss of taste response to sour taste (Huang et al. 2006). The absence of an entire subpopulation of taste cells, however, does not necessarily establish the role of any given protein expressed by the missing cells. Support for the hypothesis that TRPP3 in association with PKD1L3 might form a candidate sour taste receptor came from heterologous over-expression systems. In heterologous expression systems TRPP3 appears to physically interact with PKD1L3 (Murakami et al. 2005; Ishimaru et al. 2006; Huang et al. 2006). HEK293T cells over-expressing PKD1L3 together with TRPP3 generated an inward current upon acid stimulation (Ishimaru et al. 2006). Yet, this channel is sensitive to extracellular pH rather than a drop in cytoplasmic pH, which is known to be the proximate stimulus for sour taste (Lyall et al. 2001; Huang et al. 2008). In addition, mice lacking PKD1L3 remain capable of detecting acid taste stimuli and *Pkd2l1*$^{-/-}$ mice show only a partial reduction in sour taste transduction (Nelson et al. 2010; Horio et al. 2011). Since the PKD1L3/TRPP3 complex does not appear to be required for sour taste transduction, the search for the elusive sour taste receptor will continue.

The modular concept is not limited to TRPP channels and polycystin-1 family members. For example, it has been shown that TRPP2 interacts with other TRP channels like TRPC1 and TRPV4. TRPP2 and TRPV4 form a polymodal sensory channel complex that is involved in ciliary mechano- and thermosensation (Köttgen et al. 2008). A thermosensory role of TRPP channels appears to be evolutionarily conserved as TRPP channels have been shown to mediate cold sensation in *D. melanogaster* (Gallio et al. 2011).

In summary, TRPP channels and polycystin-1-like proteins have important physiological functions linking channel activity to fundamental developmental and sensory processes. We are beginning to understand some of the cellular mechanisms that explain the dramatic phenotypes observed upon loss of function of these proteins. Future studies will have to identify the molecular mechanisms translating channel activity into specific cellular signal transduction pathways controlling organ shape and patterning in vivo.

8 Lessons from Knockouts

The molecular analysis of TRPP2 and polycystin-1 is only the latest step of a long ADPKD research history. There has been an extensive collection of human loss of function data in ADPKD patients well before the molecular identity of the two ADPKD genes was known. However, because of the complex genetic situation in humans, animal model systems have been invaluable to elucidate the physiological function of TRPP and polycystin-1 family proteins. This is because ADPKD patients are heterozygous for the inherited PKD mutation in most of their cells

with only very few cells that are homozygous due to somatic mutations (also see Sect. 9).

A number of constitutive and conditional mouse knockout models for *PKD1* and *PKD2* have been created (Wilson 2008). They faithfully recapitulate central aspects of the human phenotype (Lu et al. 1997; Wu et al. 1998b; Piontek et al. 2004, 2007; Pennekamp et al. 2002; Garcia-Gonzalez et al. 2010; Kim et al. 2000; Boulter et al. 2001). Constitutive *Pkd1*$^{-/-}$ and *Pkd2*$^{-/-}$ mice develop cystic kidneys, oedema as well as haemorrhage and typically die in utero ~15.5 days post-coitum (Lu et al. 1997; Wu et al. 1998b, 2000; Paterson et al. 2002; Pennekamp et al. 2002; Garcia-Gonzalez et al. 2010). Like in ADPKD patients, renal cysts originate from focal dilatations along all nephron segments, including occasional glomerular cysts (Wu et al. 1998b, 2000; Pennekamp et al. 2002; Baert 1978). Heterozygous PKD animals survive to adulthood; only *Pkd1*$^{+/-}$ mice progressively develop scattered cystic disease at the age of 4–19 months, but the phenotype of heterozygous animals is rather mild and does not recapitulate the human disease (Boulter et al. 2001; Lu et al. 1999). Animals heterozygous for both *Pkd1* and *Pkd2* show a harsh progression of disease (Wu et al. 2002).

Tissue-specific and temporal conditional ablation of PKD genes was instrumental in uncovering additional functions of TRPP2 and polycystin-1 usually masked by embryonic lethality. Homozygous loss of *PKD* genes in mice paved the way to additional unexpected *PKD*-dependent phenotypes. One of these phenotypes was the impaired left–right asymmetry with randomisation of unilateral organ placement in *Pkd2* and *Pkd111* mutant mice, but not in *Pkd1*$^{-/-}$ (also see Sect. 7) (Field et al. 2011; Kamura et al. 2011; Pennekamp et al. 2002). In addition, vascular fragility with haemorrhage and skeletal malformations were reported in *Pkd1* and *Pkd2* mutant mice (Kim et al. 2000; Boulter et al. 2001; Garcia-Gonzalez et al. 2010; Khonsari et al. 2013).

The time course of ADPKD in mice was elegantly probed using an inducible *Pkd1* gene inactivation model (Piontek et al. 2004, 2007). *Pkd1* deletion before day 13 post-coitum caused rapid progressive cyst formation in mice (Piontek et al. 2007), whereas *Pkd1* inactivation after day 14 resulted in only mild PKD (Piontek et al. 2007). This notable embryonic switch in kidney development was corroborated by data from *Kif3a* and *Ift88* mice, which present a similar response to timed gene ablation (Davenport et al. 2007).

Pkd2l1$^{-/-}$ mice have no obvious phenotype, but show a 25–45 % reduced response to sour stimuli compared to wild-type (Horio et al. 2011). The suggested signalling module partner in sour tasting *Pkd1l3*, however, presents no taste phenotype upon gene inactivation (Nelson et al. 2010; Horio et al. 2011).

To date no *Pkd2l2*$^{-/-}$ mouse phenotype has been published.

The evolutionary conservation of TRPP proteins facilitated the study of these proteins in *Chlamydomonas reinhardtii*, *D. melanogaster* and *C. elegans* (Barr and Sternberg 1999; Watnick et al. 2003; Gao et al. 2003; Köttgen et al. 2011; Hu and Barr 2005; Huang et al. 2007). In *D. melanogaster*, TRPP2 is a male-specific protein exclusively expressed at the distal tip of spermatozoa. It is required for male fertility, more precisely for the directed movement of spermatozoa in the

female reproductive tract (Köttgen et al. 2011). *Amo*$^{-/-}$ (*Pkd2*$^{-/-}$) flies produce normal amounts of motile spermatozoa but are still infertile (Watnick et al. 2003; Gao et al. 2003; Köttgen et al. 2011). The TRPP homologues (*Brivido 1–3*) have been shown to be important for thermosensation in *D. melanogaster* (Gallio et al. 2011). *Brivido* mutant flies display a significant deficit in their avoidance to cold temperatures (Gallio et al. 2011). *Pkd2*$^{+/-}$ mice exhibit impaired responses to warm temperatures, supporting an evolutionarily conserved role for TRPP channels in thermosensation (Köttgen et al. 2008). The *C. elegans* homologues of *PKD1* and *PKD2*, *Lov-1* and *Pkd-2*, are expressed in ciliated neuronal cells (Barr and Sternberg 1999; Hu and Barr 2005). The proteins are required for successful male mating behaviour (Barr and Sternberg 1999; Hu and Barr 2005). Studies of TRPP channels in invertebrate model organisms have added interesting functional insights. The restricted expression pattern of invertebrate TRPP channels in ciliated neurons and sperm has the advantage that null mutants are not lethal. This can be exploited to discover components in the TRPP signalling pathways using unbiased forward genetic screens.

9 Hereditary and Acquired Disease

Mutations in *PKD1* or *PKD2* cause ADPKD (#173900 and #613095 in Online Mendelian Inheritance of Man, http://www.ncbi.nlm.nih.gov/omim/) (Mochizuki et al. 1996; Reeders et al. 1985; European Polycystic Kidney Disease Consortium 1994; International Polycystic Kidney Disease Consortium 1995; Paterson and Pei 1998; Paul et al. 2014). ADPKD is the most common potentially lethal single-gene disorder in humans affecting one in 400–1,000 live births (U.S. Renal Data System 2012). The hallmark of ADPKD is the age-dependent massive enlargement of both kidneys, which is characterised by the formation of fluid-filled cysts. The defective three-dimensional tissue organisation in ADPKD begins in utero and is slowly progressive. Ultimately, approximately 50 % of affected individuals will develop end-stage renal disease (ESRD) by the sixth decade of life (Gabow et al. 1990; Hateboer et al. 1999; King et al. 2000; Fick-Brosnahan et al. 2002). In 2010, 26,993 United States citizens were suffering from kidney failure due to ADPKD; the incidence of ADPKD ESRD was 12,755 in the period of 2006–2010 (U.S. Renal Data System 2012). With no treatment options other than dialysis and kidney transplant, ESRD remains a severe illness with poor life expectancy. The five-year probability of survival among 2004 incident PKD ESRD patients was 38.8 % (Renal Data System 2011).

In general, ADPKD is slowly progressive. But the clinical course of the disease may be highly variable, ranging from neonatal death to adequate renal function into old age (Longo and Harrison 2012; Bergmann et al. 2008). The underlying sequence alterations in *PKD1* and *PKD2* are rather heterogeneous. Hundreds of unique mutations have been associated with ADPKD, but very limited information is available on genotype-phenotype correlation (The ADPKD Mutation Database, http://pkdb.mayo.edu) (Rossetti et al. 2002; Magistroni et al. 2003; Audrézet

et al. 2012). The significant inter- and intra-familiar phenotypic variability points to the involvement of genetic and environmental modifiers to the ADPKD phenotype (Persu et al. 2004). Genetic determinants, including the level of polycystin-1 and TRPP2 together with the penetrance of pathogenic alleles, might modify the disorder's slow progression over the course of decades (Fedeles et al. 2011; Rossetti et al. 2002). Environmental factors can influence the short-term progression of renal pathology, e.g. ADPKD cyst enlargement is significantly accelerated by acute kidney injury events (Takakura et al. 2009; Bastos et al. 2009; Prasad et al. 2009).

Although the clinical manifestations overlap completely between PKD1 and PKD2, there is a significant locus effect on renal pathology. On a population scale, *PKD1* mutations cause earlier PKD with ESRD occurring at 54.3 years versus 74 years in PKD2 (Hateboer et al. 1999; Torra et al. 1996). In PKD2, gender correlates with phenotypic penetrance: men experience a faster worsening symptomatology than women, with ESRD at 68.1 years and 76 years, respectively (Hateboer et al. 1999; Magistroni et al. 2003). Whether a similar gender correlation holds true for PKD1 remains to be validated (Rossetti et al. 2002; Gabow et al. 1992; Harris et al. 2006).

Approximately 1,000,000 nephrons form a human kidney (Boron and Boulpaep 2009). Still, an ADPKD patient will develop only about a thousand cysts which will eventually disintegrate the kidney in a process associated with a decline in glomerular filtration and ESRD (Grantham 2008). The small quantity of overall cysts in relation to nephrons emphasises that only very few renal tubules undergo cystic transformation. This focal nature of ADPKD is consistent with a "two-hit" model, indicating that each cyst arises as a consequence of a distinct somatic mutation event ("second hit") on the background of an inherited germline mutation ("first hit") (Reeders 1992; Pei 2001). The acquired homozygous inactivation of *PKD1* or *PKD2* is a necessary pathogenic step leading to clonal expansion of cysts (Qian et al. 1996; Watnick et al. 1998; Menezes and Germino 2009). Thus, despite the dominant mode of inheritance, ADPKD is likely recessive at the cellular level. The "second hit" instant of time is of particular importance to cyst growth, i.e. inactivation of *Pkd1* before postnatal day 13 provokes severely cystic kidneys within 3 weeks in mice, whereas inactivation at day 14 or later results in cysts only after 5 months (Piontek et al. 2007). Thus, it is likely that the kidney's developmental stage defines the pathologic consequences of *PKD1* deletion.

Genotypes homozygous and compound heterozygous for *PKD1* or *PKD2* have been reported to be embryonic lethal with PKD, cardiac failure and gross oedema (Wu et al. 2000; Paterson et al. 2002; Pennekamp et al. 2002; Boulter et al. 2001; Lu et al. 2001; Muto et al. 2002). Individuals heterozygous for both *PKD1* and *PKD2* mutations survive to adulthood but show an accelerated progression of disease (Wu et al. 2002; Pei et al. 2001).

Renal cysts are the most prominent manifestations of ADPKD, but a wide spectrum of mostly non life-threatening abnormalities accompany the disorder. Pain is a recurrent symptom in adult patients, often caused by cyst haemorrhage, cyst infection or kidney stones (Bajwa et al. 2001, 2004). Gross and microscopic haematuria are prevalent (Grantham et al. 2006). A major contributor to the decline

in kidney function together with cardiovascular morbidity and mortality is arterial hypertension (Kelleher et al. 2004). Hepatic cysts are the primary extra-renal lesion (Bae et al. 2006). Cysts may also develop in the arachnoid membrane, pancreas and seminal tract (Danaci et al. 1998; Wijdicks et al. 2000). Cardiac and vascular manifestations include an increased incidence of valvular heart disease, coronary artery and intracranial aneurysms as well as chronic subdural haematoma (Wijdicks et al. 2000; Hossack et al. 1988; Schievink et al. 1995; Hadimeri et al. 1998; Lumiaho et al. 2001; Leung and Fan 2005).

In summary, ADPKD is a systemic disorder that represents a therapeutic challenge for clinicians across multiple disciplines. It is hoped that a better understanding of the pathogenic mechanisms will provide the basis for rational therapies of this devastating disease.

Conclusions

It has been exciting times since the identification of *PKD1* and *PKD2* as the genes mutated in ADPKD. Biological roles of the encoded proteins polycystin-1 and TRPP2 have been deduced from phenotypes in ADPKD patients, but recent insights from vertebrate and invertebrate model organisms have significantly expanded our understanding of the physiological functions of these proteins. The discovery of the central role of primary cilia and the modular assembly of TRPP channels and polycystin-1 family members are a strong foundation for future discoveries. The identification of upstream and downstream components in the enigmatic polycystin signalling pathway is one of the next important milestones. Clearly, we still have quite some work to do before we understand the molecular mechanisms of these proteins in biological processes like tubular morphogenesis and organ patterning.

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