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REVIEW

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Receptor-operated cation channels formed by TRPC4 and TRPC5

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Abstract TRPC4 and TRPC5 form cation channels that contribute to phospholipase C-dependent Ca^{2+} entry following stimulation of G-protein-coupled receptors or receptor tyrosine kinases. Surprisingly, in different studies, TRPC4 and TRPC5 have been shown to form either store-operated channels with a relatively high Ca^{2+} permeability, or nonselective cation channels activated independently of store depletion. In this review, we summarize and discuss data on the regulation and permeability properties of TR PC4 and TRPC5, and data on native channels that might be composed of these isoforms.

Introduction

For many years, it has been known that stimulation of receptors for hormones and neurotransmitters that couple to isoforms of phospholipase C (PLC) leads to increases in the concentration of intracellular Ca^{2+} that are composed, to different degrees depending on the cell type, of two components; Ca^{2+} release from intracellular stores and Ca^{2+} entry through the plasma membrane. The Ca^{2+} release component is triggered by inositol 1,4,5-trisphosphate ($InsP_3$), one product of the PLC-catalyzed breakdown of phosphatidylinositol bisphosphate (PIP_2), and has been relatively well characterized in recent years. The channels involved in the Ca^{2+} entry component show a greater diversity in their regulation and functional properties and have proved more elusive.

In the last 10 years, mammalian channels of the TRP family have emerged as molecular candidates for the cation channels involved in receptor-operated Ca^{2+} entry. Of the large number of mammalian TRP channels identified (Clapham et al. 2001; Montell et al. 2002; Clapham et al. 2003), TRP channels of the classical or canonical subfamily (TRPC), which are most closely related to their PLC-regulated *Drosophila* counterparts TRP and TRPL, are most likely to form Na^+ and Ca^{2+}-permeable channels involved in similar signaling pathways in mammalian cells. Like many other cation permeable channels, TRP channel subunits have six membrane-spanning regions (S1–S6), intracellular N and C termini, and a pore-forming loop between S5 and S6. Functional channels are presumably formed of tetrameric subunit combinations.

The TRPC subfamily comprises seven members TRPC1 to TRPC7. Based on sequence similarity, the TRPCs can be further subdivided into four groups (Clapham et al. 2001; Montell et al. 2002; Clapham et al. 2003); group 1 and group 2 contain only the isoforms TRPC1 and TRPC2, respectively. Group 3 comprises TRPC3, TRPC6 and TRP C7, and group 4 TRPC4 and TRPC5. Group 4 TRPCs are most closely related to group 1. These structural similarities have important consequences for the ability of TRPCs to form heteromultimers. Thus, in adult tissues, heteromultimers can be formed between TRPC1, TRPC4 and TRPC5, and between TRPC3, 6, 7 (Strübing et al. 2001; Goel et al. 2002; Hofmann et al. 2002; Strübing et al. 2003). TRPC2 is unable to combine with other TRPCs (Hofmann et al. 2002). In embryonic tissues and following heterologous expression of three isoforms, more complex combinations composed of isoforms from group 1, group 3 and group 4 can be formed (Strübing et al. 2003).

All of the TRPC channels have been shown to be activated in a PLC-dependent manner, but there is still considerable confusion in the literature regarding the regulatory steps distal to PLC and in some cases with respect to the biophysical properties of the channels such as the shape of the IV relation and the Ca^{2+} permeability. Pro-

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posed activation mechanisms for TRPC channels include store-dependent regulation by an unknown messenger dependent on the filling level of intracellular Ca^{2+} stores, conformational coupling by a protein–protein interaction between the channel and the $InsP_3$ receptors localized in the membrane of the intracellular Ca^{2+} stores, and direct regulation of the channel by a component of the pathway regulated by PLC. Although there are reports linking all TRPCs to store-operated currents, there is still considerable controversy regarding the molecular nature of store-operated channels and their activation mechanism (see e.g., Perspectives in Science STKE 243; http://www.stke.sciencemag.org/content/vol2004/issue243/)

Although there are a large number of publications regarding TRPC1, it is still unclear whether the subunit can form functional channels on its own or whether it can only form channels in heteromultimers with other TRPC isoforms (Strübing et al. 2001). Following heterologous expression, TRPC1 is mostly located in intracellular compartments and is transported to the membrane only after coexpression with TRPC4 (Hofmann et al. 2002). Where functional expression of TRPC1 has been observed, the channel has been reported to be constitutively active (Zitt et al. 1996), store-operated (Zhu et al. 1996; Zitt et al. 1996) or activated in the absence of Ca^{2+} by diacylycerol (DAG), the other product of PIP_2 breakdown by PLC (Lintschinger et al. 2000). TRPC1 is widely expressed. TRPC2, the only member of group 2, is able to form functional channels in rodents, where the protein is expressed in the vomeronasal organ (Liman et al. 1999) and in testis (Vannier et al. 1999), but the bovine (Wissenbach et al. 1998) and human (Wes et al. 1995; Vannier et al. 1999) isoforms are truncated and are unable to form functional channels. Data from TRPC2−/− mice indicate that TRPC2 forms a cation channel involved in responses to pheromones in the VNO (Leypold et al. 2002; Stowers et al. 2002) and may be activated by DAG (Lucas et al. 2003). For group 3 TRPCs, it is widely accepted that the channels can be regulated by DAG (Hofmann et al. 1999; Okada et al. 1999), but evidence exists for other mechanisms including conformational coupling with the $InsP_3R$ (Kiselyov et al. 1998) and store depletion-dependent regulation (Vazquez et al. 2001). For more information on TRPC2, TRPC3 and TRPC3, 6 and 7 see the reviews by Zufall, Groschner, and Rosker, and Dietrich et al. in this issue.

In this review we focus on the group 4 TRPCs, TRPC4 and TRPC5. Transcripts for TRPC4 have been shown to be expressed in the brain and a wide range of other tissues including the adrenal gland, pancreas, placenta, uterus, testis, prostate, lung, kidney, heart, endothelial cells and vascular and intestinal smooth muscle (see e.g., Philipp et al. 1996; Mori et al. 1998; McKay et al. 2000; Riccio et al. 2002). TRPC5 mRNA is most abundant in the brain, but is also present in testis, kidney, lung, heart, ovary, adrenal gland, uterus, endothelium and vascular and gastric smooth muscle (Okada et al. 1998; Philipp et al. 1998; Riccio et al. 2002; Kim et al. 2003; Beech et al. 2004). Because of considerable overlap in the expression patterns of TRPC4 and TRPC5, and between these isoforms and TRPC1, the formation of heteromeric combinations in native tissues is very likely. Here, we concentrate on the regulation and permeability properties of TRPC4 and TRPC5; two aspects for which contradictory findings exist, and on the properties of heteromeric combinations of composed of group 4 TRPCs with TRPC1. We also give an overview of studies on native channels that might involve TRPC4 and TRPC5. Other features of these channels have been reviewed recently (Plant and Schaefer 2003).

TRPC4 and TRPC5: store-operated or receptor-operated, store-independent channels?

Regulation by receptors and PLC

It is widely agreed, as expected for channels of the TRPC subfamily, that TRPC4 and TRPC5 are activated following stimulation of G-protein-coupled receptors of the $G_{q/11}$ family which couple to PLCβ, or receptor tyrosine kinases which couple to PLCγ. The channels can also be activated by application of activators of G proteins like GTPγS and AlF_4^- to the intracellular membrane surface (Philipp et al. 1996; Schaefer et al. 2000; Obukhov and Nowycky 2004). Receptor-mediated activation is prevented by inhibition of PLC (Schaefer et al. 2000). Where major differences have been found is with respect to what happens after PLC and the permeability properties of the channels formed by TRPC4 and TRPC5.

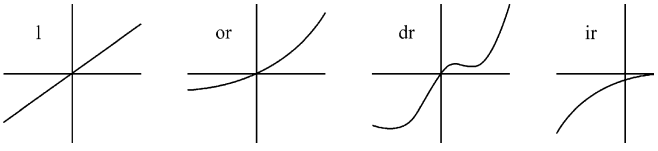
Regulation downstream of PLC

Bovine TRPC4 and rabbit TRPC5 were reported to be moderately to highly Ca^{2+} permeable channels activated by Ca^{2+} store depletion (Table 1; Philipp et al. 1996; Philipp et al. 1998; Warnat et al. 1999). These conclusions are based on the measurement of intracellular Ca^{2+} in response to store depletion and Ca^{2+} readdition, and measurements of current responses to infusion of a solution containing $InsP_3$ with Ca^{2+} buffered to a concentration of 500 nM. The currents measured in all of these studies showed inward rectification and positive reversal potentials indicative of a moderate to high Ca^{2+} permeability.

In strong contrast to the finding that TRPC4 and TRPC5 are store-operated, the first report on mouse TRPC5 described a moderately Ca^{2+} permeable, receptor-operated channel with no involvement of store depletion in channel activation (Okada et al. 1998). We, and a number of other groups have confirmed this finding for mouse, rat and human variants of TRPC4, and for mouse TRPC5, transiently expressed in mammalian cell lines (Schaefer et al. 2000; Strübing et al. 2001; Schaefer et al. 2002). Likewise, rat TRPC5 forms a receptor-operated nonselective cation channel, but the activation mechanism has not been studied in more detail (Obukhov and Nowycky 2004). For a summary see Table 1. In our experiments, no increases in cation influx were seen following store depletion protocols in fluorometric experiments. Furthermore, cation currents

Table 1 Overview of studies on heterologously expressed TRPC4 and TRPC5. Species: *h* human, *b* bovine, *rb* rabbit, *r* rat, *m* mouse; Regulation: *SA* spontaneously active, *ROC* receptor-operated channel, *SOC* store-operated channel, *CaM* calmodulin, *DAG* diacylglycerol; IV: *l* linear, *ir* inwardly rectifying, *dr* doubly rectifying, *or* outwardly rectifying; La^{3+} or Gd^{3+}: effects of micromolar lanthanides. + Potentiation, − inhibition

Channel	Regulation	P_{Ca}/P_{Na} or cation permeability	Single channel conductance	IV	La^{3+} or Gd^{3+}	References
TRPC4						
hTRPC4α	SA; no regulation	1.1		l		McKay et al. (2000)
hTRPC4α	no SA; ROC, not SOC	Nonselective	30 pS	dr	+	Schaefer et al. (2002)
bTRPC4α	SOC	7		ir		Philipp et al. (1996)
bTRPC4α	SOC	Ca^{2+} selective		ir		Warnat et al. (1999)
rTRPC4α	no SA; ROC, not SOC	Nonselective	28 pS	dr	+	Schaefer et al. (2002)
mTRPC4α	$Ca^{2+}{}_i$ (CaM?)-inhibited	Nonselective			−	Walker et al. (2002)
hTRPC4β	no SA; ROC, not SOC	Nonselective	30 pS	dr	+	Schaefer et al. (2002)
rTRPC4β	no SA; ROC, not SOC	Nonselective		dr	+	Schaefer et al. (2002)
mTRPC4β	no SA; ROC, not SOC	1.1	41 pS	dr	+	Schaefer et al. (2000)
mTRPC4β	$Ca^{2+}{}_i$ (CaM?)-inhibited	Nonselective	18 pS	or	−	Walker et al. (2002)
TRPC5						
hTRPC5	No SA; ROC and SOC	Nonselective		dr/or	+	Zeng et al. (2004)
rbTRPC5	SOC	Ca^{2+} selective		ir		Philipp et al. (1998)
rTRPC5	no SA; ROC	Nonselective		dr	+	Obukhov and Nowycky (2004)
mTRPC5	ROC, not SOC	14.3			−	Okada et al. (1998)
mTRPC5	ROC		48 pS			Yamada et al. (2000)
mTRPC5	SA; ROC, not SOC	1.8	63 pS	dr	+	Schaefer et al. (2000)
mTRPC5	SA; ROC	Nonselective	38 pS	dr	+	Strübing et al. (2001)
mTRPC5	SA; ROC	1.8	41 pS	dr	+	Jung et al. (2003)
mTRPC5	ROC, DAG	Nonselective		dr	−	Lee et al. (2003)
Heteromultimeric channels						
hTRPC1/mTRPC5	SA; ROC, not SOC	1.1	5 pS	or	+	Strübing et al. (2001)
hTRPC1/mTRPC4	ROC	Nonselective		or		Strübing et al. (2001)



were not activated by infusion of $InsP_3$ in strongly Ca^{2+}-buffered intracellular solutions, a protocol that activated the well-characterized, store-operated current, the calcium release-activated calcium current (I_{CRAC}) in Jurkat T cells. Inhibition of $InsP_3R$ activation with low molecular weight heparin (LMWH) should prevent receptor-mediated Ca^{2+} release and emptying of stores and, thus, store-operated channel activation, and, in addition, should prevent channel activation by conformational coupling to the $InsP_3R$ initiated by $InsP_3$. We found that receptor-mediated activation of currents in cells transfected with TRPC4 and TRPC5 was unaffected by infusion of LMWH. Further evidence that TRPC5 can be activated independently of $InsP_3$ receptors and intracellular stores was obtained in another study where it was shown that TRPC5 could be activated via a muscarinic receptor in DT-40 B cells lacking all three $InsP_3$ receptor subtypes (Venkatachalam et al. 2003). In contrast, a study on oocytes expressing TRPC5 found that the $InsP_3$ receptor was essential for store-independent activation (Kanki et al. 2001). Although these data do not support a store-operated mechanism of activation, the 'activator' of TRPC4 and TRPC5 has not yet been identified

(see below). Store-independent regulation of TRPC4 and TRPC5 by PLC-coupled receptors is not only a property of homomeric channels formed by these isoforms. Like homomeric TRPC4 and TRPC5, heteromers of TRPC1 with TRPC5 were not activated by infusion of $InsP_3$, but responded to subsequent muscarinic receptor activation (Strübing et al. 2001).

Until recently, store-independent regulation of TRPC4 and TRPC5 was consistently associated with channels with dramatically different permeability properties and, as a result, with a different form of IV relation to those seen for store-dependent TRPC4 and TRPC5 channels. Instead of forming highly Ca^{2+}-permeable channels, store-independent channels have similar permeabilities to Na^+, Cs^+ and Ca^{2+}. In addition to this difference in permeability which results in a reversal potential close to 0 mV, nonselective TRPC4 and TRPC5 channels displayed an unusually-shaped IV relation, with a reduction in slope, or even a negative slope at potentials in a range just positive to the reversal potential (Fig. 1). Another important feature of the IV-relation is that the shape is not constant, but depends on the extent of channel activation, particularly at potentials

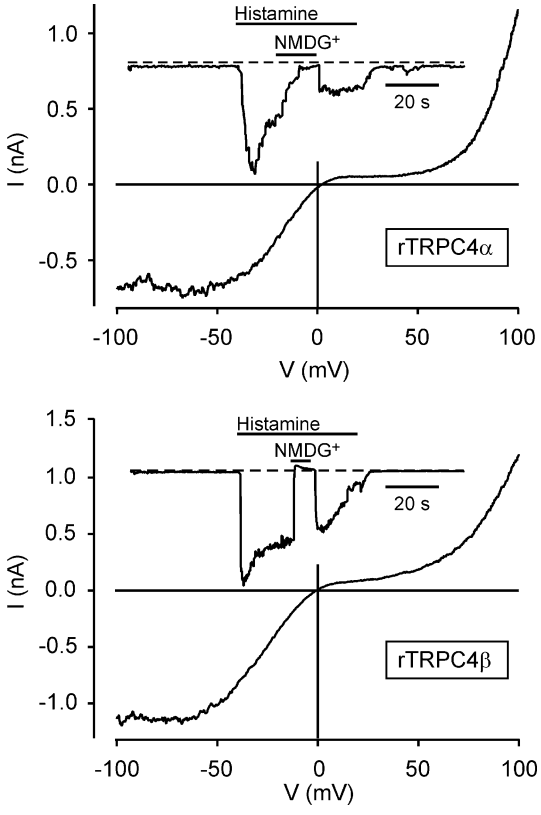


Fig. 1 Receptor-operated, nonselective cation currents in cells expressing rat TRPC4α (*upper panel*) or rat TRPC4β (*lower panel*) together with an H1 histamine receptor. Currents were recorded in the whole-cell mode at a holding potential of −60 mV and during voltage ramps from −100 to +100 mV applied at 10-s intervals. For both splice variants, application of histamine (100 μM) activated an inward current at −60 mV, which was abolished upon removal of Na^+ and Ca^{2+} from the extracellular solution (replacement by $NMDG^+$). The characteristically-shaped current–voltage relations were recorded close to the maximum of the receptor-activated current.

negative to 0 mV (Plant and Schaefer 2003). The reason for the characteristic shape of the IV relation for receptor-operated TRPC4 and TRPC5 is unclear. Experiments with Mg^{2+}-free solutions on both sides of membrane patches suggested that it might result from inhibition by Mg^{2+} (Schaefer et al. 2000), but this result could not be confirmed by other groups in whole-cell experiments (Strübing et al. 2001), nor were effects of changing the Mg^{2+} concentration on one membrane surface seen in isolated patches (Yamada et al. 2000). Very recently, however, the Mg^{2+} block of TRPC5 has been characterized in more detail (Obukhov and Nowycky 2005). Intracellular Mg^{2+} blocked outward currents through TRPC5 with an IC_{50} of 457 μM. The block was localized to a negatively-charged amino acid, D633, located in the intracellular C terminal region between S6 and the "TRP box," an invariant sequence in TRPCs which forms part of the highly conserved TRP domain. Neutralization of this amino acid abolished Mg^{2+} block and the reduction in IV slope at positive potentials. The shape of the IV relation of TRPC4 and TRPC5 is unusual, but not unique to these isoforms because a similar shape has been observed in some studies for TRPC3, TRPC6 and TRPC7. Interestingly, all group 3 and group 4 TRPCs, but not TRPC1, have a negatively charged aspartate or glutamate residue at analogous positions to D633 in TRPC5. One major difference between group 3 and group 4 TRPCs is that on strong activation the IV relation of the former shows more pronounced outward rectification.

The clear distinction between properties of channels activated store-dependently and those activated store-independently was not observed in a recent study on TRPC5. In addition, this study revived the hypothesis that species variants can determine whether group 4 TRPCs can be regulated in a store-operated fashion. Using the human isoform of TRPC5, which had not been studied previously at a functional level, it was shown that the channel could operate in a receptor-operated, store-independent mode and in a store-operated mode (Zeng et al. 2004). The channel was activated by muscarinic receptors following a long pretreatment with thapsigargin to empty intracellular Ca^{2+} stores and was thus store-independent. However, in other experiments using Gd^{3+} to inhibit endogenous store-operated channels, and probably to increase currents through TRPC5 (see below), evidence for store depletion-dependent activation was obtained in fluorometric experiments, and currents were increased by cyclopiazonic acid, an inhibitor of Ca^{2+} uptake into intracellular stores. Interestingly, human TRPC5 shares greater similarity with the rabbit isoform, originally described to be store-dependent (Philipp et al. 1998), than with the mouse variant. However, for human TRPC5, both store-dependent and store-independent currents displayed similar IV relations (Zeng et al. 2004). These reversed at around 0 mV, indicative of a nonselective cation channel, and displayed mainly outwardly rectifying IV relations with a weak hump positive to 0 mV.

Not only have laboratories studied different species variants of TRPC4, but also different splice variants (Table 1). The major variants of TRPC4 are TRPC4α and TRPC4β.

The latter is shorter and lacks an 84–85 amino acid domain in the C terminus. This domain contains an interaction site for the $InsP_3R$ (Mery et al. 2001). To investigate whether the differences in properties result from the species or splice variant studied, we compared rat and human TRP C4α and β (Schaefer et al. 2002). However, we found that both species and splice variants formed PLC-regulated nonselective cation channels and that neither showed store-operated regulation. One surprising difference between the human splice variants was a significantly reduced current density for TRPC4α. Indeed, coexpression of hTRPC4α with hTRPC4β resulted in a reduction in cation influx. Truncation experiments indicated that the inhibitory effects involve the C terminus of hTRPC4α. Further evidence against the $InsP_3R$ interaction site in the C-terminal alternatively-spliced domain of TRPC4 being the determinant of store-operated regulation, is the presence of a similar site in the C terminus of mouse TRPC5 and the absence of store-operated regulation in this isoform.

In addition to studies which showed store-dependent or store-independent regulation, there are two other reports on TRPC4 (Table 1). In the first, human TRPC4α was found to be a constitutively active, nonselective cation channel in CHO cells, but was not regulated by activation of a receptor coupling to PLC via G_q, nor by store depletion (McKay et al. 2000). No functional expression was observed in HEK cells, but, as our subsequent study showed, currents with this isoform are often very small (Schaefer et al. 2002). In a further study, the mouse TRPC4α and β splice variants were found to be activated by reduction of intracellular Ca^{2+} by infusion of BAPTA in whole-cell recordings (Walker et al. 2002). Since the calmodulin antagonist calmidazolium also increased currents, TRPC4 was suggested to be a Ca^{2+}-inhibited nonselective cation channel, possibly regulated by Ca^{2+} via calmodulin.

If not store-operated, what then? Regulatory mechanisms for TRPC4 and TRPC5

Regulation via the PLC pathway

Diacylycerol activates group 3 TRPCs (Hofmann et al. 1999; Okada et al. 1999) and, based on data from TRPC2−/− mice, can activate TRPC2 (Lucas et al. 2003). Most studies that have tested the effects of DAG on TRPC4 or TRPC5 found no increase in cation influx measured using fluorometric techniques (Hofmann et al. 1999; Schaefer et al. 2000; Schaefer et al. 2002; Venkatachalam et al. 2003). However, there is one report that DAG can activate TRPC5 (Lee et al. 2003). In this study, using the patch clamp technique, it was shown that DAG can activate a current with the characteristic IV relation of TRPC5, but activation was smaller than that in response to muscarinic receptor stimulation. However, the concentration of the membrane-permeable DAG analog used was one that is submaximally effective for activating the group 3 DAG-sensitive TRPCs (Hofmann et al. 1999). In the study of Venkatachalam et al. (2003), where DAG was found not to activate TRPC5, it was demonstrated to inhibit receptor-mediated activation of the channel by a protein kinase C (PKC)-dependent mechanism. This study also suggested that PKC-mediated effects are responsible for the decaying phase of the transient TRPC5 responses to receptor stimulation.

The PLC pathway generates or metabolizes a large number of signaling molecules that are potential channel regulators. If DAG itself is not the activator, one of the many downstream products of DAG metabolism could be. However, arachidonic acid and other fatty acids, some of which have been reported to activate the *Drosophila* photoreceptor channels TRP and TRPL (Chyb et al. 1999), were ineffective (Schaefer et al. 2000). Similarly, inhibitors of DAG metabolism did not affect the activation of TRPC4 or TRPC5 (Schaefer et al. 2000). One possibility that is still open is that the channels are activated by breakdown of a substrate of PLC like PIP_2. A reduction in PIP_2 and relief from inhibition by PIP_2 has been reported to be responsible for PLC-mediated potentiation of a member of another TRP subfamily TRPV1 (Chuang et al. 2001).

Rapid insertion from submembrane vesicles

A novel mechanism by which TRPC5 is regulated that was described recently is rapid insertion into the plasma membrane from submembrane vesicles (Bezzerides et al. 2004). This insertion is regulated by growth factors via phosphatidylinositide 3-kinase (PI(3)K), the Rho-related GTPase Rac1 and phosphatidylinositol 4-phosphate-5-kinase (PIP(5)Kα) and occurs within 2 min of stimulating the growth factor receptor. Interestingly, rapid vesicular insertion occurs with homomeric TRPC5, but not with heteromeric channels formed of TRPC1 and TRPC5. Insertion of channels increases the number of channels available for activation by $G\alpha_{q/11}$/PLCβ. Surprisingly, in this study, no activation of TRPC5 via EGFR/PLCγ was observed, in contrast to our previous observations which indicated that TRPC4 and TRPC5 can be regulated by both PLCβ and PLCγ (Schaefer et al. 2000).

Intracellular Ca^{2+}

A Ca^{2+} dependence of TRPC5 activation was described in one of the first reports on this channel (Okada et al. 1998). There, it was shown that receptor-mediated activation was weak in the absence of extracellular Ca^{2+}, and increased upon raising the Ca^{2+} concentration. In addition, the number of cells responding to agonist application decreased when the intracellular Ca^{2+} concentration was reduced. We found similar results for TRPC4 and TRPC5 (Schaefer et al. 2000). At similar Ca^{2+} concentrations, strong intracellular Ca^{2+} buffering reduced or inhibited channel activation by receptor stimulation. The most consistent current responses were observed with an intracellular solution of lower Ca^{2+} buffer capacity. However, some activation is observed with strong buffering at around 100 nM. Fewer cells responded with strong buffering and a lower Ca^{2+} concentration. In

experiments looking at channel activation by GTP-γ-S infusion, only weak channel activation over many minutes was observed in the absence of extracellular Ca^{2+}, but a dramatic current increase was seen after addition of Ca^{2+} to the extracellular solution. Infusion of solutions with micromolar concentrations of Ca^{2+} resulted in transient current increases immediately after attaining the whole cell configuration, but in most cells these were much smaller than the current increases in response to subsequent receptor stimulation. Increases in intracellular Ca^{2+} also stimulated activity of TRPC4 and TRPC5 in inside-out patches (Schaefer et al. 2000). In a recent study on human TRPC5 (Zeng et al. 2004), no currents were observed on infusion with a 0 Ca^{2+} solution, but an increase in current was observed upon infusion of 200 nM Ca^{2+}. The sensitivity to intracellular Ca^{2+} is preserved in heteromers of TRPC1 and TRPC5 (Strübing et al. 2001). Both spontaneous channel activity and receptor-mediated activation were reduced at Ca^{2+} concentrations under 10 nM.

These results clearly show that channel activation is dependent on intracellular Ca^{2+}. The effectiveness of receptor-mediated activation depends on the level of intracellular Ca^{2+}. During PLC-mediated activation, Ca^{2+} entry through the channel is an important potentiator of the response (Okada et al. 1998; Schaefer et al. 2000) and raising Ca^{2+} alone can activate the channels (Schaefer et al. 2000). It is also possible, although not yet demonstrated, that in the physiological response Ca^{2+} release from stores could contribute to potentiation of TRPC4 and TRPC5. However, Ca^{2+} is certainly not the sole activator in response to PLC-mediated activation since strong activation can occur after depletion of intracellular Ca^{2+} stores and with heparin to prevent $InsP_3$ receptor activation. The mechanism by which intracellular Ca^{2+} regulates TRPC4 and TRPC5 is unknown. It could involve a direct or an indirect action e.g., via calmodulin or Ca^{2+}-dependent enzymes on the channel proteins or an action on the PLC pathway. All TRPCs have binding sites for calmodulin (Tang et al. 2001; Trost et al. 2001), but the functional role of the CaM binding site in TRPC4 and TRPC5 is unknown. A recent study on TRPC6 and TRPC7 has indicated that regulation by Ca^{2+} and calmodulin is extremely complex and diverse, even within one group of TRPCs (Shi et al. 2004).

Extracellular Ca^{2+} and lanthanides

We reported that increasing extracellular Ca^{2+} from 2 to 20 mM increases currents through TRPC5 (Jung et al. 2003), but did not study the concentration dependence in more detail. A similar effect was found in the recent study on human TRPC5 and the concentration dependence studied in a more physiological range (Zeng et al. 2004). This study also showed that raising Ca^{2+} not only potentiates active channels, but was sufficient to increase channel activity in the absence of another agonist. Increases in current were observed on raising Ca^{2+} to 5 mM but not to 4 mM, with strong intracellular Ca^{2+} buffering to minimize changes in intracellular Ca^{2+}. The effects of raising Ca^{2+} are larger than those expected for an increase in the electrochemical gradient for Ca^{2+}. Strong evidence for an extracellular effect of Ca^{2+} was obtained using channel mutants (Jung et al. 2003). The rapid effect of Ca^{2+} is mediated by negatively-charged amino acids (E543 and E595) in the putative pore-forming loop (P-loop) between the transmembrane segments S5 and S6. These amino acids are also important for the surprising potentiation of TRPC5 by micromolar La^{3+} and Gd^{3+} (Jung et al. 2003). Neutralization of either of these amino acids leads to a loss of the rapid potentiatory effects of trivalent cations and Ca^{2+}. For E543Q, potentiation by Ca^{2+} is replaced by inhibition. For E595Q/E598Q, fast potentiation is lost, but a slower potentiation becomes more prominent. The latter most probably results from an intracellular action of Ca^{2+} (see above) whose passage through the channel pore is increased in the mutant as a result of an increased Ca^{2+} permeability. Since the concentration at which Ca^{2+} begins to act via these extracelullar sites is around 5 mM (Zeng et al. 2004), the physiological significance of this effect remains questionable.

These data indicate that the activity of TRPC4 and TRPC5 can potentially be regulated by a number of mechanisms. Of those identified, none is adequate to account for receptor-mediated activation of the channels via PLC and it remains to be seen what roles these regulatory mechanisms play for physiological channel function. A number of TRP channels do not have a single activator, but can be activated independently by multiple signals. This is particularly true for some members of the TRPV subfamily (Julius and Basbaum 2001; Vriens et al. 2004). The regulatory mechanisms identified for TRPC4 and TRPC5 differ from these in that most could contribute to PLC-mediated activation.

Possible explanations for the major differences between studies on TRPC4 and TRPC5

It is clear from this brief resumé of data on the regulation and channel properties of TRPC4 and TRPC5 that there are a number of major differences. How could some of these differences be explained?

Small changes, even down to a single amino acid exchange in a regulatory domain, which could be located in a number of regions of the protein, could explain differences in the regulatory properties between species variants. For TRPC4 variants, most sequence differences are observed in the intracellular C terminal region where several interaction sites have already been identified. The differences in selectivity, which are determined by the amino acid composition of the pore-forming region, are more difficult to explain. Like other cation channels with a similar transmembrane topology, the pore region of TRP channels is formed by the loop between S5 and S6 (Voets and Nilius 2003). The S5–S6 linker which includes the putative pore-forming region is identical in rabbit, mouse, rat and human TRPC5 and,

with the exception of one amino acid (a threonine instead of a serine) in the human isoform, are identical in rat, mouse, bovine and human TRPC4. Since amino acids in the pore-forming loop are likely to be the major determinants of cation permeability for TRP channels and related cation channels, it is extremely improbable that the isoforms should display major differences in their ion selectivity.

This reasoning holds as long as TRPC4 and TRPC5 are the pore-forming subunits and do not act as auxiliary subunits which bring previously silent endogenous subunits to life or modify endogenous channels. One piece of evidence against the involvement of an endogenous subunit is the very high current density that is often observed. This would require that the cells express high levels of silent endogenous proteins. The best direct evidence that the expressed protein really forms the channel pore is if mutation in the putative pore region changes permeability properties or the type of channel block. Although the structural determinants of permeability have not been studied systematically for any of the TRPC channel isoforms, we have obtained some evidence that indicates that TRPC5 is a pore-forming subunit. Whilst studying the complex potentiatory and inhibitory effects of lanthanides on TRPC5, we found that neutralization of two amino acids (E595 and E598) in the P-loop close to S6 resulted not only in a loss of potentiation by La^{3+}, but also in a modification of channel block by La^{3+}, a doubling of the relative permeability to Ca^{2+} and an increase in the single channel conductance (Jung et al. 2003). These amino acid residues are probably located close to the extracellular mouth of the channel pore in the short stretch of the P-loop between the pore and S6. The results with this mutant provide some evidence that TRPC5 is a pore-forming subunit. However, more detailed studies are necessary to identify to the determinants of permeability in TRPC channels.

A dual role of TRPC4 and TRPC5 as both channel-forming subunits and as modulatory subunits of endogenous channels could go some way to explain the discrepancies in the literature. That TRPC isoforms from this family can influence the activity of other channels is evidenced by the increase in currents through inward-rectifier K^+ channels following expression of TRPC4 (Zhang et al. 2001) and the absence of cystic fibrosis transmembrane conductance regulator (CFTR)-mediated currents in aortic endothelial cells from TRPC4−/− mice (Wei et al. 2001). The mechanisms by which these effects occur are unknown. Modulation of an endogenous channel resulting in an increased store-operated conductance could explain the effects of TRPC4 and TRPC5 expression in some studies and the relatively small currents obtained after overexpression (Philipp et al. 1996, 1998; Warnat et al. 1999). An effect of this type would also account for the effects of TRPC4 antisense (Philipp et al. 2000), and the loss of a store-operated channel in TRPC4−/− mice (Freichel et al. 2001). However, the idea does not explain why other groups using similar protocols could not demonstrate a store-operated conductance after expression of TRPC4 and TRPC5.

It is often argued that differences in regulatory and functional properties observed for heterologously-expressed TRPCs results from heteromultimerization of the expressed subunits with different endogenous TRPC subunits or other, as yet unidentified partners in the various expression systems used or in the same “cell line” in different laboratories. We have observed similar properties for TRPC4 and TRPC5 in CHO-K1 and HEK293 cells (Schaefer et al. 2000), and similar properties have been observed for TRPC4 following expression in neuroendocrine cells (Obukhov and Nowycky 2002). As mentioned above, recent studies have provided evidence for the ability of TRPC isoforms to heteromultimerize and have characterized some of the channel properties of heteromultimers (Strübing et al. 2001; Goel et al. 2002; Hofmann et al. 2002; Strübing et al. 2003). TRPC4 and TRPC5 can heteromultimerize with each other or with TRPC1. More complex heteromeric combinations of TRPC3 or TRPC6 with TRPC1 and TRPC4 or TRPC5 are also possible in embryonic tissues and in HEK cells. Although the shape of the IV relation is changed in heteromeric combinations (Strübing et al. 2001, 2003; Obukhov and Nowycky 2005) and is significantly different when formed of three different subunits instead of two (Strübing et al. 2003), none of them had the inwardly-rectifying IV relation expected for a highly Ca^{2+} permeable channel. Furthermore, all have reversal potentials around 0 mV, indicative of nonselective cation channels, and the relative Ca^{2+} permeability of heteromeric TRPC1/5 is similar to that of homomeric TRPC5 (Strübing et al. 2001). The difference in IV shape cannot, however, be solely attributed to the absence of a negatively charged amino acid at the position where internal Mg^{2+} blocks TRPC5 (Obukhov and Nowycky 2005), and presumably other TRPCs, because introduction of a negatively charged amino acid at this position did not change the shape of the IV relation (Obukhov and Nowycky 2005). Thus, even though TRPC4 and TRPC5 can heteromultimerize with other TRPC isoforms, and presumably coassemble with endogenous TRPC channel isoforms in expression systems, none of the heteromultimers investigated displays the properties of a store-operated, Ca^{2+}-selective channel. It is of course possible that other endogenous proteins could act as auxiliary subunits and modify the properties of the heterologously-expressed channel subunit. This sort of modification, however, normally influences channel regulation, not permeability properties.

It has recently been argued that TRPC channels show qualitative differences in properties depending on the expression level because the number of interaction partners in the cell with which complexes can be formed may be limited (see Putney 2004 for review). At low levels of expression enough endogenous partners may be available and the TRPC subunits could interact with one or more of them to form store-operated channels. In contrast, at high levels of expression, the probability of TRPC subunits finding the interaction partners may be low and channels may result that are receptor-operated and store-independent. In patch-clamp measurements of currents following transient transfection of TRPC4 and TRPC5 we have

observed a large variations in the levels of activation which reflect, to some extent, differences in the amount of channel expressed. These range from currents of a few picoamperes, where only openings of single channels were seen in whole cell recordings, to currents of several nanoamperes. Small currents sometimes showed slight changes in the shape of the IV relation, probably resulting from contamination by background currents or interaction with endogenous TRPC subunits, but we have no evidence for differences in the regulatory properties or selectivity.

Can the formation of signaling complexes influence channel properties? In *Drosophila* photoreceptors, components involved in phototransduction, including the cation channels TRP and TRPL, are brought together in a multimolecular signaling complex (signalplex), which is important for proper subcellular localization, for maintenance of component stoichiometry and for efficient signaling (Montell 2003). The signalplex is held together by the scaffolding protein InaD which has five PDZ protein–protein interaction motifs. Unlike other TRPCs, TRPC4 and TRPC5 end at their C terminus with the PDZ binding motif VTTRL. TRPC4 has been shown to interact through this motif with the scaffolding protein NHERF (regulatory factor of the Na^+/H^+ exchanger), also called EPB50 (ezrin-binding phosphoprotein-50; Tang et al. 2000; Mery et al. 2002). NHERF can also interact with PLC isozymes via a second PDZ domain and, through an additional ezrin/radixin/moesin/merlin domain, form a signaling complex linked to the actin cytoskeleton (Tang et al. 2000). In astrocytes, TRPC4 also interacted via the PDZ domain with the scaffolding protein zona occludens 1 (ZO-1) and localized to discrete sites in the cell membrane close to sites of cell–cell contact (Song et al. 2005). Deletion of the TRL or VTTRL motif in TRPC4 or TRPC5 reduces plasma membrane expression and changes their expression pattern in HEK293 cells and in astrocytes (Mery et al. 2002; Obukhov and Nowycky 2004; Song et al. 2005). It has been suggested that targeting to different subcellular locations or differences in channel complex composition dependent on PDZ interactions could influence functional properties of the channel e.g., store-dependence or -independence (Song et al. 2005). Although the channel activation mechanism was not studied, currents through TRPC5 lacking the VTTRL motif had similar amplitudes and biophysical properties to those seen for the wild-type channel in HEK293 cells (Obukhov and Nowycky 2004). The influence of EPB-50-binding was to slow current activation kinetics. A C-terminally truncated hTRPC4α, which was identical to hTRPCβ but lacked the last 108 amino acids including the PDZ domain, showed no noticeable differences in channel properties to hTRPC4β (Schaefer et al. 2002). Thus, at least in HEK293 cells, interactions with the PDZ domain have relatively minor effects on channel properties.

Endogenous channels formed by or involving TRPC4 or TRPC5

Our knowledge of the properties and physiological roles of endogenous channels formed by TRPC4, TRPC5 or heteromeric combinations including these proteins is still very limited. Evidence from TRPC4−/− mice and from experiments using TRPC4 antisense oligonucleotides suggests an essential involvement of TRPC4 in store-operated currents in endothelial cells (Freichel et al. 2001), adrenal cortical cells (Philipp et al. 2000) and mesangial cells (Wang et al. 2004). In endothelial cells from TRPC4−/− mice, a very small, highly Ca^{2+} permeable, inwardly rectifying store-operated current was lost, and Ca^{2+} entry reduced, leading to impaired agonist-induced vasorelaxation (Freichel et al. 2001) and impaired agonist-induced increases in microvascular permeability (Tiruppathi et al. 2002). TRPC4 antisense treatment reduced a similar current in adrenal cortical cells (Philipp et al. 2000). Freichel et al. (2001) were careful to point out that TRPC4, whilst an essential component of these responses, may not form the pore of the store-operated channel. It is interesting that the TRPC4-dependent endothelial cell current is inhibited by La^{3+} (Freichel et al. 2001). Transcripts for TRPC1 and TR PC4α were found in mouse mesangial cells, smooth muscle-like cells involved in the regulation of the filtration surface area in the glomerulus, and TRPC4 shown using antisense oligonucleotides to be involved in store-operated Ca^{2+} entry (Wang et al. 2004).

Receptor-operated nonselective cation currents with strong similarities to those that we observed with heterologously expressed TRPC4 and TRPC5 are seen in response to activation of muscarinic acetylcholine receptors in intestinal smooth muscle. These native cation channels are activated in a G-protein- and Ca^{2+}-dependent manner, have a similar, doubly rectifying IV relation and a single channel conductance in outside out patches (25 pS) close to that of TRPC4 (30 pS; Inoue et al. 1987; Inoue and Isenberg 1990a,b; Zholos and Bolton 1996). A distinctive feature of these intestinal channels is their potentiation by La^{3+} (Inoue et al. 1998), a property shared with TRPC4 and TRPC5 in some studies (Table 1). These channels are probably formed by TRPC4 because transcripts, particularly those for TRPC4β, but not for TRPC5 or TRPC1 were identified in this tissue (Walker et al. 2001). TRPC5 has also been suggested to play a role in acetylcholine-induced nonselective cation currents in the stomach. Transcripts for all TRPCs, except TRPC2, were found in the stomach, but the acetylcholine-activated currents share most similarity with TRPC5, when compared to all TRPCs expressed heterologously in the same study (Lee et al. 2003). Interestingly, both the native channel and TR PC5 were activated by DAG in this study. In spite of showing a doubly rectifying IV relationship, neither the endogenous channel nor TRPC5 were potentiated, but rather inhibited by La^{3+}.

TRPC5 transcripts or proteins have also been identified in smooth muscle cells from some vessels (see Beech et al. 2004 for a review). Furthermore, an antibody against the outer pore region of TRPC5 inhibited store-operated currents in arterioles. Inhibitory effects of an anti-TRPC1 antibody in the same tissue together with a single channel conductance like that of receptor-operated, store-independent heteromultimers of TRPC1 and TRPC5 (Strübing et al. 2001) led the authors to suggest that the store-operated nonselective cation current in these arterioles is mediated by heteromultimers of TRPC1 and TRPC5 (Beech et al. 2004).

In the brain, high levels of TRPC4 and TRPC5 are observed in the hippocampus, where their expression overlaps with that of TRPC1 (Mori et al. 1998; Otsuka et al. 1998; Philipp et al. 1998; Strübing et al. 2001). A role for TRPC4 in the brain, identified using TRPC4−/− mice, is in the serotonin-mediated regulation of GABA release from thalamic interneuronal dendrites (Munsch et al. 2003). In wild-type mice, spontaneous inhibitory postsynaptic current (sIPSC) activity was increased by serotonin in a PLC- and Ca^{2+} entry-dependent manner, but was independent of Ca^{2+} release and voltage-gated Ca^{2+} channels. sIPSC activity was increased by micromolar La^{3+} and Gd^{3+}, a feature consistent with a channel composed of TRPC4 (see Table 1). The effect of serotonin on sIPSC activity was drastically reduced in TRPC4−/− mice. The exact mechanism of the effect and the properties of the channels involved are still unclear. One role of TRPC5 in neurones may be in the regulation of growth cone morphology and motility. Greka et al. (2003) demonstrated the expression of TRPC5 and a nonselective cation channel with a single channel conductance of 50 pS, which was not inhibited by La^{3+}, in growth cones. The single channel conductance is consistent with that of TRPC5 alone and not heteromeric combinations of TRPC1 and TRPC5 (Table 1). Supporting this, immunocytochemical evidence showed that TRPC1 is expressed in the cell body and neuronal processes, but not in the growth cone, and, further, shows that subcellular differences in the subunit composition of TRPC cation channels are possible. In these hippocampal neurones, TRPC5 interacts with the growth cone-enriched protein stathmin 2 and is transported in vesicles to growth cones, where, as shown using a dominant negative isoform, it influences morphology and is involved in the inhibition of neurite extension (Greka et al. 2003). In addition to these studies, there are a number of reports of nonselective cation channels involved in current responses to G-protein-coupled receptor activation in hippocampal (Crepel et al. 1994; Pozzo Miller et al. 1995; Congar et al. 1997; Gee et al. 2003) or cortical neurones (Alzheimer 1994; Haj-Dahmane and Andrade 1996, 1999). A comparison of the properties of these currents in neurones with heterologously expressed TRPCs is complicated by the presence of large currents contributed by other ionic channels. However, the outwardly rectifying shape of the IV relationship with a region of negative slope at negative membrane potentials in some studies and some of the regulatory properties resemble those of heteromeric combinations of TRPC1 with TRPC4 or TRPC5 (Strübing et al. 2001; Obukhov and Nowycky 2005). Other properties, like the inhibition of responses by La^{3+} (Gee et al. 2003), do not support to an involvement of these isoforms. In general, nonselective cation currents coupled to metabotropic receptors activate more slowly than currents mediated by ionotropic receptors and often only in response to repetitive presynaptic activity, however rapid EPSCs mediated by G-protein-coupled receptors have been reported (Pozzo Miller et al. 1995). The currents will contribute to slow excitatory postsynaptic potentials (EPSCs) and to increased neuronal excitability. In this context, TRPC1, which may be unable to form functional channels as homomultimers (Hofmann et al. 2002), has been shown to be involved in glutamatergic slow EPSCs in response to parallel fibre stimulation in cerebellar Purkinje neurones (Kim et al. 2003). Current responses were inhibited by transfection of a dominant negative TRPC1 and by infusion of TRPC1 antibodies. It remains to be seen whether other TRPC isoforms are involved with TRPC1 in the formation of functional channels in these neurones in which there is little evidence for the expression of TRPC4 or TRPC5. Interestingly, agonist-induced responses were not inhibited by micromolar La^{3+}.

It is clear from this brief presentation that, despite the studies performed, we still know relatively little about TR PC4 and TRPC5, and that major questions still need to be answered. Two of particular importance are: do TRPC4 and TRPC5 form receptor-operated, store-independent nonselective channels in native tissues like intestinal smooth muscle or central neurones, and what is the activator of TRPC4 and TRPC5 in the PLC pathway?

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