Advances in Experimental Medicine and Biology 1051
Protein Reviews

M. Zouhair Atassi *Editor*

Protein Reviews

Volume 19

Springer

Advances in Experimental Medicine and Biology

Protein Reviews

Volume 1051

Subseries Editor
M. Zouhair Atassi

More information about this series at http://www.springer.com/series/14330

M. Zouhair Atassi
Editor

Protein Reviews

Volume 19

Springer

Editor
M. Zouhair Atassi
Biochem and Mol Biol
Baylor College of Medicine
Houston, TX, USA

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISSN 2520-1891 ISSN 2520-1905 (electronic)
Protein Reviews
ISBN 978-981-10-7610-7 ISBN 978-981-10-7611-4 (eBook)
https://doi.org/10.1007/978-981-10-7611-4

Library of Congress Control Number: 2017938742

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Protein Reviews is a book series, which has been published by Springer since 2005. It has published 18 printed volumes to date (to see previous volumes, please go to http://www.springer.com/series/6876). To speed up the publication process and enhance accessibility, all articles will appear online before they are published in a printed book. The book series appears as a subseries of Advances in Experimental Medicine and Biology (http://www.springer.com/series/14330). They are published in volumes, each of which will focus on a theme, or in volumes that contain reviews of an assortment of topics, in order to stay up-to-date and to publish timely reviews in an efficient manner.

The aim of the *Protein Reviews* is to serve as a publication vehicle for reviews that focus on crucial contemporary and vital aspects of protein structure, function, evolution, and genetics. Publications will be selected based on their importance to the understanding of biological systems, their relevance to the unraveling of issues associated with health and disease, or their impact on scientific or technological advances and developments. Proteins linked to diseases or to the appearance and progress of diseases will obviously provide essential topics that may be covered in this series. Moreover, proteins that are, or can be, used as potential biomarkers or as candidates for treatment and/or for the design of distinctive, new therapeutics will receive high attention in this book series.

The issues may include biochemistry, biophysics, immunology, structural and molecular biology, genetics, molecular and cellular mechanisms of action, clinical studies, and new pioneering therapies. A given volume may be focused on a particular theme or may contain a selected assortment of different current topics.

The authors of the articles are selected from leading basic or medical scientists in academic or industrial organizations. The invited authors are nominated by the editorial board or by experts in the scientific community. However, interested individuals may suggest a topic for review and/or may propose a person to review a current important topic. Colleagues interested in writing a review or in guest-editing a special thematic issue are encouraged to submit their proposals and list of authors of the suggested chapter/topics to the editor before submitting a manuscript.

It should be emphasized that *Protein Reviews* will publish within 6 weeks accepted review articles online before they appear in print. And there will be no page or color charges and no page or color image limitations.

Volume 19 focuses on purinergic receptors, also termed purinoceptors. They are plasma membrane proteins that are present in nearly all mammalian tissues. They participate in a number of cell functions that include proliferation and migration of neural stem cells, vascular reactivity, apoptosis, and cytokine secretion and have been associated with learning and memory, feeding conduct, movement, and sleep. They facilitate relaxation of smooth muscle of the gut in response to adenosine (P1 receptors) or ATP (P2 receptors). The latter has five subclasses: P2X, P2Y, P2Z, P2U, and P2T.

The chapters in this volume are authored by experts in the field. They deal with aspects of structure and biological activity of selected receptor proteins. The first chapter is an introduction to this special issue on purinergic receptors. Chapter 2 deals with ATP-gated P2X3 receptors as specialized sensors of the extracellular environment. Chapter 3 describes the P2X7 receptor, and Chap. 4 deals with the activation of P2X receptor. Chapter 5 deals with properties and functional activities of $P2Y_1$ receptors. This is followed by Chap. 6 that reviews $P2Y_2$ receptor functions in cancer: with a special perspective in the context of colorectal cancer. Chapter 7 discusses properties, distribution, and functions of $P2Y_{11}$ receptors. It is followed by Chap. 8 which discusses the structure, pharmacology, and roles in physiology of the $P2Y_{12}$ receptor. Chapter 9 presents an update on signaling and function of $P2Y_{13}$ receptor. It is followed by Chap. 10 on the pharmacological properties and biological functions of the GPR17 receptor and discusses it as a potential target for neuro-regenerative medicine. Chapter 11 handles the biochemical and pharmacological role of A_1 adenosine receptors and their modulation as novel therapeutic strategy, and, finally, Chap. 12 treats the molecular mechanism of plant recognition of extracellular ATP. The volume is intended for research scientists, clinicians, physicians, and graduate students in the fields of biochemistry, cell biology, molecular biology, immunology, and genetics.

I hope that this volume of *Protein Reviews* will continue to serve the scientific community as a valuable vehicle for dissemination of vital and essential contemporary discoveries on protein molecules and their immensely versatile biological activities.

M. Zouhair Atassi
Editor in Chief

Contents

Adv Exp Med Biol - Protein Reviews (2017) 19:1–6
DOI 10.1007/5584_2017_12

Published online: 22 February 2017

Introduction to the Special Issue on Purinergic Receptors

Geoffrey Burnstock

Abstract

In this Introduction to the series of papers that follow about purinergic receptors, there is a brief history of the discovery of purinergic signalling, the identity of purinoceptors and the current recognition of P1, P2X and P2Y subtypes. An account of key functions mediated by purinoceptors follows, including examples of both short-term and long-term (trophic) signalling and a table showing the selective agonists and antagonists for the purinoceptor subtypes. References to evolution and roles of purinoceptors in pathological conditions are also presented.

Keywords

Neurotransmission • Secretion • Development • Regeneration • Pathophysiology

Purinergic signalling was proposed in 1972 (Burnstock 1972) and purinergic receptors defined in 1976 (Burnstock 1976). In 1978, it was recognised that there were two families of purinoceptors, named P1 (adenosine) and P2 (nucleotide) receptors (Burnstock 1978). An edited book about purinergic receptors was published in 1981 (Burnstock 1981). Based on pharmacology, P2 receptors were later divided into P2X and P2Y subtypes (Burnstock and Kennedy 1985). P2Z (later named P2X7) receptors (Gordon 1986), P2T (later named $P2Y_{12}$) receptors (Gordon 1986) and P2U (later named $P2Y_2$ and/or $P2Y_4$) receptors (O'Connor et al. 1991) followed. Important advances were made in the early nineties, when transduction mechanisms were identified (Dubyak and El Moatassim 1993) and P1, P2Y and P2X receptors cloned and characterised. Four subtypes of P1 receptors, A_1, A_{2A}, A_{2B} and A_3 receptors (Maenhaut et al. 1990; Fredholm et al. 1994), initially $P2Y_1$ (Webb et al. 1993) and $P2Y_2$ (Lustig et al. 1993) receptors and a year later

G. Burnstock (✉)
University College London Medical School,
London, UK
e-mail: g.burnstock@ucl.ac.uk

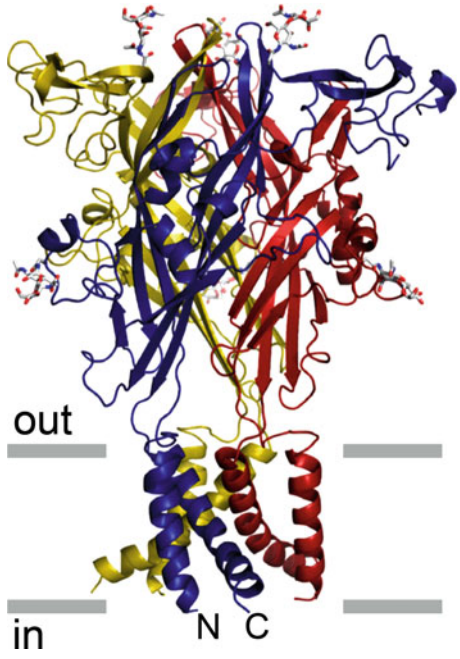

Fig. 1 The architecture of P2X receptors. Stereoview of the homotrimeric ΔzfP2X4 structure viewed parallel to the membrane. Each subunit is depicted in a different colour. N-acetylglucosamine (NAG) and glycosylated asparagine residues are shown in *stick* representation. The *grey bars* suggest the boundaries of the outer (*out*) and inner (*in*) leaflets of the membrane bilayer (Reproduced from Kawate et al. 2009, with permission from Nature Publishing Group)

P2X1 (Valera et al. 1994) and P2X2 (Brake et al. 1994) receptors were identified. These findings were rationalised by Abbracchio and Burnstock (Abbracchio and Burnstock 1994) by defining seven P2X ion channel receptor subtypes and eight P2Y G protein-coupled receptors. A popular comprehensive review about purinoceptors was published by Ralevic and Burnstock (1998) (4094 citations). An important study showed that three P2X receptor subtypes were combined to form trimer ion channels (Nicke et al. 1998) either as homomultimers and heteromultimers (Burnstock 2007a). Reviews about the expression and functions of purinergic receptors for many different cell types (Burnstock and Knight 2004) and the molecular pharmacology of P2X receptors (North 2002) are available and the elegant identification of the crystal structure of the P2X4 receptors (Fig. 1) (Kawate et al. 2009; Hattori and Gouaux 2012). Reviews about P1 (Fredholm et al. 2011; Chen et al. 2014), P2Y (Abbracchio et al. 2006; Erlinge 2011; Jacobson et al. 2015) and P2X (Müller 2015; Habermacher et al. 2016) receptors are available. A valuable book edited by Jacobson and Linden, entitled 'Pharmacology of Purine and Pyrimidine Receptors', was published in 2011 (Jacobson and Linden 2011).

Purinoceptors modulate both short-term signalling in neurotransmission, neuromodulation and secretion and long-term (trophic) signalling in cell proliferation, differentiation and death in development and regeneration (see Burnstock 2016). Selective agonists and antagonists to purinoceptor subtypes currently available are summarised in Table 1. The evolution of purinoceptors (Burnstock 1996; Fountain and Burnstock 2009; Burnstock and Verkhratsky 2012) and the plasticity of expression and roles of purinoceptors in pathological conditions (Burnstock 2006, 2007b, 2013) have been reviewed. The intracellular expression of purinoceptors is being explored (Burnstock 2015) and the expression of purinoceptors during development and ageing has also been reviewed (Burnstock and Dale 2015). Detailed analysis of various aspects of purinoceptors will be presented in the following articles.

Table 1 Characteristics of purine-regulated receptors

Receptor		Main distribution	Agonists	Antagonists	Transduction mechanisms
P1 (Ado)	**A_1**	Brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA>R-PIA=S-ENBA; CVT-510; GR79236, 2′-MeCCPA, SDZ WAG 994, INO-8875, MRS 5474	DPCPX, N-0840, MRS1754, WRC-0571, PSB36, SLV320, CGS 16943, PQ-69	G_i/G_o ↓cAMP
	A_{2A}	Brain, heart, lungs, spleen	HENECA>CGS 21680=CVT-3146; ATL-146e; Regadenoson, apadenoson, UK-432.097	KF17837, SCH58261, ZM241385, KW 6002	G_S ↑cAMP
	A_{2B}	Large intestine, bladder	Bay60-6583, NECA	PSB603, MRE-2029-F20, MRS1754, PSB0788 MRS1706, PSB1115, Alloxazine, GS-6201	G_S ↑cAMP
	A_3	Lung, liver, brain, testis, heart	IB-MECA> MRS5698>MRS5168> 2-Cl-IB-MECA; DBXRM; VT160; HEMADO, MRS5980	MRS1220, L-268605, MRS1191, MRS1523(rat), VUF8504, VUF5574, MRS1334(human), PSB10	G_i/G_o, G_q/G_{11}, ↓cAMP, PLC-β activation
P2X	**P2X1**	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	BzATP > ATP = 2-MeSATP≥α,β-meATP=L-β,γ-meATP (rapid desensitization); PAPET-ATP	NF864>NF449>IP_5I≥TNP-ATP> RO 0437626> NF279, NF023, RO1, MRS2159	Intrinsic cation channel (Ca^{2+} and Na^+)
	P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia, pancreas	ATP≥ATPγS≥2-MeSATP>>α,β-meATP (pH + zinc sensitive); β,γ-CF_2ATP	PSB-1011>RB2, isoPPADS>PPADS>Suramin, NF770, NF778, Aminoglycoside	Intrinsic ion channel (particularly Ca^{2+})
	P2X3	Sensory neurons, NTS, some sympathetic neurons	2-MeSATP≥ATP≥ Ap_4A ≥ α,β-meATP (rapid desensitization); PAPET-ATP; BzATP	TNP-ATP, AF353, A317491, RO3, isoPPADS > NF110 > PPADS, Ip_5I, phenol red, RN-1838, Spinorphin	Intrinsic cation channel
	P2X4	CNS, testis, colon, endothelial cells, microglia	ATP>>α,β-meATP>> CTP, 2-MeSATP Ivermectin potentiation	5-BDBD>>TNP-ATP, PPADS>BBG, Paroxetine, phenolphthalein, CO donor (CORM 2), 5MPTP	Intrinsic ion channel (especially Ca^{2+})
	P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord, heart, adrenal medulla	ATP=2-MeSATP=ATPγS>>α,β-meATP>AP_4A	BBG>PPADS, Suramin	Intrinsic ion channel
	P2X6	CNS, motor neurons in spinal cord	– (only functions as a heteromultimer)	–	Intrinsic ion channel
	P2X7	Immune cells including dendritic cells (mast cells, macrophages), pancreas, skin, microglia	BzATP>ATP ≥2-MeSATP >>α,β-meATP (clemastine potentiates)	KN62, BBG, KN04, MRS2427, O-ATP, RN-6189, Perazine, AZ10606120, A740003, A-438079, A-804598, GSK-1370319, Comp 31 (GSK), AZD-9056, CE-224,535, JNJ-47965567, JNJ-42253432 (penetrates BBB),	Intrinsic cation channel and a large pore with prolonged activation

(continued)

Table 1 (continued)

Receptor		Main distribution	Agonists	Antagonists	Transduction mechanisms
				decavanadate, AZ11657312, A-839977, GSK1482160	
P2Y	**$P2Y_1$**	Epithelial and endothelial cells, platelets, immune cells, osteoclasts, brain	MRS2365>2-MeSADP=Ap_5(γB)>> ADPβS>ATP >2-MeSATP=ADP, 2-Cl-ADP	MRS2500 > MRS2279 > MRS2179, PIT, A3P5P, BPTU	G_q/G_{11}; PLC-β activation
	$P2Y_2$	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	2-thio-UTP>UTP, MRS2698≥ATP, INS 365> INS 37217, UTPγS>Ap_4A>MRS 2768, Up_4-phenyl ester	AR-C126313>Suramin> RB2, PSB-716, MRS2576, PSB-0402, AR-C118925, AR-C118925	G_q/G_{11} and possibly G_i/G_o; PLC-β activation
	$P2Y_4$	Endothelial cells, placenta, spleen, thymus	2′-azido-dUTP> UTPγS, UTP≥ATP≥ Ap_4A Up_4U MRS4062	ATP (human)>Reactive Blue 2>Suramin, MRS2577, PPADS	G_q/G_{11} and possibly G_i; PLC-β activation
	$P2Y_6$	Airway and intestinal epithelial cells, placenta, T cells, thymus, microglia (activated)	MRS2693> UDPβS , PSB0474> INS48823, Up_3U, 3-phenacyl-UDP>>UDP>UTP>>ATP, α,β-meUDP, MRS2957, MRS4129, 5-OMe-UDP αB	MRS2578>Reactive Blue 2, PPADS, MRS2567, MRS2575 (human)	G_q/G_{11}; PLC-β activation
	$P2Y_{11}$	Spleen, intestine, granulocytes	ATPγS>AR-C67085MX>BzATP≥ATP, NF546, NAD^+, $NAADP^+$, S*p*-2-propylthio-ATP-α-B	NF157>Suramin>RB2, 5′-AMPS, NF340, AMP-α-5,	G_q/G_{11} and G_S; PLC-β activation
	$P2Y_{12}$	Platelets, glial cells	2-MeSADP≥ADP>ATP, ADP-β-S	AR-C69931MX> AZD6140 (Ticagrelor), INS50589>RB2>2-MeSAMP AR-C66096, CT50547,PSB-0413, Carba-nucleosides, MRS2395, AR-C67085, [^{3}H] PSB-0413; clopidogrel, AZD1283; ACT-246475	$G\alpha_i$; inhibition of adenylate cyclase
	$P2Y_{13}$	Spleen, brain, lymph nodes, bone marrow, erythrocytes	ADP=2-MeSADP>2-MeSATP, ATP	AR-C69931MX>AR-C67085>MRS2211, 2-MeSAMP	G_i/G_o
	$P2Y_{14}$	Placenta, adipose tissue, stomach, intestine, discrete brain regions, mast cells	MRS2690>UDP>UDP glucose≥UDP-galactose, UDP-glucosamine, MRS2905, MRS4183	PPTN, MRS4174	G_q/G_{11}
	GPR17	Oligodendrocytes	Uracil nucleotides/cysteinyl-leukotrienes, MDL29,951	PZB01415033	G_i, adenylate cyclase inhibition

Reproduced from Burnstock (2003), with permission

Abbreviations: Ap_5(γB), Adenosine pentaphosphate γ-boranophosphate; BBG, Brilliant blue green; 5-BDBD, 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one BzATP, 2'-&3'-*O*-(4-benzoyl-benzoyl)-ATP; cAMP, cyclic AMP; CCPA, chlorocyclopentyl adenosine; CTP, cytosine triphosphate; IP_3, inosine triphosphate; Ip_5I, di-inosine pentaphosphate; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5'-*N*-ethylcarboxamido adenosine; PLC, phospholipase C; RB2, reactive blue 2; P2X receptor subtype agonist potencies based on rat preparations, while P1 and P2Y receptor subtype agonist potencies are based on human preparations

Compliance with Ethical Standards

Conflicts of Interest The author declares that he has no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

References

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P_{2X} and P_{2Y} purinoceptors? Pharmacol Ther 64(3):445–475

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology. Update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58(3):281–341

Brake AJ, Wagenbach MJ, Julius D (1994) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 371(4697):519–523

Burnstock G (1972) Purinergic nerves. Pharmacol Rev 24 (3):509–581

Burnstock G (1976) Do some nerve cells release more than one transmitter? Neuroscience 1:239–248

Burnstock G (1978) A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L (eds) Cell membrane receptors for drugs and hormones: a multidisciplinary approach. Raven Press, New York, pp 107–118

Burnstock G (1981) Purinergic receptors. Receptors and recognition, series B, vol 12. Chapman & Hall, London

Burnstock G (1996) Purinoceptors: ontogeny and phylogeny. Drug Dev Res 39(3–4):204–242

Burnstock G (2003) Introduction: ATP and its metabolites as potent extracellular agonists. In: Schwiebert EW (ed) Current topics in membranes, vol 54. Purinergic receptors and signalling. Academic, San Diego, pp 1–27

Burnstock G (2006) Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 58 (1):58–86

Burnstock G (2007a) Purine and pyrimidine receptors. Cell Mol Life Sci 64(12):1471–1483

Burnstock G (2007b) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87 (2):659–797

Burnstock G (2013) Purinergic signalling: pathophysiology and therapeutic potential. Keio J Med 62 (3):63–73

Burnstock G (2015) Intracellular expression of purinoceptors. Purinergic Signal 11(3):275–276

Burnstock G (2016) Short- and long-term (trophic) purinergic signalling. Philos Trans R Soc B: Biol Sci 371(1700):20150422

Burnstock G, Dale N (2015) Purinergic signalling in development and ageing. Purinergic Signal 11 (3):277–305

Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P_2-purinoceptor? Gen Pharmacol 16(5):433–440

Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 240:31–304

Burnstock G, Verkhratsky A (2012) Evolution of P2X receptors. WIREs Membr Transp Signal 1(2):188–200

Chen JF, Lee CF, Chern Y (2014) Adenosine receptor neurobiology: overview. Int Rev Neurobiol 119:1–49

Dubyak GR, El Moatassim C (1993) Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol 265(3 Pt 1):C577–C606

Erlinge D (2011) P2Y receptors in health and disease. Adv Pharmacol 61:417–439

Fountain SJ, Burnstock G (2009) An evolutionary history of P2X receptors. Purinergic Signal 5(3):269–272

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. Pharmacol Rev 46(2):143–156

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors – an update. Pharmacol Rev 63(1):1–34

Gordon JL (1986) Extracellular ATP: effects, sources and fate. Biochem J 233(2):309–319

Habermacher C, Dunning K, Chataigneau T, Grutter T (2016) Molecular structure and function of P2X receptors. Neuropharmacology 104:18–30

Hattori M, Gouaux E (2012) Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature 485(7397):207–212

Jacobson KA, Linden J (2011) Pharmacology of purine and pyrimidine receptors. Adv Pharmacol 61:1–558

Jacobson KA, Gao ZG, Paoletta S, Kiselev E, Chakraborty S, Jayasekara PS, Balasubramanian R, Tosh DK (2015) John Daly lecture: structure-guided drug design for adenosine and P2Y receptors. Comput Struct Biotechnol J 13:286–298

Kawate T, Michel JC, Birdsong WT, Gouaux E (2009) Crystal structure of the ATP-gated $P2X_4$ ion channel in the closed state. Nature 460(7255):592–598

Lustig KD, Shiau AK, Brake AJ, Julius D (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci U S A 90 (11):5113–5117

Maenhaut C, Van Sande J, Libert F, Abramowitz M, Parmentier M, Vanderhaegen JJ, Dumont JE, Vassart G, Schiffmann S (1990) RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. Biochem Biophys Res Commun 173 (3):1169–1178

Müller CE (2015) Medicinal chemistry of P2X receptors: allosteric modulators. Curr Med Chem 22(7):929–941

Nicke A, Baumert HG, Rettinger J, Eichele A, Lambrecht G, Mutschler E, Schmalzing G (1998) $P2X_1$ and $P2X_3$ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. EMBO J 17(11):3016–3028

North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82(4):1013–1067

O'Connor SE, Dainty IA, Leff P (1991) Further subclassification of ATP receptors based on agonist studies. Trends Pharmacol Sci 12(4):137–141

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50(3):413–492

Valera S, Hussy N, Evans RJ, Adani N, North RA, Surprenant A, Buell G (1994) A new class of ligand-gated ion channel defined by P_{2X} receptor for extracellular ATP. Nature 371(6497):516–519

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, Barnard EA (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett 324(2):219–225

Adv Exp Med Biol - Protein Reviews (2017) 19:7–16
DOI 10.1007/5584_2017_56

Published online: 22 June 2017

ATP-Gated P2X3 Receptors Are Specialised Sensors of the Extracellular Environment

Elsa Fabbretti

Abstract

P2X3 receptors are ion channels expressed by autonomic and sensory nerves and specialised in transducing extracellular ATP signals. Structural data, together with functional and biochemical studies, suggest that conformational changes of P2X3 receptors upon agonist binding influence downstream intracellular molecular mechanisms relevant for neuronal responses. Activity of P2X3 receptors is implicated in pain, itch, asthma, cardiovascular dysfunction and other pathologies. The study of these receptors has therefore a large potential in the field of drug development and interdisciplinary efforts could clarify molecular mechanisms controlling P2X3 receptor function in different physiological or pathological contexts.

Keywords

Carotid body • Cough • Itch • Pain • Purinergic • Urinary bladder

Abbreviations

CGRP	Calcitonin gene related peptide
FRET	fluorescence resonance energy transfer
NGF	Nerve growth factor
α,β-meATP	α,β-methylene ATP

E. Fabbretti (✉)
Department of Life Sciences, University of Trieste, via Giorgieri 5, 34127 Trieste, Italy
e-mail: e.fabbretti.bf@gmail.com

1 Introduction

Extracellular ATP is a signalling molecule sensed by P2Y and P2X purinergic receptors. While P2Y receptors are metabotropic, ATP-gated P2X receptors are trimeric cation-selective channels. ATP-gated P2X receptor family members have high degree of homology. Nevertheless, they are characterised by different length of intracellular N- and C-terminal domains and molecular effectors conferring to these channels distinct pharmacology and kinetic properties (North 2016). Although the occurrence of ionic current represents the most evident

outcome, a body of evidence suggests that there are other crucial aspects that might be considered as new components conferring individual specialization to different P2X receptors: (1) P2X are part of molecular complexes that control machineries involved in basic physiologic responses, like the release of soluble mediators; (2) the molecular effects downstream of agonist binding are cell-specific, often poorly reconstituted in recombinant expression systems; (3) sub-threshold agonist concentrations could be sufficient to initiate molecular responses producing a priming effect. The demonstration of specific P2X-related mechanisms is, however, intrinsically complicated by pharmacological limitations, the large variety of endogenous purinergic receptor expression in different cells, the choice of human/mouse tissues, primary cultures or recombinant expression systems. While certain models are useful for ion channel studies, others poorly represent what occurs in nature, or globally fail to produce results useful for translational medicine. For all these reasons, integration of interdisciplinary sciences might help to design the best strategies for further progress in the purinergic field.

2 P2X3 Receptors Are Sensors of Ambient ATP

P2X3 receptors are ion channels expressed by sensory or autonomic neurons: their function has been studied in primary neurons, immortalised cell lines, or oocytes (Sokolova et al. 2004; North and Jarvis 2013; Kowalski et al. 2014). Their main characteristic is very high sensitivity to ambient ATP ($EC50 = 0.5\,\mu M$), their prompt desensitisation after few milliseconds from channel opening and their delayed recovery (Khakh and North 2012). In addition, exposure to low nanomolar concentrations (<1 nM) of ATP or its synthetic non-hydrolysable agonist α,β-methylene ATP (α,β-meATP) does not evoke any functional current response, but it is sufficient to produce high affinity desensitization (Giniatullin and Nistri 2013; Grote et al. 2008; Pratt et al. 2005; Sokolova et al. 2006), a priming process due to prevalence of agonist-bound desensitized receptors endowed with strong agonist affinity (Sokolova et al. 2004). The existence of a variable population of desensitized P2X3 receptors is likely to impact cell/tissue responsiveness to larger ATP concentrations and it might exert important consequences for the initial phases of a pathological response. For these reasons, P2X3 receptors are considered sensors able to transduce ambient ATP into neuronal responses, either considering ATP as neurotransmitter, neuromodulator or sensory signalling molecule (Ford 2012). Nevertheless, the fast kinetic properties typical of neuronal P2X3 receptors are remarkably common to P2X1 receptors expressed in endothelial cells (Rettinger and Schmalzing 2003; Fryatt et al. 2016), suggesting functional similarities in different cell types.

It is now clear that P2X3 receptors are expressed by neurons that transduce different sensorial modalities, including touch or taste (Vandenbeuch et al. 2015) or sensors at the level of the carotid body (Pijacka et al. 2016). Evidence from transgenic animals has demonstrated that reduced pain-related behaviour and urinary bladder hyporeflexia are the major phenotypes associated to P2X3 receptor deficiency in mice (Cockayne et al. 2000). Accordingly, P2X3 receptors are attractive therapeutic targets (Burnstock 2006) for their modulatory role in different chronic sensitisation processes of peripheral neurons, relevant to neuropathic pain, itch, cough, urinary bladder defects, and hypertension. The recently developed P2X3 receptor antagonists AF-353 (Gever et al. 2010) was tested in clinical trials for its safety and efficacy in patients affected by chronic cough (Abdulqawi et al. 2015). The positive outcome of this study confirms that this compound, orally administered, is sufficient to block P2X3-mediated sensitisation of neuronal afferents innervating the lungs. Interestingly, all treated subjects reported also taste abnormalities (Abdulqawi et al. 2015), suggesting that taste is an important readout for testing P2X3

antagonism efficiency in human subjects. Block of P2X3 receptors likely interferes with the interplay among different soluble sensitizers of peripheral afferents, such as the Nerve growth factor (NGF), the Calcitonin gene related peptide (CGRP) and ATP itself (Giniatullin et al. 2008). CGRP, in particular, is released by sensory and autonomic afferents, has vascular and immune-modulator properties in the respiratory and skin tissue and is strongly associated to P2X3 receptor expression and function (Fabbretti et al. 2006; Simonetti et al. 2008; Vilotti et al. 2016). While NGF transiently increases P2X3 receptor function via phosphorylation mechanisms (D'Arco et al. 2007, 2009), CGRP induces new P2X3 gene expression (Simonetti et al. 2008) and trafficking of receptors to the surface membrane, thus, persistently potentiating the function of P2X3 receptors (Fabbretti et al. 2006). Evidences from CGRP null mice furthermore demonstrated the occurrence of CGRP-dependent and independent P2X3 gene expression, since exogenous CGRP is sufficient to increase the number of neurons expressing P2X3 receptors. Conversely, to contrast peripheral sensitisation, the activation of CB1 receptors (Oliveira-Fusaro et al. 2017) and the Brain natriuretic peptide (BNP) acting on natriuretic peptide receptor-A expressed by sensory neurons (Marchenkova et al. 2015) are endogenous mechanisms to limit P2X3 receptor function (Vilotti et al. 2015).

3 P2X3 Receptors Are Probably Exerting Different Roles in Peripheral or Central Neuronal Endings

The P2X receptor interactome is likely segregated according to cell-polarity concepts within active membrane nanodomains. Cell polarity promotes compartmentalisation of cell signalling and provides specific responses related to tissue requirements. For example, the apical, basal and lateral domains of endothelial cells are specialised to transduce cell-to-cell contacts and lateral inhibition via mechanisms involving gap junctions and MAGUK-family proteins. How the organisation of P2X receptor signalling is arranged in different tissues remains a matter for future study. In bipolar sensory neurons (where P2X3 receptors are expressed), key protein complexes are segregated to distal or central endings, internode spaces, or close to the synaptic and peri-synaptic regions (Bentley and Banker 2016). These considerations suggest that in vivo distribution of P2X receptors might be discretely confined to specific functional domains possessing unique molecular and functional spatial requirements that perhaps are lost in vitro. As it occurs for other brain receptors (Horton and Ehlers 2003), it is possible that the functional significance of activation of distal or central P2X3 receptors is different in health or disease. This consideration has important pharmacological consequences and might be addressed by differential targeting of these receptors. For example, in sensory neurons, functional association of presynaptic P2X3 receptors with synaptic scaffold machinery in dorsal horn could generate wanted or unwanted effects accordingly to the disease type/stage. In addition, targeting of P2X3 receptors expressed at central endings is subjected to the restriction of the blood brain barrier.

One of the first attempts to distinguish between different clusters of P2X receptors, was obtained from the study of cholesterol-enriched membrane fractions, namely lipid rafts. These membrane domains are involved in lateral diffusion of receptors before internalisation and recycling. Since temperature and pH influence lipid distribution, membrane stiffness and protein mobility at surface membrane, interpretation of lipid rafts data is complex. Notably, P2X3 receptor recovery and recycling are temperature dependent (Pryazhnikov et al. 2011). Upon gating, P2X3 receptors re-localise toward lipid rafts before internalisation (Vacca et al. 2009; Chen et al. 2012; Gnanasekaran et al. 2011). Patch clamp recording after cholesterol depletion with methyl-β-cyclodextrin (me-βCD), has confirmed the role of lipid rafts in effective segregation of

different functional P2X receptor clusters. Me-βCD treatment depresses P2X3 receptor function, suggesting that receptors clustered in lipid rafts are more readily active than receptors sparse in non-raft domains (Gnanasekaran et al. 2011). Interestingly, this effect is common to P2X1 receptors, but opposite for P2X4 receptors (Vial and Evans 2005; Allsopp et al. 2010), suggesting the occurrence of different cholesterol content and membrane stiffness of endothelial cells, neurons or microglia where these receptors are expressed.

Mobility of P2X receptors in different membrane domains could correlate with their activation state and their internalisation, suggesting different trafficking routes of P2X receptors. Time course of membrane biotinylation experiments performed following application of α,β-meATP to recombinant P2X3 receptors expressed in HEK cells (Vacca et al. 2009), or to naive receptors (Chen et al. 2012) shows rapid P2X3 receptor endocytosis via caveolin-enriched raft domains (Chen et al. 2014). After internalisation, subunit composition and assembly in heteromeric P2X receptors are regulated in a tissue- and activity-dependent manner to support development, cell maturation and plasticity. In particular, exposure to NGF controls the conditions for heteromeric assembly of P2X2/3 receptors (D'Arco et al. 2007). These data suggest the existence of distinct pools of active/primed and inactive/desensitised receptors in different membrane domains. The lateral mobility of P2X3 receptors cannot, however, occur without controlled interaction with scaffold and cytoskeleton elements operating via trafficking molecular motors.

4 Functional Impact of P2X3 Receptor Phosphorylation

Phosphorylation is the result of rapid signal transduction mechanisms upon stimuli having an impact on cell activity or P2X receptor function. Phosphorylation implies kinase activation and protein-protein interaction with transfer of a phosphate group. The kinase-substrate binding domain is highly specific. Unspecific binding is prevented by structural constrains, dynamic conformational changes, adaptor elements, and segregation of signalling molecules in specific cellular domains. Negative charges conferred by phosphorylation induce further conformational changes, and exposure of binding sites for new interactors. Pharmacological manipulation of cell signalling was used to study the involvement of kinases and phosphatases in P2X3 receptor function, producing either direct or indirect effects. Since cell signalling is substantially different in proliferating cells (i.e. stable cell lines) and in resting differentiated cells (i.e. neurons), it is not surprising that certain mechanisms are not always correctly expressed in recombinant systems. This is the case of PKC because in primary neurons P2X3 receptor phosphorylation is associated to NGF-mediated PKC signalling (D'Arco et al. 2007), Ecto-PKC kinases (Wirkner et al. 2005), or Epac-protein kinase C alpha signalling controlling P2X3-mediated hyperalgesia (Gu et al. 2016).

5 Protein-Protein Interactions

The most advanced imaging techniques, like optogenetics and fluorescence resonance energy transfer (FRET) microscopy, are useful to identify functional protein-protein interactions, despite certain technical limitations related to the expression of recombinant proteins in primary cells and structural constrains restricting the use of fluorescent tags only to the P2X C-terminal domains (Khakh et al. 2001). FRET studies confirmed conformational spread that mediate cross-inhibition between P2X and other 'cys-loop' receptors, such as nicotinic receptors. In addition, whole-cell patch-clamp recordings and intracellular Ca^{+2} imaging of primary sensory neurons, demonstrated negative functional cross talk between GABA(A) and P2X receptors (Toulmé et al. 2007; Sokolova et al. 2001),

suggesting that receptor transactivation might concur to the modulation of pre-synaptic release mechanism at spinal cord level. Interestingly, this phenomenon depends on a discrete intracellular QST(386–388) motif of P2X3 subunits (Toulmé et al. 2007). The possibility that P2X3 receptors have a modulatory role at presynaptic level in spinal cord is very attractive. More recent data from co-immunoprecipitation and western blot analysis of P2X3 receptors have demonstrated the dynamic agonist-dependent interaction of P2X3 receptors with the Calcium/Calmodulin-dependent serine protein kinase (CASK) (Gnanasekaran et al. 2013; Bele and Fabbretti 2016), a magnesium-independent scaffold protein belonging to the MAGUK family, involved in synaptic modulation. CASK/P2X3 binding is reinforced by NGF signalling, intracellular calcium and active CaMKII. After agonist application, CASK/P2X3 binding is lost and P2X3 receptors interact with Panx1, concomitantly with ATP release (Bele and Fabbretti 2016). The detergent n-octyl β-D-glucopyranoside (ODG) was used to extract specific clusters of P2X3 receptors associated to different signalling complexes. The need for different detergent requirements to purify P2X3/CASK or P2X3/Panx1 complexes suggests distinct membrane localisation of P2X3 receptors when interacting with CASK or with Panx1 (Bele and Fabbretti 2016). These data support the hypothesis that P2X3 receptor alternative structures might influence active receptor re-localisation to new membrane compartments and binding to different partners following receptor opening. Data from X-ray crystallography of P2X3 receptors confirm that conformational changes upon receptor opening are transferred to the P2X3 intracellular C-terminal regions exposing structural gating requirements (Mansoor et al. 2016), and therefore possibly involving transactivation of membrane receptors, reaction with lipids or other intracellular signalling elements. In this view, correct stoichiometry of molecular partners governs proper time of cell signalling downstream receptor activation. The expression of different effectors is strongly influenced by pathological conditions and extracellular signals, that control new gene expression, splicing variants, and new activation/repression of mechanisms possibly involved in P2X3 receptor partnerships.

6 P2X3 Receptors and Release Mechanisms

Release of different soluble mediators is a common hallmark downstream P2X receptor function demonstrated in different cell types, such as macrophages (IL-1 release), microglia (BDNF release) and pancreatic beta cells (Insulin release) (Sanz and Di Virgilio 2000; Trang et al. 2009; Jacques-Silva et al. 2010). At meningeal level, innervation of trigeminal sensory fibers is sensitive to application of α,β-meATP and results in CGRP release (Yegutkin et al. 2016). A key question is whether the expression of P2X3 receptors in the proximity of permissive regions for the release of soluble mediators might influence it. In sensory neurons, P2X3 receptors are expressed in different peptidergic and non-peptidergic neuronal subpopulations (Saeed and Ribeiro-da-Silva 2012; Usoskin et al. 2015) and possibly in close vicinity to the release-associated machinery in the periphery or in spinal cord with different outcomes in relation to the intensity of peripheral stimuli (Giniatullin et al. 2008). Indeed, activation of P2X3 receptors contribute to neuronal firing (Hullugundi et al. 2013) and evoke ATP release (Bele and Fabbretti 2016), possibly influencing cell-to-cell crosstalk and feed-forward signalling within peripheral tissues, ganglia or neuronal networks in the spinal cord (Grosche et al. 1999). Notably, CASK is associated to lipid rafts at synapses (Fallon et al. 2002) and controls pre-synaptic release mechanisms in the central nervous system (Atasoy et al. 2007). Globally, CASK/P2X3 interaction and CASK properties support the

hypothesis that scaffold proteins and gap junctions are involved in proper clustering of P2X3 receptors in hot membrane regions devoted to cell-to-cell signalling (Grosche et al. 1999; Vial and Evans 2005; Bele and Fabbretti 2016). In analogy, clusters of P2X1 receptors expressed on arteries have been found close to sympathetic nerve varicosities, where they could modulate transmitter release (Hansen et al. 1999; Vial and Evans 2005).

In addition to scaffold proteins, also membrane lipids might govern P2X receptor clustering (Bernier et al. 2013). In particular, lipid rafts, cholesterol and sphingolipid-enriched membrane domains play an essential role in regulated exocytosis pathways, whereas cholesterol depletion inhibits exocytosis (Salaün et al. 2004). Furthermore, the major protein raft-targeting signal is palmitoylation and the CASK homologous Membrane palmitoylated protein-1 (MPP1) possesses cholesterol binding motifs (Listowski et al. 2015). Although a systematic analysis of P2X palmitoylation is missing, altered P2X7 palmitoylation results in intracellular pooling of these receptors and delayed trafficking toward the plasma membrane (Gonnord et al. 2009). It is possible that mechanisms controlled by CASK and related molecules, such as MPP1, might organise proper targeting of cluster of P2X3 receptors to control their appropriate functional responses.

7 Conclusions

Purinergic signalling is transduced in a cell- and receptor-specific manner depending on ambient ATP concentrations (North 2016). In summary, P2X3 receptors are neuron-specific ATP sensors, that behave as modulators of neuronal responses in an array of different physiological contexts. How their expression and function are modulated by molecular mechanisms activated during a chronic pathological process is not clear. Further studies aimed at identifying new subthreshold molecular readouts will be useful not only for screening new synthetic compounds but also to understand and prevent maladaptive P2X3-mediated processes occurring in disease conditions.

Technical Tips 1: Working with P2X3 Membrane Receptors

P2X receptor extraction from cell membranes requires incubation in a hypotonic cell lysis buffer with detergents for 30 min. In absence of specific inhibitors, proteins are rapidly degraded by lysosomal proteases released during cell lysis. In particular, the P2X3 receptor sequence is very sensitive to N-terminal protease cleavage, generating 30 kDa peptides, a process prevented by blockers of Asp-N proteases. Antibodies targeting the P2X3 C-terminal epitope (Alomone, cat.n. APR-16) recognise a P2X3-specific pattern in western blot (45–55 kDa), consisting in differentially glycosylated isoforms sensitive to PNGase and EndoH deglycosidase treatments. In some circumstances, an unspecific band at 60 kDa is also detected. Ultracentrifugation of crude cell extracts (100,000 rpm, 1 h) results in a pellet of membrane-associated proteins of different origin (endoplasmic reticulum, Golgi and cell surface membranes). Biotinylation of surface membrane proteins (EZ-Link Sulfo-NHS-LC-biotin, Pierce) and streptavidin pulldown are used to purify surface membrane P2X3 subunits, a procedure necessary to study the time course of the internalisation of P2X3 receptors after agonist application (Vacca et al. 2009; Chen et al. 2012). TritonX-100 allows to segregate triton-insoluble and -soluble membrane proteins on the basis of their membrane cholesterol content, namely raft or non-raft membrane domains. A more systematic analysis of receptors expressed in membrane fractions having different density may be obtained by ultracentrifugation of crude protein extracts on

(continued)

a sucrose cushion using a swinging-bucket rotor at 100,000 rpm for 4 h (Gnanasekaran et al. 2011). Notably, the lipid raft-associated flotilin and the non-raft transferrin receptors are used as western blot markers to identify different membrane compartments.

Technical Tips 2: Co-immunoprecipitation Studies

Co-immunoprecipitation studies are used to identify specific protein complexes. Antibodies used in this procedure might compete for the same antigen sequence with other binding proteins, displacing weak or indirect protein-protein interactions, resulting in low pulldown efficiency. Polyclonal antibodies targeting a long sequence, such as the P2X3 C-terminal domain H-60 (S.Cruz, cat-n. sc-25,694) efficiently pulldown P2X3 receptor subunits regardless structural constrains, hidden motifs, binding to lipids or binding to other proteins. Specificity is tested in western blot with more stringent anti-P2X3 antibodies (APR-16, Alomone) and by inverted pull down, recombinant expression of tagged proteins (Myc-tag proteins), or RNA silencing of protein of interests. Unless mutagenesis confirms a direct interaction, like in the case of mouse P2X3/Csk interaction (D'Arco et al. 2009; Sundukova et al. 2012), the correct interpretation of pull down experiments is the occurrence of a macromolecular complex, where also unknown structural adaptors (peptides or RNA) may have functional relevance, likely before or downstream of receptor opening. The intrinsic stickiness of certain disordered protein domains (like PDZ domains) when they are extracted from their naïve environment, strongly increases self-aggregation or unspecific binding of these molecules. For this reason, chemical crosslinking can fix protein complexes in their naive conditions possibly increasing pulldown specificity. Crosslinking reagents are available in membrane permeable or non-permeable forms, are reactive towards protein amino-groups, have different length of reactive spacer arms, and have reducible disulfide bonds for further analysis of individual proteins by western blot procedures.

Technical Tips 3: Phosphorylation Studies

To approach receptor phosphorylation studies, serum starvation (2 h) prior cell lysis can lower the background signalling and can unmask phosphorylation signals evoked by acute stimulation of specific pathways. Inhibitors of serine/threonine (sodium fluoride) and phospho-tyrosine phosphatases (ortovanadate) prevent artefactual de-phosphorylation during the lysis procedure. Notably, many protein-protein interactions are controlled by the occurrence of phosphorylated residues. Detection of protein phosphorylation by western blot assays requires careful procedure optimisation, short incubation periods, the use of fresh blocking buffer (containing serum albumin rather than milk), and low detergent strength. P2X receptor immunoprecipitation is, therefore, processed by western blotting with anti-phospho-serine/threonine or anti-phospho-tyrosine specific antibodies that nominally distinguish phosphorylated from non-phosphorylated aminoacids. They are very sensitive and often with a limited lifespan, that prompt their use within few weeks from purchase.

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abdulqawi R, Dockry R, Holt K, Layton G, McCarthy BG, Ford AP, Smith JA (2015) P2X3 receptor antagonist (AF-219) in refractory chronic cough: a randomised, double-blind, placebo-controlled phase 2 study. Lancet 385:1198–1205

Allsopp RC, Lalo U, Evans RJ (2010) Lipid raft association and cholesterol sensitivity of P2X1-4 receptors for ATP: chimeras and point mutants identify intracellular amino-terminal residues involved in lipid regulation of P2X1 receptors. J Biol Chem 285:32770–32777

Atasoy D, Schoch S, Ho A, Nadasy KA, Liu X, Zhang W, Mukherjee K, Nosyreva ED, Fernandez-Chacon R, Missler M, Kavalali ET, Südhof TC (2007) Deletion of CASK in mice is lethal and impairs synaptic function. Proc Natl Acad Sci USA 104:2525–2530

Bele T, Fabbretti E (2016) The scaffold protein calcium/calmodulin-dependent serine protein kinase controls ATP release in sensory ganglia upon P2X3 receptor activation and is part of an ATP keeper complex. J Neurochem 138:587–597

Bentley M, Banker G (2016) The cellular mechanisms that maintain neuronal polarity. Nat Rev Neurosci 17:611–622

Bernier L-P, Ase AR, Séguéla P (2013) Post-translational regulation of P2X receptor channels: modulation by phospholipids. Front Cell Neurosci 7:226

Burnstock G (2006) Purinergic P2 receptors as targets for novel analgesics. Pharmacol Ther 110:433–454

Chen X-Q, Wang B, Wu C, Pan J, Yuan B, Su YY, Jiang XY, Zhang X, Bao L (2012) Endosome-mediated retrograde axonal transport of P2X3 receptor signals in primary sensory neurons. Cell Res 22:677–696

Chen X-Q, Zhu J-X, Wang Y, Zhang X, Bao L (2014) CaMKIIα and caveolin-1 cooperate to drive ATP-induced membrane delivery of the P2X3 receptor. J Mol Cell Biol 6:140–153

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature 407:1011–1015

D'Arco M, Giniatullin R, Simonetti M, Fabbro A, Nair A, Nistri A, Fabbretti E (2007) Neutralization of nerve growth factor induces plasticity of ATP-sensitive P2X3 receptors of nociceptive trigeminal ganglion neurons. J Neurosci 27:8190–8201

D'Arco M, Giniatullin R, Leone V, Carloni P, Birsa N, Nair A, Nistri A, Fabbretti E (2009) The C-terminal Src inhibitory kinase (Csk)-mediated tyrosine phosphorylation is a novel molecular mechanism to limit P2X3 receptor function in mouse sensory neurons. J Biol Chem 284:21393–21401

Fabbretti E, D'Arco M, Fabbro A, Simonetti M, Nistri A, Giniatullin R (2006) Delayed upregulation of ATP P2X3 receptors of trigeminal sensory neurons by calcitonin gene-related peptide. J Neurosci 26:6163–6171

Fallon L, Moreau F, Croft BG, Labib N, Gu WJ, Fon EA (2002) Parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain. J Biol Chem 277:486–491

Ford AP (2012) In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization. Purinergic Signal 8:3–26

Fryatt AG, Dayl S, Cullis PM, Schmid R, Evans RJ (2016) Mechanistic insights from resolving ligand-dependent kinetics of conformational changes at ATP-gated P2X1R ion channels. Sci Rep 6:32918

Gever JR, Soto R, Henningsen RA, Martin RS, Hackos DH, Panicker S, Rubas W, Oglesby IB, Dillon MP, Milla ME, Burnstock G, Ford AP (2010) AF-353, a novel, potent and orally bioavailable P2X3/P2X2/3 receptor antagonist. Br J Pharmacol 160:1387–1398

Giniatullin R, Nistri A (2013) Desensitization properties of P2X3 receptors shaping pain signaling. Front Cell Neurosci 7:245

Giniatullin R, Nistri A, Fabbretti E (2008) Molecular mechanisms of sensitization of pain-transducing P2X3 receptors by the migraine mediators CGRP and NGF. Mol Neurobiol 37:83–90

Gnanasekaran A, Sundukova M, van den Maagdenberg AMJM, Fabbretti E, Nistri A (2011) Lipid rafts control P2X3 receptor distribution and function in trigeminal sensory neurons of a transgenic migraine mouse model. Mol Pain 7:77

Gnanasekaran A, Sundukova M, Hullugundi S, Birsa N, Bianchini G, Hsueh YP, Nistri A, Fabbretti E (2013) Calcium/calmodulin-dependent serine protein kinase (CASK) is a new intracellular modulator of P2X3 receptors. J Neurochem 126:102–112

Gonnord P, Delarasse C, Auger R, Benihoud K, Prigent M, Cuif MH, Lamaze C, Kanellopoulos JM (2009) Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. FASEB J 23:795–805

Grosche J, Matyash V, Möller T, Verkhratsky A, Reichenbach A, Kettenmann H (1999) Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. Nat Neurosci 2:139–143

Grote A, Hans M, Boldogkoi Z, Zimmer A, Steinhäuser C, Jabs R (2008) Nanomolar ambient ATP decelerates P2X3 receptor kinetics. Neuropharmacology 55:1212–1218

Gu Y, Wang C, Li G, Huang L-YM (2016) F-actin links Epac-PKC signaling to purinergic P2X3 receptors

sensitization in dorsal root ganglia following inflammation. Mol Pain 12
Hansen MA, Dutton JL, Balcar VJ, Barden JA, Bennett MR (1999) P2X (purinergic) receptor distributions in rat blood vessels. J Auton Nerv Syst 75:147–155
Horton AC, Ehlers MD (2003) Neuronal polarity and trafficking. Neuron 40:277–295
Hullugundi SK, Ferrari MD, van den Maagdenberg AMJM, Nistri A (2013) The mechanism of functional up-regulation of P2X3 receptors of trigeminal sensory neurons in a genetic mouse model of familial hemiplegic migraine type 1 (FHM-1). PLoS One 8:e60677
Jacques-Silva MC, Correa-Medina M, Cabrera O, Rodriguez-Diaz R, Makeeva N, Fachado A, Diez J, Berman DM, Kenyon NS, Ricordi C, Pileggi A, Molano RD, Berggren PO, Caicedo A (2010) ATP-gated P2X3 receptors constitute a positive autocrine signal for insulin release in the human pancreatic beta cell. Proc Natl Acad Sci USA 107:6465–6470
Khakh BS, North RA (2012) Neuromodulation by extracellular ATP and P2X receptors in the CNS. Neuron 76:51–69
Khakh BS, Smith WB, Chiu CS, Ju D, Davidson N, Lester HA (2001) Activation-dependent changes in receptor distribution and dendritic morphology in hippocampal neurons expressing P2X2-green fluorescent protein receptors. Proc Natl Acad Sci USA 98:5288–5293
Kowalski M, Hausmann R, Dopychai A, Grohmann M, Franke H, Nieber K, Schmalzing G, Illes P, Riedel T (2014) Conformational flexibility of the agonist binding jaw of the human P2X3 receptor is a prerequisite for channel opening. Br J Pharmacol 171:5093–5112
Listowski MA, Leluk J, Kraszewski S, Sikorski AF (2015) Cholesterol interaction with the MAGUK protein family member, MPP1, via CRAC and CRAC-like motifs: an in silico docking analysis. PLoS One 10:e0133141
Mansoor SE, Lü W, Oosterheert W, Shekhar M, Tajkhorshid E, Gouaux E (2016) X-ray structures define human P2X3 receptor gating cycle and antagonist action. Nature 538:66–71
Marchenkova A, Vilotti S, Fabbretti E, Nistri A (2015) Brain natriuretic peptide constitutively downregulates P2X3 receptors by controlling their phosphorylation state and membrane localization. Mol Pain 11
North RA (2016) P2X receptors. Philos Trans R Soc Lond Ser B Biol Sci 371(1700)
North RA, Jarvis MF (2013) P2X receptors as drug targets. Mol Pharmacol 83:759–769
Oliveira-Fusaro MCG, Zanoni CIS, Dos Santos GG, Manzo LP, Araldi D, Bonet IJ, Tambeli CH, Dias EV, Parada CA (2017) Antihyperalgesic effect of CB1 receptor activation involves the modulation of P2X3 receptor in the primary afferent neuron. Eur J Pharmacol 798:113–121
Pijacka W, Moraes DJA, Ratcliffe LEK, Nightingale AK, Hart EC, da Silva MP, Machado BH, McBryde FD, Abdala AP, Ford AP, Paton JF (2016) Purinergic receptors in the carotid body as a new drug target for controlling hypertension. Nat Med 22:1151–1159
Pratt EB, Brink TS, Bergson P, Voigt MM, Cook SP (2005) Use-dependent inhibition of P2X3 receptors by nanomolar agonist. J Neurosci 25:7359–7365
Pryazhnikov E, Fayuk D, Niittykoski M, Giniatullin R, Khiroug L (2011) Unusually strong temperature dependence of P2X3 receptor traffic to the plasma membrane. Front Cell Neurosci 5:27
Rettinger J, Schmalzing G (2003) Activation and desensitization of the recombinant P2X1 receptor at nanomolar ATP concentrations. J Gen Physiol 121:451–461
Saeed AW, Ribeiro-da-Silva A (2012) Non-peptidergic primary afferents are presynaptic to neurokinin-1 receptor immunoreactive lamina I projection neurons in rat spinal cord. Mol Pain 8:64
Salaün C, James DJ, Chamberlain LH (2004) Lipid rafts and the regulation of exocytosis. Traffic 5:255–264
Sanz JM, Di Virgilio F (2000) Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. J Immunol 164:4893–4898
Simonetti M, Giniatullin R, Fabbretti E (2008) Mechanisms mediating the enhanced gene transcription of P2X3 receptor by calcitonin gene-related peptide in trigeminal sensory neurons. J Biol Chem 283:18743–18752
Sokolova E, Nistri A, Giniatullin R (2001) Negative cross talk between anionic GABAA and cationic P2X ionotropic receptors of rat dorsal root ganglion neurons. J Neurosci 21:4958–4968
Sokolova E, Skorinkin A, Fabbretti E, Masten L, Nistri A, Giniatullin R (2004) Agonist-dependence of recovery from desensitization of P2X(3) receptors provides a novel and sensitive approach for their rapid up or downregulation. Br J Pharmacol 141:1048–1058
Sokolova E, Skorinkin A, Moiseev I, Agrachev A, Nistri A, Giniatullin R (2006) Experimental and modeling studies of desensitization of P2X3 receptors. Mol Pharmacol 70:373–382
Sundukova M, Vilotti S, Abbate R, Fabbretti E, Nistri A (2012) Functional differences between ATP-gated human and rat P2X3 receptors are caused by critical residues of the intracellular C-terminal domain. J Neurochem 122:557–567
Toulmé E, Blais D, Léger C, Landry M, Garret M, Séguéla P, Boué-Grabot E (2007) An intracellular motif of P2X(3) receptors is required for functional cross-talk with GABA(A) receptors in nociceptive DRG neurons. J Neurochem 102:1357–1368
Trang T, Beggs S, Wan X, Salter MW (2009) P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. J Neurosci 29:3518–3528
Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P (2015) Unbiased classification of sensory

neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 18:145–153

Vacca F, Giustizieri M, Ciotti MT, Mercuri NB, Volonté C (2009) Rapid constitutive and ligand-activated endocytic trafficking of P2X receptor. J Neurochem 109:1031–1041

Vandenbeuch A, Larson ED, Anderson CB, Smith SA, Ford AP, Finger TE, Kinnamon SC (2015) Postsynaptic P2X3-containing receptors in gustatory nerve fibres mediate responses to all taste qualities in mice. J Physiol 593:1113–1125

Vial C, Evans RJ (2005) Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. J Biol Chem 280:30705–30711

Vilotti S, Fabbretti E, Nistri A (2015) Role of calcitonin gene-related peptide and brain natriuretic peptide to modulate the excitability state of trigeminal neurons: relevance to migraine pathology and treatment. J Recept Ligand Channel Res Dove Press 8:31–41

Vilotti S, Vana N, Van den Maagdenberg AMJM, Nistri A (2016) Expression and function of calcitonin gene-related peptide (CGRP) receptors in trigeminal ganglia of R192Q Cacna1a knock-in mice. Neurosci Lett 620:104–110

Wirkner K, Stanchev D, Köles L, Klebingat M, Dihazi H, Flehmig G, Vial C, Evans RJ, Fürst S, Mager PP, Eschrich K, Illes P (2005) Regulation of human recombinant P2X3 receptors by ecto-protein kinase C. J Neurosci 25:7734–7742

Yegutkin GG, Guerrero-Toro C, Kilinc E, Koroleva K, Ishchenko Y, Abushik P, Giniatullina R, Fayuk D, Giniatullin R (2016) Nucleotide homeostasis and purinergic nociceptive signaling in rat meninges in migraine-like conditions. Purinergic Signal 12:561–574

Adv Exp Med Biol - Protein Reviews (2017) 19:17–53
DOI 10.1007/5584_2017_59

Published online: 5 July 2017

The P2X7 Receptor

Ronald Sluyter

Abstract

The P2X7 receptor is a trimeric ion channel gated by extracellular adenosine 5′-triphosphate. The receptor is present on an increasing number of different cells types including stem, blood, glial, neural, ocular, bone, dental, exocrine, endothelial, muscle, renal and skin cells. The P2X7 receptor induces various downstream events in a cell-specific manner, including inflammatory molecule release, cell proliferation and death, metabolic events, and phagocytosis. As such this receptor plays important roles in heath and disease. Increasing knowledge about the P2X7 receptor has been gained from studies of, but not limited to, protein chemistry including cloning, site-directed mutagenesis, crystal structures and atomic modeling, as well as from studies of primary tissues and transgenic mice. This chapter focuses on the P2X7 receptor itself. This includes the *P2RX7* gene and its products including splice and polymorphic variants. This chapter also reviews modulators of P2X7 receptor activation and inhibition, as well as the transcriptional regulation of the *P2RX7* gene via its promoter and enhancer regions, and by microRNA and long-coding RNA. Furthermore, this chapter discusses the post-translational modification of the P2X7 receptor by *N*-linked glycosylation, adenosine 5′-diphosphate ribosylation and palmitoylation. Finally, this chapter reviews interaction partners of the P2X7 receptor, and its cellular localisation and trafficking within cells.

R. Sluyter (✉)
School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia

Centre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia

Illawarra Health and Medical Research Institute, Wollongong, NSW 2522, Australia
e-mail: rsluyter@uow.edu.au

Keywords
Extracellular ATP • Gene transcription • Interaction partner • Ionotropic receptor • Ligand-gated ion channel • *P2RX7* gene • P2X7 interactome • P2X7 receptor • Post-translational modification • Purinergic receptor • Purinergic signaling • Receptor localisation • Receptor trafficking • Single nucleotide polymorphism • Splice variant

Abbreviations

ADP	adenosine 5′-diphosphate
Ano6	anoctamin 6
ART	ADP-ribosyltransferase
ATP	adenosine 5′-triphosphate
BzATP	2′(3′)-*O*-(4-benzoylbenzoyl) ATP
HEK	human embryonic kidney
HSP90	heat shock protein HSP90-β
IL	interleukin
lncRNA	long-coding RNA
LPS	lipopolysaccharide
miRNA	microRNA
NAD	nicotinamide adenine dinucleotide
$NMDG^+$	*N*-methyl-D-glucamine
rs	reference SNP
SNP	single nucleotide polymorphism

1 Introduction

The P2X7 receptor belongs to the P2X receptor family, a group of trimeric ligand-gated cation channels (North 2016). Receptors within this family are activated by extracellular adenosine 5′-triphosphate (ATP) to mediate the rapid flux of Na^+, K^+, Ca^{2+} and other cations (Burnstock and Kennedy 2011). Activated P2X7 receptors can also open large pores to facilitate the passage of organic ions including *N*-methyl-D-glucamine ($NMDG^+$), $choline^+$ and fluorescent dyes such as $ethidium^+$ and YO-PRO-1^{2+} (Alves et al. 2014). P2X2, P2X4 and P2X5 receptors can also display this property (Bo et al. 2003; Khakh et al. 1999; Virginio et al. 1999), indicating that this feature is not unique to P2X7 receptors and potentially complicating the analysis of P2X receptors in native cells and tissues.

The P2X7 receptor was originally coined the P2Z receptor due to its requirement to be activated by higher concentrations of ATP (~100 μM) compared to P2X and P2Y receptors (Gordon 1986). The P2Z receptor was first cloned from a rat brain cDNA library in 1996 (Surprenant et al. 1996). The first 395 amino acid residues of this cloned receptor possessed 35–40% sequence identity with the other six known P2X receptors, which led to the reclassification of the P2Z receptor as the P2X7 receptor (Surprenant et al. 1996).

The earliest evidence for the presence of functional P2X7 receptors in cells is commonly attributed to the presence of a receptor on rat mast cells activated by the free or tetrabasic form of ATP (ATP^{4-}) (Cockcroft and Gomperts 1979, 1980). However the optimal concentrations of ATP required to activate mast cells in these former studies are lower than that required typically to activate the rat P2X7 receptor (Surprenant et al. 1996), whilst it is now known that free ATP can also activate other P2X receptors (Li et al. 2013). Thus, initial evidence for the presence of functional P2X7 receptors in cells is (perhaps) better reflected in earlier studies of rat mast cells (Keller 1966), canine erythrocytes (Parker and Snow 1972), rabbit renal tubules (Rorive and Kleinzeller 1972), hamster astrocytes (Trams 1974) and virus-transformed murine fibroblasts (Rozengurt and Heppel 1975). In these studies extracellular ATP induced events with features typical of P2X7 receptors including activation by high ATP concentrations (>100 μM) (Surprenant et al. 1996), inhibition by Ca^{2+} or Mg^{2+}

(Acuna-Castillo et al. 2007), or sensitivity to changes in temperature (Wiley et al. 1998) or pH (Liu et al. 2009). Moreover, it is now well established that functional P2X7 receptors are present in many cell types including haematopoietic stem cells (Rossi et al. 2012), mesenchymal stem cells (Jiang et al. 2017), leukocytes (Jacob et al. 2013), erythrocytes (Sluyter 2015), ocular cells (Sanderson et al. 2014), bone cells (Agrawal and Gartland 2015), dental pulp cells (Jiang et al. 2015a; Shiozaki et al. 2017), exocrine cells (Novak et al. 2010; Woods et al. 2012), endothelial cells (Burnstock and Ralevic 2014), muscle cells (Young et al. 2012), renal cells (Booth et al. 2012) and skin cells (Geraghty et al. 2016), as well as various malignant cell types (Roger et al. 2015). The presence of P2X7 receptors in the central nervous system has been long debated (Anderson and Nedergaard 2006), but increasing evidence supports the presence of this receptor on various glial and neural cells including neurons (Stokes et al. 2015; Sperlagh and Illes 2014). Further studies however are required to fully address this debate (Sperlagh and Illes 2014).

As a result of the broad distribution of the P2X7 receptor, this receptor is involved in a number of physiological and pathophysiological processes, many of which have been reviewed extensively elsewhere. In brief, these processes include the activation of the inflammasome and subsequent release of interleukin (IL)-1β and IL-18 (Dubyak 2012), the stimulation of metalloproteases to cause the shedding of cell surface molecules such as CD23, CD27 and CD62L (Pupovac and Sluyter 2016), and phagosome-lysosome fusion, autophagy, and reactive oxygen and nitrogen species generation to mediate the destruction of intracellular pathogens including mycobacteria, chlamydiae and toxoplasma (Miller et al. 2011). P2X7 receptor activation can (paradoxically) drive proliferation and death of various cell types (Adinolfi et al. 2005), and can cause excitotoxicity of glial and neural cells by inducing glutamate release (Matute 2011). Less examined roles of the P2X7 receptor include: the stimulation of phospholipase A_2 and cyclooxygenase and subsequent release of lipid mediators (Barbera-Cremades et al. 2012; Jiang et al. 2007; Norris et al. 2014); the activation of tissue factor and release of microparticles bearing tissue factor or phosphatidylserine (Baroni et al. 2007; Furlan-Freguia et al. 2011; Moore and MacKenzie 2007); and the release of various enzymes including cathepsins (Lopez-Castejon et al. 2010), metalloprotease-9 (Gu and Wiley 2006), nicotinamide phosphoribosyltransferase (Schilling and Hauschildt 2012) and transglutaminase-2 (Adamczyk et al. 2015). P2X7 receptor activation also has roles in the metabolism of glucose (Amoroso et al. 2012; Grol et al. 2012) and lipids (Beaucage et al. 2014), as well as alterations in gut microbiota (Perruzza et al. 2017). Finally, the P2X7 receptor may function as a scavenger receptor to mediate phagocytosis of cells independently of activation by extracellular ATP (Wiley and Gu 2012).

The current chapter focuses on the P2X7 receptor itself including its gene and gene products, activation and inhibition, splice and polymorphic variants, transcriptional regulation, post-translational modifications, interaction partners, cellular localisation and trafficking. This chapter discusses these points mostly in the context of mammalian P2X7 receptors, with occasional reference to P2X7 receptors from other species. Detailed reviews about P2X7 receptors regarding its structure (Costa-Junior et al. 2011; Jiang et al. 2013), pharmacology (Bartlett et al. 2014; Bhattacharya and Biber 2016; Park and Kim 2017), roles in heath and disease (De Marchi et al. 2016; Tewari and Seth 2015), and cellular distribution and functions (see references above) can be found elsewhere as indicated. Whilst, a comprehensive overview of P2X receptor activation by Toshimitsu Kawate is found as a separate chapter within this volume.

2 The *P2RX7* Gene

The gene coding the P2X7 subunit (commonly designated as *P2RX7*) has been reported in at

Table 1 Recombinant P2X7 receptors

Species	Gene location (chromosome)	Protein length (amino acid residues)	UniProtKB	Identity to human P2X7 (%)	First recombinant receptor (year)
Human	12	595	Q99572	100	1997
Rhesus macaque	11	595	F6TSC2	97	2011
Dog	26	595	F1P9Y2	86	2009
Giant panda		595	G1M6C4	85	2016
Mouse	5	595	Q9Z1M0, Q8CHP4	81	1998
Rat	12	595	Q64663	80	1996
Guinea pig		594	H0V491	77	2008
Ayu sweetfish		574	M5BFT2	47	2013
Gilthead seabream		576	A1INL4	46	2007
Japanese flounder		580	A0A059T2C6	46	2014
African clawed frog		553	Q8UUP5	45	2002
Zebrafish	8	596	F1QJ17	42	2003

Sources: http://asia.ensembl.org and http://www.uniprot.org/ (last accessed 9 February 2017)

least 55 species (http://asia.ensembl.org, last accessed 9 February 2017), but the number of species in which P2X7 receptors have been further investigated is much smaller (Table 1). Recombinant P2X7 receptors have been characterised from seven mammalian species: human (*Homo sapiens*) (Rassendren et al. 1997), rhesus macaque (*Macaca mulatta*) (Bradley et al. 2011a), dog (*Canis lupus familiaris*) (Roman et al. 2009), giant panda (*Ailuropoda melanoleuca*) (Karasawa and Kawate 2016); rat (*Rattus norvegicus*) (Surprenant et al. 1996), mouse (*Mus musculus*) (Chessell et al. 1998) and guinea pig (*Cavia porcellus*) (Fonfria et al. 2008). Recombinant P2X7 receptors have also been characterised from non-mammalian species including the African clawed frog (*Xenopus laevis*) (Paukert et al. 2002), ayu sweetfish (*Plecoglossus altivelis*) (He et al. 2013), Japanese flounder (*Paralichthys olivaceus*) (Li et al. 2014), gilthead seabream (*Sparus aurata*) (Lopez-Castejon et al. 2007) and zebrafish (*Danio rerio*) (Kucenas et al. 2003).

The genomic organisation and chromosomal localisation of a *P2RX7* gene was first reported in humans (Buell et al. 1998a). The human *P2RX7* gene is comprised of 13 exons and localised to chromosome 12q24. Online data (http://asia.ensembl.org, last accessed 9 February 2017) demonstrate that rhesus monkey, dog, giant panda, rat, mouse and guinea pig *P2RX7* genes each comprise 13 exons but (unsurprisingly) differ in chromosomal localisation, while the zebrafish *P2RX7* gene comprises 14 exons and is present on chromosome 8 (Table 1).

3 The P2X7 Protein

The first recombinant P2X7 subunit was cloned from the rat (Surprenant et al. 1996) and subsequently obtained from other mammalian species (Rassendren et al. 1997; Bradley et al. 2011a; Roman et al. 2009; Karasawa and Kawate 2016; Chessell et al. 1998; Fonfria et al. 2008). Of the recombinant P2X7 subunits obtained to date, non-human mammalian and non-mammalian subunits have 77–97% and 42–47% sequence identity, respectively, to the human P2X7 subunit (Table 1). Mammalian P2X7 subunits are typically 595 (or 594) amino acid residues in length, while non-mammalian P2X7 subunits vary in length depending on the species (Table 1). Each

P2X7 subunit comprises two transmembrane domains, intracellular N- and C-termini, and a large ectodomain (which contains two ATP-binding sites) (Jiang et al. 2013).

P2X7 subunits assemble predominately as homotrimeric receptors (Boumechache et al. 2009; Nicke 2008; Torres et al. 1999), but in some instances, P2X7 subunits can coassemble with P2X4 subunits to form heterotrimeric receptors (Boumechache et al. 2009; Antonio et al. 2011; Guo et al. 2007; Hung et al. 2013; Perez-Flores et al. 2015). The P2X4 receptor can also participate in inflammatory mediator release (Kawano et al. 2012a; Sakaki et al. 2013) and cell death (Kawano et al. 2012b) downstream of P2X7 receptor activation, highlighting further the complex relationship between these two purinergic receptors. Finally, P2X2 and P2X5 subunits can also form heterotrimeric receptors that display P2X7 receptor-like properties

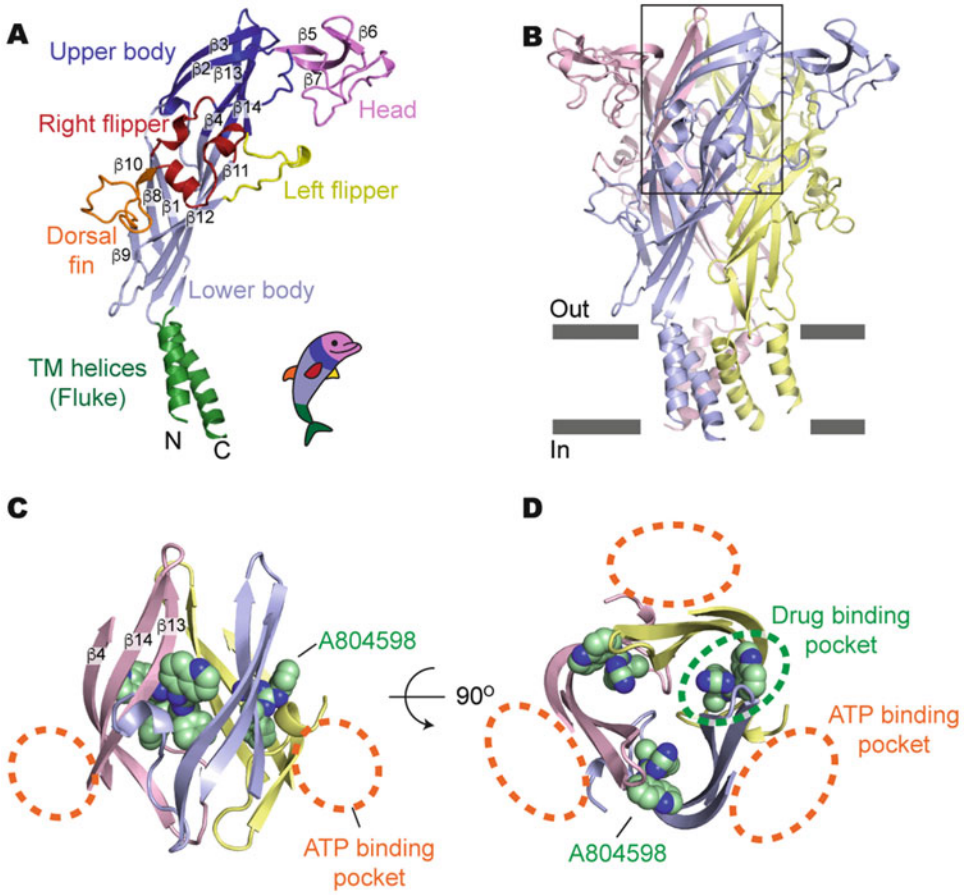

Fig. 1 The P2X7 receptor. (**A**) Cartoon representation of a 'dolphin-like' single subunit of the apo giant panda P2X7 receptor structure. Fourteen β-strands are labeled β1-14. Each domain is coloured consistent with the previous studies for better comparison (Hattori and Gouaux 2012; Kawate et al. 2009). (**B**) Cartoon representation of the trimeric giant panda P2X7 receptor structure from the side. The *black box* indicates an approximate location of the upper body domains shown in (**C**) and (**D**). (**C**) Side view of the upper body domains exhibiting A804598 binding sites with respect to the ATP-binding pockets (*orange dashed lines*). A804598 is shown as CPK spheres. (**D**) Top view of the apo giant panda P2X7 receptor structure with respect to the ATP-binding pockets (*orange dashed lines*) and one of the drug-binding pockets (*green dashed lines*). Figure and legend from Karasawa and Kawate (Karasawa and Kawate 2016) and used with permission from the authors

(Compan et al. 2012), thereby complicating the identification of P2X7 receptors in some cells and tissues via functional studies.

The crystal structure of the giant panda P2X7 receptor was recently revealed (Karasawa and Kawate 2016), with individual subunits similar in structure to crystal structures of the zebrafish P2X4 (Hattori and Gouaux 2012; Kawate et al. 2009), Gulf Coast tick P2X (Kasuya et al. 2016) and human P2X3 (Mansoor et al. 2016) subunits. The crystal structure of the panda P2X7 receptor also demonstrated the trimeric structure of this receptor (Karasawa and Kawate 2016), as observed for crystal structures of other P2X receptors (Hattori and Gouaux 2012; Kawate et al. 2009; Kasuya et al. 2016; Mansoor et al. 2016). Previously, atomic modeling, based on the zebrafish P2X4 receptor, had also revealed similar structures for various P2X7 receptors (Jiang et al. 2013; Ahmadi et al. 2015; Bradley et al. 2011b; Gu et al. 2015a). As comprehensively evaluated (Jiang et al. 2013) and directly shown (Karasawa and Kawate 2016), the subunits are in the shape of a 'dolphin', with the ectodomain (containing 14 β-strands) and the two transmembrane domains representing the body (with head and fins) and tail, respectively (Fig. 1A). The trimeric P2X7 receptor forms a 'chalice-like shape', with the three ectodomains interacting at several points, and the transmembrane domains forming individual helices, with the second transmembrane domains located centrally to form the channel pore and the first transmembrane domains located at the periphery (Fig. 1B). The crystal structure of the panda P2X7 receptor also revealed a 'turret-like' structure composed by the β13 and β14 strands (Karasawa and Kawate 2016) (Fig. 1C). Closing of this 'turret-like' structure appears necessary for the full opening of the lower body domain and subsequent channel opening, a process prevented by antagonist binding (Karasawa and Kawate 2016) (Fig. 1C–D). The crystal structure of the panda P2X7 receptor is in the closed (apo) state (Karasawa and Kawate 2016), but analysis of the zebra P2X4 receptor in the open state (Hattori and Gouaux 2012) reveals that P2X7 and other P2X receptors may need to open more widely to allow the permeation of fluorescent cations (Wei et al. 2016). Thus, structures of P2X7 receptors in the open state are required to confirm whether organic cations larger than $NMDG^+$ (195 Da) can permeate through these receptors (Wei et al. 2016) as indicated by functional studies of the rat P2X7 receptor (Browne et al. 2013).

Complete structures of P2X subunits with N- and C-termini remain to be resolved, but both termini play important roles in P2X7 receptor biology. The N-terminus regulates, in part, the flow of Ca^{2+} through the channel (Liang et al. 2015) and the activation of extracellular signal-regulated kinases (Amstrup and Novak 2003), as well as control of P2X7 receptor gating and facilitation (Allsopp and Evans 2015). A conserved protein kinase C consensus site (T*X* (K/R)) is located in the N-terminus and modulates P2X2 receptor densensitisation (Boue-Grabot et al. 2000), but potential roles of this consensus site (residues 15T*X*17K) in P2X7 receptor phosphorylation are yet to be reported. However, mutation of the 15K residue alters permeability of the rat P2X7 receptor to organic cations (Yan et al. 2008), whilst mutation of the K17 and V18 residues impairs the enhanced sensitivity of the human P2X7 receptor following plasma membrane cholesterol depletion, but not pore formation *per se* (Robinson et al. 2014). The C-terminus is essential for receptor expression and function, and contains a number of established and putative motifs or domains, and has been reviewed in detail (Costa-Junior et al. 2011). Of most relevance to discussions below, the P2X7 subunit C-terminus contains a cysteine-rich domain (residues C362-V379) within the juxtamembrane region (Chaumont et al. 2004) and a lipopolysaccharide (LPS)-binding domain (residues C573-G590) (Denlinger et al. 2001), unique domains amongst the P2X subunit members.

4 Activation and Inhibition of P2X7 Receptors

4.1 Activation

P2X7 receptors are activated by extracellular ATP, with half maximal effective concentrations in the range of ~50 μM to ~2.5 mM depending on the species (Surprenant et al. 1996; Rassendren et al. 1997; Bradley et al. 2011a; Roman et al. 2009; Karasawa and Kawate 2016; Chessell et al. 1998; Fonfria et al. 2008; Paukert et al. 2002; He et al. 2013; Li et al. 2014; Lopez-Castejon et al. 2007). Attempts to observe ATP binding to crystal structures of P2X7 receptors have proved unsuccessful to date (Karasawa and Kawate 2016). Atomic modeling of P2X7 receptors however reveals the ATP binding sites to involve nine amino acid residues from two adjacent subunits (LK64, K66, T187 and K197; Q292, R294 and K311) (Jiang et al. 2013). Whilst functional studies (Adriouch et al. 2009; Liu et al. 2008; Young et al. 2007; Stokes et al. 2010), have demonstrated that other residues (K127, K145, R276, R277 and N284) surrounding the ATP binding sites may also be important for receptor activation (Jiang et al. 2013).

P2X7 receptors can also be activated by the synthetic ATP analogue, 2′(3′)-*O*-(4-benzoylbenzoyl) ATP (BzATP) with reported half maximal effective concentrations about tenfold lower than that required for ATP (Surprenant et al. 1996; Rassendren et al. 1997; Bradley et al. 2011a; Roman et al. 2009; Chessell et al. 1998; Paukert et al. 2002; Lopez-Castejon et al. 2007), except in the guinea pig where BzATP has minimal effect (Fonfria et al. 2008) and in the Japanese flounder where ATP and BzATP are equipotent (Li et al. 2014). Human, canine, rat and murine P2X7 receptors can also be activated partially by the synthetic ATP analogue, adenosine 5′-(γ-thio)-triphosphate (Donnelly-Roberts et al. 2009a; Spildrejorde et al. 2014). Murine P2X7 receptors can also be activated by nicotinamide adenine dinucleotide (NAD) (discussed further below). Adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate do not generally activate P2X7 receptors (Donnelly-Roberts et al. 2009a). Priming of murine or *Xenopus* P2X7 receptors by 1 mM ATP for 10–20 s however renders these receptors sensitive to activation by high concentrations of either ADP or adenosine 5-′-monophosphate (half maximal effective concentrations of ~5 mM) (Chakfe et al. 2002). A finding that has not been widely pursued, possibly due to the high concentrations of ADP or adenosine 5′-monophosphate required, thus reducing the potential physiological relevance of these nucleotides to P2X7 receptor activation.

The cathelicidin-derived antimicrobial peptide, LL-37, has been repeatedly purported to activate P2X7 receptors since its initial discovery to directly activate this receptor (Elssner et al. 2004). However other studies demonstrate that this peptide most likely acts as a positive modulator of basal and agonist-induced P2X7 receptor activation (Pochet et al. 2006; Tomasinsig et al. 2008). Consistent with this, other compounds such as tenidap (Sanz et al. 1998), polymyxin B (Ferrari et al. 2004), clemastine (Norenberg et al. 2011), ivermectin (Norenberg et al. 2012) and ginsenosides (Helliwell et al. 2015) can act as positive modulators of P2X7 receptor activation.

Intracellular LPS can also facilitate P2X7 receptor activation. Intracellular LPS can stimulate murine caspase-11 (the paralogue to human caspase-4 and caspase-5), which then cleaves the ATP release channel, pannexin-1, to increase extracellular ATP and resulting in subsequent P2X7 receptor activation (Yang et al. 2015). In this context, intracellular LPS can also serve to decrease the threshold required for P2X7 receptor activation, possibly by binding to the putative LPS-binding domain (Denlinger et al. 2001) to induce conformational changes and increase sensitivity to ATP (Yang et al. 2015), although direct evidence for this is lacking. Pannexin-1 can also be activated by caspase-3 (and possibly caspase-7) (Chekeni et al. 2010) following the caspase-mediated cleavage of its pore-associated

C-terminal autoinhibitory region (Sandilos et al. 2012). However, any relationship of this process to P2X7 receptor activation remains to be described.

4.2 Inhibition

P2X7 receptors can be inhibited by a number of non-selective and selective antagonists; and have been the subject of recent reviews (Bartlett et al. 2014; Bhattacharya and Biber 2016; Park and Kim 2017). In brief, a variety of small molecule antagonists are now commercially available for use in research laboratories (Table 2). Other antagonists have been used safely in past clinical trials (Eser et al. 2015; Keystone et al. 2012; Stock et al. 2012) or are part of new clinical trials (Park and Kim 2017), but none are currently used as therapeutic drugs. P2X7 receptors can also be blocked by specific biologics, either monoclonal antibodies (Buell et al. 1998b; Kurashima et al. 2012) or nanobodies (Danquah et al. 2016), providing experimental and therapeutic alternatives to small molecule antagonists. For example, anti-murine P2X7 monoclonal antibodies or nanobodies can ameliorate experimental colitis (Kurashima et al. 2012), or experimental glomerulonephritis and allergic contact dermatitis (Danquah et al. 2016), respectively. Lastly, monoclonal or polyclonal antibodies that bind non-functional but not fully functional P2X7 receptors have been described (Barden et al. 2003; Gilbert et al. 2017). Topical application of these antibodies can reduce both B16 melanoma tumour growth in mice and the lesion area in human basal cell carcinoma (Gilbert et al. 2017). The mechanisms by which these antibodies display therapeutic action remain to be determined, but may possibly act via antibody-mediated cell lysis.

A number of P2X7 receptor antagonists have been systematically examined against human and rodent P2X7 receptors (Donnelly-Roberts et al. 2009a; Bhattacharya et al. 2013; Hibell et al. 2001), providing the most comprehensive analyses to date. Notably, these and other studies have demonstrated that some antagonists are specific for P2X7 receptors, while others can inhibit additional P2 receptors (Table 2). Conversely, some presumed P2X1 receptor antagonists (MRS2159, NF279 and NF449) inhibit P2X7 receptors (Donnelly-Roberts et al. 2009a; Klapperstuck et al. 2000; Sophocleous et al. 2015). Whilst, the P2X7 receptor antagonists inhibitors, A438079 and Brilliant Blue G, can impair pannexin-1 (Qiu and Dahl 2009), and conversely the pannexin-1 inhibitor, probenecid, can impair the P2X7 receptor (Bhaskaracharya et al. 2014), confounding the relationship between these two channels. Finally, it should be noted that Brilliant Blue G, but not A438079, can stimulate certain intracellular signaling enzymes (Hedden et al. 2011), further complicating its use in studies of P2X7 receptor biology.

Crystal structure analyses of giant panda P2X7 receptors have demonstrated an antagonist-binding pocket formed between two adjacent P2X7 subunits and juxtaposed to the putative ATP-binding pocket (Karasawa and Kawate 2016) (Fig. 1C–D). The antagonist-binding pocket comprises 13 amino acid residues, with antagonist binding mainly mediated via amino acid residues F95, F103, M105, F293 and V312 (Karasawa and Kawate 2016). Of these, residue F103 was most critical for the inhibitory actions of all five antagonists examined (A740003, A804598, AZ10606120, GW791343 and JNJ47965567) (Karasawa and Kawate 2016). Whilst residue F95 had been shown previously to be important for P2X7 receptor antagonist binding including differences in P2X7 receptor blockade between species (Michel et al. 2008a, 2009). More recent mutagenesis and atomic modeling studies of the human P2X7 receptor have identified the binding pocket for AZ10606120, which is largely in agreement with the antagonist-binding pocket identified in the giant panda P2X7 receptor (Allsopp et al. 2017). The equivalent pocket in the P2X4 receptor is too narrow to accommodate P2X7 receptor antagonists (Karasawa and Kawate 2016), providing a possible explanation for antagonist specificity. Curiously, three of the examined compounds (A740003, A804598, and

Table 2 Commercially available P2X7 receptor antagonists

Antagonist	P2X7 receptor species selectivity[a]	P2 receptor selectivity	
		Inhibition	No or weak inhibition
A438079	h, rm, d, r, m		P2X1, P2X2, P2X2/3, P2X4, P2Y1, P2Y2
A740003	h, p, r, m, f		P2X1, P2X2, P2X2/3, P2X4, P2Y1, P2Y2
A804598	h, rm, p, d, r, m		P2X1, P2X2, P2X2/3, P2X4, P2Y1, P2Y2
AZ10606120	h, rm, p, d, r, m		
AZ11645373	h, rm, d, m, gp (not r)		P2X1, P2X2, P2X2/3, P2X4, P2X5
BBG[b]	h, d, r, m, gp, s, f, x, z	P2X1, P2X2, P2X4, P2X5	P2X2/3, P2X1/5, P2Y1, P2Y2
GW791343	h, p (not r[e])		
JNJ47965567	h, rm, p, d, r, m		P2X1, P2X2, P2X3, P2X2/3, P2X4
KN62	h, rm, d, m, gp (not r, x)		
MRS2159[c]	h, r, m	P2X1, P2X2, P2X2/3, P2Y1	P2X4, P2Y2
NF279[c]	h, r, m	P2X1, P2X2, P2Y1	P2X2/3, P2X4, P2Y2
NF449[cd]	h, r, m	P2X1	P2X2, P2X2/3, P2X4, P2Y1, P2Y2
Oxidised ATP	h, r, m, a	P2X1, P2X2, P2X2/3, P2Y1	P2Y2
PPADS	h, r, m, gp, s, x, z	P2X1, P2X2, P2X3, P2X2/3, P2X5, P2Y1, P2Y2	P2X4
PPNDS	h, r, m	P2X1, P2X2, P2X2/3, P2Y1, P2Y2	P2X4

Information obtained from the following references (Bo et al. 2003; Bradley et al. 2011a; Roman et al. 2009; Karasawa and Kawate 2016; Fonfria et al. 2008; Paukert et al. 2002; He et al. 2013; Li et al. 2014; Lopez-Castejon et al. 2007; Donnelly-Roberts et al. 2009a; Spildrejorde et al. 2014; Bhattacharya et al. 2013; Hibell et al. 2001; Sophocleous et al. 2015; Michel et al. 2009; Donnelly-Roberts et al. 2009b; Jiang et al. 2000; Seyffert et al. 2004; Michel et al. 2008b; Beigi et al. 2003; Bianchi et al. 1999; Evans et al. 1995; Stokes et al. 2006)

Abbreviations: *ATP* adenosine 5′-triphosphate, *BGG* Brilliant Blue G, *PPADS* pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid, *PPNDS* pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulphonate)

[a]Species: *h* human, *rm* rhesus macaque, *p* giant panda, *d* dog, *r* rat, *m* mouse, *gp* guinea pig, *a* ayu sweetfish, *s* gilthead seabream, *f* Japanese flounder, *x* African clawed frog, *z* zebrafish

[b]Conflicting evidence regarding P2X1 receptor inhibition (Donnelly-Roberts et al. 2009a; Jiang et al. 2000; Seyffert et al. 2004), whilst P2X2 and P2X4 receptor inhibition is species dependent (Jiang et al. 2000)

[c]Marketed as P2X1 receptor antagonists

[d]Conflicting evidence regarding P2X7 receptor inhibition (Donnelly-Roberts et al. 2009a; Sophocleous et al. 2015)

[e]Positive allosteric modulator of the rat P2X7 receptor (Michel et al. 2008b)

JNJ47965567) were reported previously as competitive antagonists (Bhattacharya et al. 2013; Donnelly-Roberts et al. 2009b; Honore et al. 2006), but further analysis demonstrated that each of these compounds impaired P2X7 receptor activation in a non-competitive manner (Karasawa and Kawate 2016). Thus, crystal structures of other mammalian P2X7 receptors (particularly human and rodent) and further functional studies are required to reconcile these differences.

5 P2X7 Receptor Splice Variants

Numerous splice variants are reported for P2X7 subunits (http://asia.ensembl.org, last accessed 9 February 2017), of which only a few have been functionally characterised. These variants are the result of various truncations or insertions, and more commonly result in shorter length subunits.

5.1 Human Splice Variants

Nine splice variants have been observed for the human P2X7 subunit (P2X7B to P2X7J), with the common P2X7 subunit defined as P2X7A. Of these, protein subunits have only been reported for P2X7B, P2X7H and P2X7J (Adinolfi et al. 2010; Feng et al. 2006). A now defunct variant (GenBank NM_177427) was originally designated as the P2X7I variant (Feng et al. 2006), but the P2X7I variant was redesignated (Sluyter and Stokes 2011) as that resulting from a rare single nucleotide polymorphism (SNP) in intron 1 of the *P2RX7* gene (reference SNP(rs) 35933842) (Skarratt et al. 2005). It remains to be determined if this variant results in a protein product or if other SNPs give rise to novel P2X7 splice variants. The P2X7B variant is 364 amino acid residues in length and can form functional channels but not large pores, with putative roles in cell proliferation (Adinolfi et al. 2010). The P2X7H variant is 274 amino acid residues in length and is unlikely to assemble as a functional receptor (Cheewatrakoolpong et al. 2005). The P2X7J variant is 258 amino acid residues in length and can assemble with P2X7A subunits to form non-functional heterotrimeric receptors (Feng et al. 2006), which may function to protect certain cell types, such as ocular and malignant epithelial cells, from ATP-induced cell death (Feng et al. 2006; Guzman-Aranguez et al. 2017).

5.2 Murine Splice Variants

Four splice variants of the murine P2X7 subunit (P2X7B, P2X7C, P2X7D and P2X7K) have been observed, with the common P2X7 subunit defined as P2X7A. The P2X7B (P2X7 13B or P2X7R-v3) and P2X7C (13C or P2X7R-v2) variants are 431 and 442 amino acid residues in length, respectively (Haanes et al. 2012; Kido et al. 2014; Masin et al. 2012), and form receptors with low channel activity (Masin et al. 2012). The P2X7D (P2X7R-v4) variant is 153 amino acid residues in length and, as also observed for the P2X7B variant, can negatively modulate the basal activity of full-length P2X7 subunits (Kido et al. 2014). The P2X7K variant is 592 amino acid residues in length (Nicke et al. 2009), and assembles into receptors with increased activity and higher sensitivity to agonists compared to P2X7A receptors (Schwarz et al. 2012; Xu et al. 2012). Similar findings were originally observed with the rat P2X7K variant (Nicke et al. 2009), but other variants of the rat P2X7 subunit are yet to be described. Of note, the P2X7K variant escapes deletion in the GlaxoSmithKline P2X7 knockout mouse (Nicke et al. 2009), rendering some cell types in which it is preferentially expressed, such as T lymphocytes (Schwarz et al. 2012; Xu et al. 2012), with functional P2X7 receptors (Taylor et al. 2009). Finally, a fifth P2X7 variant (hybrid) is present in the Pfizer P2X7 knockout mouse (Masin et al. 2012). The hybrid variant consists of a truncated P2X7 subunit and part of the targeting vector (used to disrupt the *P2RX7* gene), and also forms receptors with low channel activity (Masin et al. 2012). It remains unknown if P2X7 variants escape deletion in the Lexicon Pharmaceuticals P2X7 knockout mouse (Basso et al. 2009), a line generated by targeting exons 2 and 3. In contrast, the GlaxoSmithKline (Chessell et al. 2005) and Pfizer (Solle et al. 2001) mice were generated by targeting exon 1 and 13, respectively. Thus, the Lexicon Pharmaceuticals P2X7 knockout mouse may not express any P2X7 variants. Furthermore, all known versions of the murine P2X7 receptor remain absent in a humanised P2X7 receptor mouse following Cre recombinase-mediated inactivation of the humanised receptor (Metzger et al. 2017).

6 Polymorphic Variants of P2X7 Receptors

6.1 Human Polymorphisms

The P2X7 receptor is polymorphic, with a number of missense (or non-synonymous) mutations

present in this receptor in humans. Early studies of P2X7 receptor activity in lymphocytes (Wiley et al. 1992) and macrophages (Lammas et al. 1997) revealed human subjects with negligible P2X7 receptor-mediated responses in these cell types (one of eight and two of 19 subjects, respectively). While other early studies showed that P2X7 activity in leukocytes varied between human individuals (Gu et al. 2000; Wiley and Dubyak 1989), a variation not readily explained by differences in cell-surface P2X7 expression (Gu et al. 2000). Subsequent sequencing of the human *P2RX7* gene identified the presence of a missense SNP coding for a glutamic acid to alanine substitution at amino acid residue position 496 (E496A) and resulting in a loss of receptor function (Gu et al. 2001). Since this initial discovery, a total of 16 SNPs coding for missense mutations have been characterised in the coding region of human *P2RX7* gene (Fig. 2). Most of these SNPs alter P2X7 receptor activity (Fig. 2), and many are associated with increased or decreased susceptibility to disease as reviewed previously (Jiang et al. 2013; De Marchi et al. 2016; Sluyter and Stokes 2011; Caseley et al. 2014).

The presence of SNPs in human *P2RX7* genes is complicated by the inheritance of heterozygous alleles. For example, co-expression of human P2X7 receptors containing the Q460R mutation, which have wild-type-like or partly reduced activity (Stokes et al. 2010; Roger et al. 2010a), with wild-type P2X7 receptors in human embryonic kidney (HEK)-293 cells results in receptors with greatly reduced activity (Aprile-Garcia et al. 2016). Conversely, co-inheritance of the mutant Q460R allele with alleles coding for loss-of-function mutations, E496A or I568N, increases P2X7 receptor activity in human monocytes compared to co-inheritance of the wild-type Q460 allele with these loss-of-function alleles (Denlinger et al. 2006).

Another consideration regarding P2X7 receptor activity is the inheritance of SNPs in linkage disequilibrium or haplotypes. Five haplotypes, accounting for 99% of subjects, were originally identified in Caucasians (Fuller et al. 2009) (Table 3), and subsequently shown in other Caucasian cohorts (Husted et al. 2013; Wesselius et al. 2013), with another study extending this to six haplotypes (Backlund et al. 2012), to account for the rare SNP coding for the loss-of-function mutation, I568N (Wiley et al. 2003). Of the original five haplotypes (Fuller et al. 2009), P2X7-1 was defined as wild-type, P2X7-2 and P2X7-4 as gain-of-function (due mainly to the A348T mutation), and P2X7-3 and P2X7-5 as loss-of-function (due to the E496A and T357S mutations, respectively) (Stokes et al. 2010). These five haplotypes can be further subdivided into 17 haplotypes, each with a frequency of more than 1% and with predicted functional effects (Jorgensen et al. 2012) (Table 3). Finally, 10 haplotypes, which comprises six SNPs in the non-coding region of the *P2RX7* gene spanning from the 5′-untranslated region to intron 9, have been described in a Japanese population (Ide et al. 2014). Associations between these haplotypes and P2X7 receptor activity are lacking.

6.2 Canine Polymorphisms

As observed in humans, P2X7 receptor activity also varies between dogs (Spildrejorde et al. 2014; Faulks et al. 2016). This variation can be partly explained by SNPs. Four SNPs coding for missense mutations have been characterised in the canine P2X7 receptor (Fig. 2). Of these, the R270C mutation has the greatest impact on P2X7 receptor activity, resulting in a near-complete loss of channel and pore activity (Spildrejorde et al. 2014). To date, this mutation has been observed in Cocker spaniels, but not other dog breeds (Spildrejorde et al. 2014). This mutation is yet to be associated with any disorder. A missense mutation (R270H) is present at the analogous site in human P2X7 receptor and also impairs receptor activity (Stokes et al. 2010). This mutation in humans is associated with reduced pain sensitivity in humans (Sorge et al.

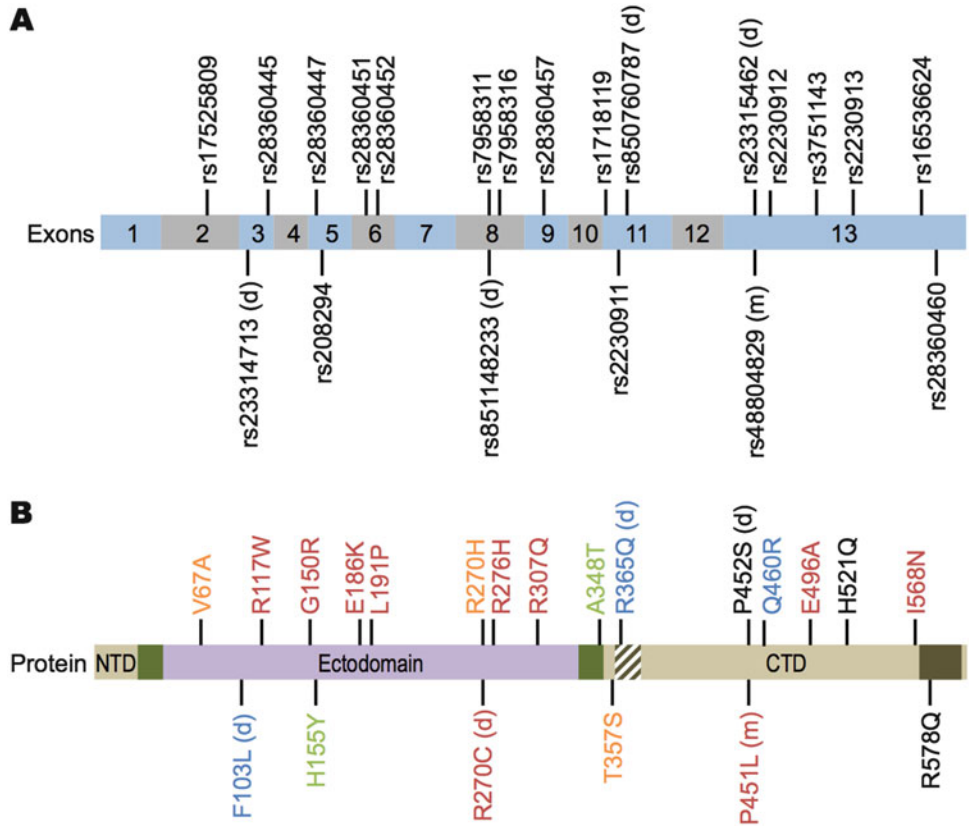

Fig. 2 The mammalian *P2RX7* gene and P2X7 subunit. (**A**) The *P2RX7* gene contains 13 exons (*blue* and *grey*), as well as 5′- and 3′-untranslated regions, and introns (not shown). Reference SNP (rs) numbers represent respective SNPs in either the human, dog (d) or mouse (m) *P2RX7* gene that have been functionally characterised. A SNP (rs35933842) is also present in intron 1 of the human *P2RX7* gene (not shown) and results in a null allele (Skarratt et al. 2005). (**B**) The P2X7 subunit comprises two transmembrane domains (*green*), an N-terminus (NTD) and C-terminus (CTD) (*tan*), and an ectodomain (*purple*) containing two ATP-binding sites (not shown). Location of the cysteine-rich domain (hatched) and LPS-binding domain (*dark tan*) are shown. Amino acid residue substitutions, in either the human, dog (d) or mouse (m) P2X7 subunits and corresponding to their respective SNP (**A**), are shown. Substitutions that cause possible loss, partial loss, loss or gain of P2X7 receptor activity are marked in *blue, orange, red or green*, respectively, as determined previously (Stokes et al. 2010; Spildrejorde et al. 2014; Roger et al. 2010a; Adriouch et al. 2002). The P452S substitution in the dog P2X7 subunit corresponds to the equivalent residue position as the P451L substitution in the mouse P2X7 subunit

2012), but whether the R270C mutation is also associated with reduced pain sensitivity in Cocker spaniels remains unknown. In contrast, the F103L mutation in the canine P2X7 receptor (Spildrejorde et al. 2014), which may partly reduce receptor activity (Sluyter and Stokes 2014), is associated with increased susceptibility to glioma in brachycephalic dog breeds (Truve et al. 2016).

6.3 Murine Polymorphisms

One SNP coding for a missense mutation (P451L) has been characterised in the murine P2X7 receptor (Fig. 2). This mutation results in reduced channel and pore activity (Adriouch et al. 2002; Young et al. 2006). This SNP is of relevance to common laboratory strains of mice. For example, the P451 allele is present in BALB/c, NOD and 129 strains, whilst the L451 allele is present in C57BL, DBA and C3H strains

Table 3 Common variants of the human P2X7 receptor arising from the 17 most frequent haplotypes (H1–H17) in a Danish cohort[a]

	V76A	G150R	H155Y	R270H	R276H	R307Q	A348T	T357S	Q460R	E496A	I568N	Frequency (%)	Activity
H1	V	G	H	H▽	R	R	A	T	Q	E	I	16.2	Wild-type
H2	V	G	H	R	R	R	A	T	Q	E	I	5.3	Wild-type
H3	V	G	Y▲	H▽	R	R	A	T	Q	E	I	4.4	Wild-type
H4	V	G	H	R	R	R	A	T	Q	E	N▼	2.6	Loss
H5	V	G	Y▲	R	R	R	A	T	Q	E	I	2.3	Wild-type
H6	V	G	Y▲	H▽	H▼	R	A	T	Q	E	I	1.4	Loss
H7	V	G	H	H▽	R	Q▼	A	T	Q	E	I	1.1	Loss
H8	V	G	H	R	R	R	T▲	T	Q	E	I	15.3	Gain
H9	A▽	G	H	R	R	R	T▲	T	Q	E	I	4.5	Gain
H10	V	G	Y▲	R	R	R	T▲	T	Q	E	I	2.4	Gain
H11	V	G	Y▲	R	R	R	A	T	Q	A▼	I	11.8	Loss
H12	V	G	H	R	R	R	A	T	Q	A▼	I	2.6	Loss
H13	V	R▼	Y▲	R	R	R	A	T	Q	A▼	I	1.0	Loss
H14	V	G	Y▲	R	R	R	T▲	T	R▽	E	I	14.0	Gain
H15	V	G	H	R	R	R	T▲	T	R▽	E	I	1.2	Gain
H16	V	G	H	R	R	R	A	S▽	Q	E	I	5.0	Loss
H17	V	G	Y▲	R	R	R	A	S▽	Q	E	I	3.4	Loss

[a]Adapted from Jorgensen et al. (Jorgensen et al. 2012)

Haplotypes H1-H17 include five haplotypes (P2X7-1–P2X7-5; A348T-E496A) as originally defined (Stokes et al. 2010; Fuller et al. 2009): H1–H7 (P2X7-1), H8–H10 (P2X7-2), H11–H13 (P2X7-3), H14 and H15 (P2X7-4) and H16 and H17 (P2X7-5)

Amino acid residue substitutions that cause possible loss (▽), partial loss (▽), loss (▼) or gain (▲) of human P2X7 receptor activity based on previous studies (Stokes et al. 2010; Roger et al. 2010a) are indicated

(Adriouch et al. 2002; Syberg et al. 2012a). However, it should be noted that others have observed similar maximal responses to BzATP and pharmacological profiles between recombinant BALB/c and C57BL/6 P2X7 receptors (Donnelly-Roberts et al. 2009a). Nevertheless, the P451L mutation is associated with reduced bone strength and density (Syberg et al. 2012a). The P451L mutation also influences bone phenotype in GlaxoSmithKline P2X7 receptor knockout mice, with greater differences observed between BALB/c wild-type and knockout littermates, compared to that of C57BL/6 wild-type and knockout littermates (Syberg et al. 2012b). Finally, the P451L mutation impairs glucose homeostasis, with impaired glucose tolerance and insulin responsiveness in P451L congenic mice compared to wild-type 129Sv mice (Todd et al. 2015). The mechanism by which the P451L mutation changes P2X7 receptor activity is yet to be fully established, but this same mutation in the human P2X7 receptor fails to alter activity of this receptor (Adamczyk et al. 2015). Moreover, the P452S mutation in the canine P2X7 receptor, which occurs at the analogous cite to P451L in the mouse, does not alter receptor activity (Spildrejorde et al. 2014).

6.4 Other Mutations

It remains to be determined if some mutations characterised in the human (V80M, A166G and N187D) (Chong et al. 2010; Sun et al. 2010), dog (L440F) (Spildrejorde et al. 2014) and murine (L11F, T221A and T283M) (Young et al. 2006) P2X7 receptors represent true SNPs, or mutations arising from cloning or cell culture. These mutations are yet to be assigned an rs number in the public domain Single Nucleotide Polymorphism database (or dbSNP) (https://www.ncbi.nlm.nih.gov/projects/SNP/). Furthermore, this database contains many other missense mutations, as well as other types of mutations in *P2RX7* genes from various species, which have not been characterised.

7 Transcriptional Regulation of P2X7 Receptors

7.1 Promoter and Enhancer Regions

The promoter regions of the genes coding human (and murine) but not other P2X7 receptors have been characterised. The promoter region of the human *P2RX7* gene (GenBank Y12851) resides between nucleotides −158 to +32 surrounding the transcription start site (+1) within the 5′-untranslated region (Zhou et al. 2009). Notably, CCAAT/enhancer binding proteinβ can bind to a region between nucleotides −213 to +6 and enhance human *P2RX7* gene transcription, a process impaired by CCAAT/enhancer binding proteinα and CCAAT/enhancer binding protein-β-LIP (Bilodeau et al. 2015). Human *P2RX7* gene transcription can also be regulated by (unknown) enhancers binding to a region located at nucleotides +222 to +323 and +401 to +573, which is controlled by cytosine hypermethylation at cytosine-phosphodiester-guanosines that cluster or colocalise to these regions (Zhou et al. 2009).

The promoter region of the murine *P2RX7* gene (GenBank NM_011027) resides between nucleotides −249 to +17 surrounding the transcription start site (+1) within the 5′-untranslated region (Garcia-Huerta et al. 2012). A number of putative transcription factor binding sites are found within this site, including four functional sites for specificity protein 1, with this transcription factor able to up-regulate P2X7 receptor expression (Garcia-Huerta et al. 2012). This up-regulation by specificity protein 1 has been mostly observed in the nervous system (Garcia-Huerta et al. 2012; Engel et al. 2017; Gomez-Villafuertes et al. 2015), although similar effects have been reported in murine macrophages (Garcia-Huerta et al. 2012) indicating potential roles for this transcription factor in P2X7 receptor expression in the periphery.

An enhancer region has also been identified between exons 2 and 3 of the murine *P2RX7* gene. Following exposure of cells to retinoic acid (vitamin A), retinoic acid receptor-α binds

this enhancer region to increase *P2RX7* gene expression (Hashimoto-Hill et al. 2017). This mechanism is observed in murine lymphocytes (Hashimoto-Hill et al. 2017), consistent with an earlier study demonstrating that retinoic acid increases P2X7 receptor expression in such cells (Heiss et al. 2008). Retinoic acid also increases P2X7 receptor expression in murine skin mast cells, a process suppressed by the retinoic acid-degrading enzyme, Cyp26b1, which is highly expressed in murine skin fibroblasts and serves to maintain skin-barrier homeostasis (Kurashima et al. 2014). In contrast, retinoic acid decreases P2X7 receptor expression in human and murine neural cells (Glaser et al. 2014; Orellano et al. 2010; Wu et al. 2009). These differences observed between immune and neural cells remain to be explored.

7.2 MicroRNA

P2RX7 gene transcription can also be regulated within the 3′-untranslated region. Most notably, P2X7 receptor expression can be modulated by microRNAs (miRNAs) interacting with this region (Ferrari et al. 2016). miR-150 and miR-186 can down-regulate human P2X7 receptor expression, and may serve to reduce P2X7 receptor in breast, cervical and bladder cancers where these miRNAs are up-regulated (Zhou et al. 2008; Huang et al. 2013). Similarly, low amounts of P2X7 receptor mRNA expression correspond with higher amounts of miR-21 expression in human non-small cell lung cancer (Boldrini et al. 2015). Conversely, others have reported increased P2X7 receptor mRNA expression and reduced miR-216b expression in human breast cancer, with direct evidence showing that miR-216b can directly down-regulate P2X7 receptor expression (Zheng et al. 2014).

The regulation of P2X7 receptor expression by miRNAs is not only restricted to malignancies. miR-22 can down-regulate murine P2X7 receptor expression and modulate disease in a murine model of epilepsy (Jimenez-Mateos et al. 2015). Similarly miR-150 can down-regulate murine P2X7 receptor expression in lung epithelial cells to reduce BzATP-induced surfactant secretion, while miR-150 and P2X7 receptor expression are inversely expressed in rat lung epithelial cells (Weng et al. 2012) supporting a role for these molecules in lung function. Moreover, down-regulation of P2X7 receptor expression by miR-150 may have a cardioprotective role in a murine model of cardiac ischaemia (Tang et al. 2015). miR-186 can down-regulate human P2X7 receptor expression in renal podocytes, and down-regulation of miR-186 and subsequent increased P2X7 receptor expression may contribute to kidney disease (Sha et al. 2015). Finally, a SNP (rs1653625) in the 3′-untranslated region of the human *P2RX7* gene, which is in linkage disequilibrium with the mood disorder-associated SNP (Q460R), resides within a putative target site for miR-625 and miR-1302 (Rahman et al. 2010). Although it remains to be determined if these miRNAs directly regulate P2X7 receptor expression, it raises the possibility that this SNP or others within the 3′-untranslated regions of *P2RX7* genes may modify P2X7 receptor expression and activity to alter physiology and contribute to disease.

7.3 Long-Coding RNA

Long-coding RNAs (lncRNAs) can also regulate P2X7 receptor expression, mostly evidenced in various disease states. lncRNAs are RNAs greater than 200 base pairs in length that can alter gene expression via various mechanisms, but do not code for specific proteins (Deniz and Erman 2017). Small inhibitory RNA to lncRNA NONRATT021972 can down-regulate rat P2X7 receptor expression in neurons following myocardial ischemia (Tu et al. 2016; Zou et al. 2016), metabolic stress (Li et al. 2016; Xu et al. 2016) and neuropathic pain (Liu et al. 2016). Similarly, small inhibitory RNA against the lncRNA uc.48+ down-regulates P2X7 receptor expression in neurons of diabetic rats (Wu et al. 2016). Collectively, this indicates that some lncRNAs are up-regulated in disease states to increase P2X7 receptor expression. The role of

lncRNAs in P2X7 receptor expression under resting states remains to be established.

8 Post-Translational Modifications of P2X7 Receptors

The P2X7 receptor is subject to post-translational modifications including *N*-linked glycosylation, palmitoylation and ADP-ribosylation. Human and rodent P2X7 subunits are glycosylated resulting in protein products typically 75 to 78 kDa in size compared to the predicted non-glycosylated size of 68 kDa (Nicke 2008; Young et al. 2007; Lenertz et al. 2010). The ectodomain of the human P2X7 receptor is glycosylated on five asparagine residues (N187, 202, 213, 241 and 284) (Lenertz et al. 2010). *N*-linked glycosylation at these sites, and in particular N187, facilitates P2X7 receptor trafficking to the cell surface and subsequent activity (Lenertz et al. 2010). Furthermore, with the exception of residue N284 in the murine P2X7 subunit (where an aspartic acid residue resides), these five asparagine residues are conserved between mammalian P2X7 subunits (Jiang et al. 2013) suggesting that P2X7 receptors from other species may also be glycosylated at these sites. Consistent with this, mutation of residue N284 to aspartic acid residue in the murine P2X7 subunit increases its molecular weight (Young et al. 2007) indirectly confirming residue N284 as a glycosylation site. Lastly, a natural mutation (R578Q) alters *N*-linked glycosylation of the human P2X7 receptor resulting in a receptor of higher molecular weight and impaired function indicating that the C-terminus plays a role in glycosylation of the P2X7 receptor possibly through altered trafficking of the receptor (Wickert et al. 2013).

The murine P2X7 receptor is palmitoylated on cysteine residues within the C-terminus spanning four regions (C371, 373 and 374; C477, 479 and 482; C498, 499 and 506; C572 and 573) (Gonnord et al. 2009). Reduced palmitoylation of the murine P2X7 receptor reduces plasma membrane expression due to increased receptor retention in the endoplasmic reticulum and reduced protein half-life (Gonnord et al. 2009). These cysteine residues are largely conserved between mammalian P2X7 subunits (Jiang et al. 2013) suggesting that P2X7 receptors from other species may also be palmitoylated at these sites. In this regard, high palmitate in combination with high glucose can increase P2X7 receptor expression in human endothelial cells, but this may be related to increased *P2RX7* mRNA expression rather than a direct effect on the receptor itself (Sathanoori et al. 2015).

The murine P2X7 receptor can be ADP-ribosylated by the ADP-ribosyltransferase (ARTs), ART2.1 and ART2.2 (Hong et al. 2009; Seman et al. 2003). This process results in the transfer of the ribose moiety from NAD to R125 in the ectodomain of the P2X7 receptor and the subsequent activation of the receptor (Adriouch et al. 2008). Thus, NAD is an alternate P2X7 receptor agonist to ATP in mice, a process not observed in humans due to the absence of ART2.1 and ART2.2 (Rissiek et al. 2015). Importantly, the murine P2X7K variant but not the P2X7A receptor is far more sensitive to activation by ADP-ribosylation, providing an explanation for the preferential activation of the P2X7 receptor by NAD in murine T cells (which predominately express P2X7K) over macrophages (which predominately express P2X7A) (Schwarz et al. 2012; Xu et al. 2012; Hong et al. 2009). It remains to be reported if humanised P2X7 receptors of transgenic mice (Metzger et al. 2017) can be activated by ADP-ribosylation.

Collectively, these results highlight the potential importance of post-translational modifications to P2X7 receptor expression and function. However such observations are mostly limited to P2X7 receptors from one species. It remains to be determined if these post-translational events, particularly palmitoylation and ADP-ribosylation, apply to P2X7 receptors of other species.

9 Interaction Partners of P2X7 Receptors

The P2X7 receptor directly interacts with over 50 different molecular partners (Table 4). This list includes peripheral and integral membrane proteins, as well as soluble proteins including chaperone, cytoskeletal, signaling and other proteins. The majority of these interactions are summarised in the P2X7 Interactome database collated by the laboratories of Mark Young and Amanda MacKenzie (http://www.p2x7.co.uk/). Many of the interaction partners were initially identified through mass spectrometry of immunoprecipitation preparations of rat and human P2X7 receptors (Gu et al. 2009; Kim et al. 2001). Additional interaction partners have been identified through candidate molecule approaches using immunoprecipitation and immunoblotting (see Table 4 and references therein), or through peptide array target screening (Wu et al. 2007) or yeast two-hybrid technology (Wang et al. 2011; Wilson et al. 2002).

9.1 Tyrosine Kinases

Activation of the P2X7 receptor results in the stimulation of multiple signaling pathways involving many different types of enzymes (Lenertz et al. 2011). Conversely, enzymes may also regulate P2X7 receptor activation (Liang and Schwiebert 2005), although direct evidence is limited. The rat P2X7 receptor can be tyrosine phosphorylated (Kim et al. 2001; Feng et al. 2005), predominately at amino acid residue Y343 (Kim et al. 2001). Although this residue lies within the second transmembrane domain, potentially limiting access by enzymes, the rat P2X7 receptor interacts with receptor protein tyrosine phosphatase-β and becomes dephosphorylated upon activation (Kim et al. 2001). Moreover, tyrosine phosphatase inhibitors can prevent run-down of rat P2X7 receptors, a process in which current magnitude decreases in a stepwise manner upon short intermittent agonist applications (Kim et al. 2001). This suggests that tyrosine phosphorylation can modulate receptor function. P2X7 receptors can also interact with tyrosine-protein kinase ABL1 (Wu et al. 2007) or Fyn (Feng et al. 2015), or tyrosine-protein phosphatase non-receptor type 6 (Gu et al. 2009). Further studies are required to confirm functional relationships between these enzymes and P2X7 receptors.

9.2 Calmodulin

The rat but not human P2X7 receptor interacts with calmodulin (Roger et al. 2008, 2010b). Calmodulin constitutively binds to the rat P2X7 receptor, with binding increasing upon receptor activation to mediate Ca^{2+}-dependent facilitation of the receptor (Roger et al. 2010b). Calmodulin binds to a C-terminal domain spanning residues I541-560S of the rat P2X7 receptor (Roger et al. 2008), but this domain is absent in the human P2X7 receptor and which fails to undergo Ca^{2+}-dependent facilitation (Roger et al. 2010b). This domain is also absent in the murine P2X7 receptor (Costa-Junior et al. 2011). In contrast, both rat and human P2X7 receptors can undergo Ca^{2+}-independent facilitation, which is mediated by the C-terminal cysteine-rich domain (residues C362-V379) (Roger et al. 2010b). Curiously, deletion of this cysteine-rich domain prevents permeability of the rat P2X7 receptor to $NMDG^{+}$ but not YO-PRO-1^{2+}, suggesting that these two cations may enter cells through alternative pathways (Jiang et al. 2005). Alternatively, this domain may simply be necessary for $NMDG^{+}$ but not YO-PRO-1^{2+} uptake through the P2X7 receptor pore. Further complicating these findings, deletion of this cysteine-rich domain in the human P2X7 receptor reduces $ethidium^{+}$ uptake (Allsopp and Evans 2015; Robinson et al. 2014). Whether these contrasting results represent differences between species or cation dyes remains to be explored.

Table 4 Interaction partners of the P2X7 receptor[a]

Partner	Partner species	P2X7 species	Reference
Integral membrane proteins			
Anoctamin 6 (Ano6)	Human	Human	Ousingsawat et al. (2015)
Ephrin B3	Human	Human	Wang et al. (2011)
Epithelial membrane protein 1	Human	Rat	Wilson et al. (2002)
Epithelial membrane protein 2	Human	Rat	Wilson et al. (2002)
Epithelial membrane protein 3	Human	Rat	Wilson et al. (2002)
Integrin β2	Human	Rat	Kim et al. (2001)
P2X4 receptor	Human, rat, mouse	Human, rat, mouse	Boumechache et al. (2009), Antonio et al. (2011), Guo et al. (2007), Hung et al. (2013) and Perez-Flores et al. (2015)
Pannexin-1	Human, rat, mouse	Human, rat, mouse	Hung et al. (2013), Iglesias et al. (2008), Kanjanamekanant et al. (2014), Li et al. (2011), Pelegrin and Surprenant (2006) and Poornima et al. (2012)
Peripheral myelin protein 22	Human	Rat	Wilson et al. (2002)
Receptor-type tyrosine-protein phosphatase β	Human	Rat	Kim et al. (2001)
Toll-like receptor 2	Mouse	Mouse	Babelova et al. (2009)
Toll-like receptor 4	Mouse	Mouse	Babelova et al. (2009)
Transmembrane 9 superfamily member 1	Human	Human	Wang et al. (2011)
Peripheral membrane proteins			
Caveolin-1	Mouse	Mouse	Barth et al. (2008), Pfleger et al. (2012) and Weinhold et al. (2010)
Caveolin-3	Mouse	Mouse	Pfleger et al. (2012)
CD14	Human	Human	Dagvadorj et al. (2015)
CD44	Hamster	Hamster	Moura et al. (2015a)
Clathrin	Human	Human	Feng et al. (2005)
MAGUK p55 subfamily member 3	Human	Rat	Kim et al. (2001)
Nitric oxide synthase, neuronal	Mouse	Mouse	Pereira et al. (2013)
Nucleoprotein TPR	Human	Human	Gu et al. (2009)
Peripheral plasma membrane protein CASK	Human	Human	Wang et al. (2011)
Supervillin	Human	Rat	Kim et al. (2001)
Chaperone proteins			
Heat shock 70 kDa protein	Human	Human, rat	Gu et al. (2009) and Kim et al. (2001)
Heat shock cognate 71 kDa protein	Human	Rat	Kim et al. (2001)
Heat shock protein HSP90-β	Human, rat	Human, rat	Gu et al. (2009), Kim et al. (2001), Adinolfi et al. (2003) and Franco et al. (2013)
Cytoskeletal proteins			
α-Actinin 4	Human	Rat	Kim et al. (2001)
Actin, cytoplasmic 1 (β actin)	Human	Human, rat	Gu et al. (2009) and Kim et al. (2001)
Calmodulin	Human	Rat	Roger et al. (2008)
Dynamin	Human	Human	Feng et al. (2005)
Myosin-9	Human	Human	Gu et al. (2009)
Tubulin β chain	Human	Human	Gu et al. (2009)
Unconventional myosin-Va	Human	Human	Gu et al. (2009)

(continued)

Table 4 (continued)

Partner	Partner species	P2X7 species	Reference
Signaling proteins			
β-Adrenergic receptor kinase 2	Human	Human	Feng et al. (2005)
β-Arrestin 2	Human	Human	Feng et al. (2005)
Growth factor receptor-bound protein	Human	Human	Wu et al. (2007)
MyD88	Human	Mouse	Liu et al. (2011)
Tyrosine-protein kinase ABL1	Human	Human	Wu et al. (2007)
Tyrosine-protein kinase Fyn	Rat	Rat	Feng et al. (2015)
Tyrosine-protein phosphatase non-receptor type 6	Human	Human	Gu et al. (2009)
Other proteins			
Apoptosis-associated speck-like protein containing a CARD (ASC)	Human, rat	Human, rat	Minkiewicz et al. (2013) and Silverman et al. (2009)
Biglycan	Mouse	Mouse	Babelova et al. (2009)
Cytoplasmic protein NCK1	Human	Human	Wu et al. (2007)
E3 ubiquitin-protein ligase TRIM21 (52 kDa Ro protein)	Human	Human	Gu et al. (2009)
Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1	Human	Human	Gu et al. (2009)
Laminin subunit α-3	Human	Rat	Kim et al. (2001)
Myosin regulatory light chain 12	Human	Human	Gu et al. (2009)
NACHT, LRR and PYD domains-containing protein 2 (NLRP2)	Human	Human	Minkiewicz et al. (2013)
NACHT, LRR and PYD domains-containing protein 3 (NLRP3)	Mouse	Mouse	Franceschini et al. (2015)
Neutrophil defensin 1	Human	Human	Chen et al. (2014)
Nucleoside diphosphate kinase B	Human	Human	Gu et al. (2009)
Phosphatidylinositol 4-kinase α	Human	Rat	Kim et al. (2001)
α-Synuclein	Mouse	Mouse	Jiang et al. (2015b)

[a]Adapted from the P2X7 Interactome (http://www.p2x7.co.uk/, last accessed 16 February 2017)

9.3 Chaperones

As expected for proteins, the P2X7 receptor interacts with chaperones (Gu et al. 2009; Kim et al. 2001; Adinolfi et al. 2003; Franco et al. 2013), but heat shock protein HSP90-β (HSP90) can also regulate P2X7 receptor function. HSP90 associated with human or rat P2X7 receptors is tyrosine phosphorylated (Adinolfi et al. 2003). Moreover, the HSP90 inhibitor, geldanamycin, decreases tyrosine phosphorylation of HSP90, and increases the sensitivity of the rat P2X7 receptor to BzATP (Adinolfi et al. 2003), but not ATP (Migita et al. 2016). In contrast, geldanamycin (and other HSP90 inhibitors) enhance the initial phase of ATP-induced inward currents, but decrease permeability to $NMDG^+$ (Migita et al. 2016). Further data indicates the HSP90 interacts through the C-terminus of the P2X7 receptor (Adinolfi et al. 2003; Migita et al. 2016). Combined, this data indicates that HSP90 may function as a negative regulator of the P2X7 receptor channel.

HSP90 may also regulate P2X7 receptor-mediated events. Geldanamycin can increase basal membrane blebbing in HEK-293 cells expressing heterologous rat P2X7 receptors, although this effect appears to be mediated by increased basal ATP release (Adinolfi et al. 2003). Nitrated HSP90 can also associate with

the P2X7 receptor in motor neurons resulting in P2X7 receptor activation and stimulating Fas-mediated cell death (Franco et al. 2013). Importantly, nitrated HSP90 is observed in spinal cords from amyotrophic lateral sclerosis patients and rodents, and in spinal cords following experimental injury (Franco et al. 2013), conditions in which the P2X7 receptor plays important roles (Apolloni et al. 2014; Bartlett et al. 2017; Peng et al. 2009; Wang et al. 2004). Finally, HSP90 is required for P2X7 receptor-induced pore formation to promote autophagy and cell death in muscle cells (Young et al. 2015). This pathway may be important in Duchenne muscular dystrophy, where P2X7 receptor expression and signaling is increased in muscle cells (Young et al. 2012).

9.4 Inflammasome-Associated Molecules

The P2X7 receptor also interacts with a number of proteins associated with the synthesis, and functional maturation and release of IL-1β and IL-18. This includes CD14, Toll-like receptors 2 and 4, and MyD88 (Babelova et al. 2009; Dagvadorj et al. 2015; Liu et al. 2011), which are associated with the synthesis of inflammasome and IL-1 cytokine members (Bauernfeind et al. 2009; Mehta et al. 2001); inflammasome components, NACHT, LRR and PYD domains-containing protein 2 and 3, and apoptosis-associated speck-like protein containing a CARD (Franceschini et al. 2015; Minkiewicz et al. 2013; Silverman et al. 2009); and pannexin-1 (Hung et al. 2013; Iglesias et al. 2008; Kanjanamekanant et al. 2014; Li et al. 2011; Pelegrin and Surprenant 2006; Poornima et al. 2012), which helps promote K^+ efflux to promote IL-1β and IL-18 maturation and release (Pelegrin and Surprenant 2007). Collectively this reiterates the importance of P2X7 receptor in the maturation and release of IL-1β and IL-18 (Dubyak 2012; Giuliani et al. 2017), which arguably remains the most explored role of this receptor.

Pannexin-1 can also contribute to some but not all forms of P2X7 receptor-mediated cell death (Hanley et al. 2012; Locovei et al. 2007; Shoji et al. 2014). Moreover, pannexin-1, originally thought to be a pore forming unit of the P2X7 receptor (Pelegrin and Surprenant 2007) but now redundant in this process (Hanley et al. 2012; Qu et al. 2011), may serve as an ATP release channel (Qiu and Dahl 2009) to promote P2X7 receptor activation (Dubyak 2009). P2X7 receptor activation may also induce ATP-induced internalisation of pannexin-1 (Boyce et al. 2015) to potentially limit ATP release. However a role for the P2X7 receptor is this process was established through the use of A438079 at 100 μM (Boyce et al. 2015), a concentration approximate to the half maximal inhibitory concentration for pannexin-1 (Qiu and Dahl 2009). Thus, A438079 may have been directly acting on pannexin-1, rather than the P2X7 receptor, to impair its internalisation.

9.5 Myosin

The P2X7 receptor interacts with a number of cytoskeletal and associated proteins. In particular the human P2X7 receptor is tightly associated with myosin-9 (non-muscle myosin heavy chain IIA), an ATPase that provides the energy for cytoskeletal processes including phagocytosis (Gu et al. 2009). As such, human, murine and rat P2X7 receptors, in the absence of serum, can mediate the uptake of latex beads (Fang et al. 2009; Gu et al. 2010; Yamamoto et al. 2013), live and dead bacteria (Gu et al. 2010), and apoptotic lymphocytes (Gu et al. 2011) and neural cells (Lovelace et al. 2015). These particles can bind the ectodomain of the P2X7 receptor suggesting that it can function as a scavenger receptor (Gu et al. 2011). P2X7 receptor-mediated phagocytosis can be achieved by macrophages (Gu et al. 2009), microglia (Fang et al. 2009), astrocytes (Yamamoto et al. 2013), and neural precursors and neuroblasts (Lovelace et al. 2015), as well as HEK-293 cells expressing P2X7 receptors (Gu et al. 2010). Notably, this process takes place in the absence of extracellular ATP, whilst ATP activation causes the dissociation between the P2X7 receptor and myosin-9

to prevent P2X7 receptor-mediated phagocytosis (Gu et al. 2009). Conversely, the presence of myosin-9 and myosin Va limits P2X7 receptor activation (Gu et al. 2009). Besides potential roles in immune surveillance (Wiley and Gu 2012), P2X7 receptor-mediated phagocytosis may also be important in the clearance of apoptotic neuroblasts during neurogenesis (Gu et al. 2015b). These findings have potential implications in neurological disorders including age-related macular degeneration (Gu et al. 2013).

9.6 Anoctamin 6 and CD44

Other notable interaction partners with the P2X7 receptor include anoctamin 6 (Ano6) and CD44. Ano6 is a Ca^{2+}-dependent phospholipid scramblase and putative Ca^{2+}-activated Cl^- channel and non-selective cation channel (Kunzelmann et al. 2014). Ano6 can associate with the human P2X7 receptor (Ousingsawat et al. 2015). Moreover, Ano6 mediates P2X7 receptor-induced events including pore formation, phospholipid exposure, membrane blebbing, killing of intracellular bacteria and cell death (Ousingsawat et al. 2015). Collectively, this indicates Ano6 as a central effector molecule downstream of P2X7 receptor activation. However it should be noted that a second study failed to demonstrate an interaction or functional relationship between Ano6 and the human P2X7 receptor (Stolz et al. 2015). Thus, further clarification is required concerning the relevance of Ano6 to P2X7 receptor-mediated responses.

The glycosaminoglycan chains of soluble CD44 can also bind to the hamster P2X7 receptor to function as a positive allosteric modulator of P2X7 receptor activation (Moura et al. 2015a). Notably, P2X7 receptor activation can induce the ADAM10-mediated shedding of CD44 from murine cells (Lin et al. 2012). Thus, collectively providing a potential positive feedback mechanism to promote P2X7 receptor-mediated responses (Moura et al. 2015b). It remains unknown if other types of molecules that are shed by ADAM10 downstream of P2X7 receptor activation, such as CD23 (Pupovac et al. 2015) and E-cadherin (Sommer et al. 2012), can regulate the P2X7 receptor in a manner similar to that of CD44.

10 Cellular Localisation of P2X7 Receptors

10.1 Plasma membrane

The P2X7 receptor is widely described as a cell-surface receptor, but there is increasing evidence that the P2X7 receptor is also present within intracellular compartments of cells. The P2X7 receptor is unquestionably present at the cell-surface of many cell types. In addition to the plethora of functional studies, use of specific anti-P2X7 receptor antibodies (Buell et al. 1998b; Kurashima et al. 2012; Adriouch et al. 2005; Collo et al. 1997) has confirmed the presence P2X7 receptors on the plasma membranes of many cell types. Furthermore, use of P2X7 subunit N- and C-termini-tagged fusion proteins demonstrates the localisation of P2X7 receptors at the plasma membrane with both termini residing with the cytoplasm (Amstrup and Novak 2003; Smart et al. 2002) and indirectly confirming that the ectodomain is exposed to the extracellular milieu. Further understanding of the distribution of P2X7 receptors in cell types and tissues will continue to be aided through the use of P2X7 receptor reporter mice (Garcia-Huerta et al. 2012) and conditional humanised P2X7 receptor knockout mice (Metzger et al. 2017), especially in the central nervous system were the precise distribution of P2X7 receptors has been long debated (Anderson and Nedergaard 2006). However, it is conceded that such mice are unlikely to yield any information about the subcellular localisation of the receptor (Metzger et al. 2017).

10.2 Lipid Rafts

In some cell types, P2X7 receptors are found in lipid rafts, which can be defined as localised

plasma membrane regions, which are enriched in cholesterol and sphingolipids, and more rigid compared to more fluid parts of the plasma membrane (Garcia-Marcos et al. 2009). P2X7 receptors have been observed in lipid rafts, as well as in non-lipid raft fractions from murine lymphoma cells (Bannas et al. 2005), rat submandibular glands (Garcia-Marcos et al. 2006a), murine lung epithelial cells (Barth et al. 2007) and murine osteoblasts (Gangadharan et al. 2015). The role of P2X7 receptors within lipid rafts remains to be fully explored, but data from rat submandibular gland cells suggests that activation of lipid raft-associated P2X7 receptors stimulates lipid-signaling pathways involving neutral sphingomyelinase and phospholipase A_2 (Garcia-Marcos et al. 2006b). In contrast, non-lipid raft-associated P2X7 receptors mediate the cation channel activity of the receptor (Garcia-Marcos et al. 2006b). Importantly, plasma membrane cholesterol impairs human and murine P2X7 receptor channel and pore activity (Robinson et al. 2014). Regions within the N-terminus (V10-K17) and C-terminus (L354-R364 and V378-K387) proximal to the transmembrane domains contain putative cholesterol recognition amino acid consensus motifs, each of which contribute to cholesterol sensitivity of the P2X7 receptor (Robinson et al. 2014). Finally, lipin-2, a phosphatidic acid phosphohydrolase, can promote a lipid environment that leads to increased plasma membrane cholesterol and impaired P2X7 receptor activation (Lorden et al. 2017). Thus, lipin-2 can function as a negative modulator of P2X7 receptor activation. Collectively, this data indicates that cholesterol can regulate P2X7 receptor function within plasma membranes especially those containing lipid rafts.

10.3 Apical and Basolateral Membranes

Whilst early studies demonstrated uniform P2X7 receptor distribution on the plasma membrane of various cell types expressing endogenous P2X7 receptors such as leukocytes (Sluyter et al. 2001) or in HEK-293 cells expressing heterologous P2X7 receptors (Amstrup and Novak 2003; Smart et al. 2002), other studies reveal localised cell-surface P2X7 receptor expression in asymmetric or polarised cell types. For example, P2X7 receptors are localised to the apical membrane of resting human T84 colon cells, but partially redistribute to the basolateral membrane following neutrophil translocation (Cesaro et al. 2010). In contrast, heterologous P2X7 receptors expressed within Madin-Darby canine and LCC-PK1 porcine kidney epithelial cells are localised to the basolateral, but not apical membranes (Bradley et al. 2010). Notably, residues spanning P570-Q587 (and particularly P582-Q587) are critical in targeting P2X7 receptors to basolateral membranes (Bradley et al. 2010). These residues almost entirely overlap within the LPS-binding domain (residues C573-G590) (Denlinger et al. 2001), but any role for LPS in targeting the P2X7 receptor to the basolateral membrane remains unknown.

10.4 Intracellular Compartments

Some cell types, such as leukocytes and platelets, contain large pools of intracellular P2X7 receptors (Gu et al. 2000). Although the functional significance of these intracellular receptors remains unknown, pools of intracellular P2X7 receptors may contribute to increased cell-surface receptor expression during cellular differentiation, as observed during the differentiation of human monocytes to macrophages (Gudipaty et al. 2001). In contrast, cell-surface P2X7 receptor expression in human monocytes is unaltered following P2X7 receptor activation, suggesting the receptors from these intracellular pools are unlikely to be rapidly inserted following receptor activation (Gudipaty et al. 2001). Subcellular P2X7 receptors have also been observed in cerebellar granular neurons (Sanchez-Nogueiro et al. 2014). This further supports the concept that intracellular P2X7 receptors are present with various intracellular compartments (Burnstock 2015).

The P2X7 receptor is present in the nuclei of various cell-types. P2X7 receptors have been

observed in the nuclei of corneal and oesophageal epithelia (Groschel-Stewart et al. 1999), urinary bladder and ureter epithelia (Lee et al. 2000), and visceral smooth muscle cells (Menzies et al. 2003) and prostate glandular epithelium (Slater et al. 2005). Notably, immunolabelling demonstrated the P2X7 receptor on the nuclear envelope within hippocampal neurons, with the 'ectodomain' of the receptor facing the cytoplasm (Atkinson et al. 2002). It remains unknown how P2X7 receptors traffic to the nucleus and what is their role at this site, but nuclear P2X7 receptors may be functional, with P2X7 receptor-like channel activity reported in *Xenopus* oocytes (Mazzanti et al. 1994) and murine liver nuclei (Assandri and Mazzanti 1997).

The P2X7 receptor is also present in the phagosomes of murine macrophages, where it demonstrates activity (Kuehnel et al. 2009a). ATP synthesised from ADP via adenylate kinase, within the phagosome lumen can activate P2X7 receptors to assemble actin (Kuehnel et al. 2009a), a process that can also occur on the plasma membrane of macrophages (Kuehnel et al. 2009b). Although the physiological roles of phagosome P2X7 receptors remain to be defined, these receptors may contribute to known functions of the P2X7 receptor in phagocytosis (Gu et al. 2010), phagosome-lysosome fusion (Fairbairn et al. 2001) and autophagy (Biswas et al. 2008).

11 Trafficking of P2X7 Receptors

Amino acid residues or domains within the C-terminus regulate trafficking of P2X7 receptors to the cell surface. A domain spanning amino acid residues R551-P582 of the C-terminus regulates cell surface expression of the rat P2X7 receptor (Smart et al. 2003). This domain is thought to contain one region that impairs receptor trafficking to the cell surface, and another that can override the first region to facilitate receptor trafficking to the cell surface (Smart et al. 2003). Although the precise sequences of these two regions are to be defined, introduced mutations within these regions alter P2X7 receptor trafficking to the cell surface (Smart et al. 2003; Denlinger et al. 2003). Moreover, the I568N and R578Q SNPs within this domain also impair trafficking of the human P2X7 receptor to the cell surface (Wiley et al. 2003; Wickert et al. 2013). Notably, the R578Q SNP alters oligomerisation and *N*-linked glycosylation of P2X7 subunits providing a possible mechanism for this mutation in preventing P2X7 receptor trafficking (Wickert et al. 2013). As discussed above, palmitoylation of the C-terminus of the P2X7 receptor can also effect cell surface expression of this receptor (Gonnord et al. 2009).

A conserved C-terminal motif (Y*XXX*K) that stabilises various P2X receptors in the plasma membrane is also present in the P2X7 subunit (residues Y383-K387) (Chaumont et al. 2004). The role of this motif in P2X7 receptor expression and trafficking is yet to be elucidated. Given that it juxtaposed to the cysteine-rich domain (residues C362-V379) (Chaumont et al. 2004) and resides within a putative cholesterol recognition amino acid consensus motif (residues V378-K387) (Robinson et al. 2014), the possibility remains that the Y*XXX*K motif in the P2X7 receptor does not share the same function as in other P2X receptors. In contrast, a C-terminal motif (Y*XX*GL) that is facilitates basal and ATP-induced endocytosis of the P2X4 receptor is absent in the P2X7 receptor (Royle et al. 2002), which may explain differences in their respective rates of internalisation. Nevertheless internalisation of P2X7 receptors has been observed in response to low agonist concentrations (Feng et al. 2005; Khadra et al. 2013), a process that may involve β-adrenergic receptor kinase 2 and β-arrestin 2 (Feng et al. 2005).

Amino acid residues within the ectodomain also regulate trafficking of P2X7 receptors. Ten conserved cysteines, which form five disulfide bonds (C119-C168, C129-C152, C135-C162, C216-C226 and C260-C269), are essential for trafficking of the rat P2X7 to the cell surface (Jindrichova et al. 2012). Whilst, the H155Y SNP increases cell surface expression of the

human P2X7 receptor (Bradley et al. 2011b), providing a possible explanation for the increased activity of this mutant receptor (Cabrini et al. 2005). Glycosylation of the ectodomain of the P2X7 receptor can also impact its expression at the cell surface (Lenertz et al. 2010; Wickert et al. 2013) as discussed in detail above.

Finally, as indicated by earlier studies in human monocytes (Gudipaty et al. 2001), P2X7 receptors do not insert into the plasma membranes or form clusters during agonist-induced pore formation (Smart et al. 2002; Connon et al. 2003), although clusters can form after extended periods of receptor activation (Connon et al. 2003). Differing results are observed in neurons. Single particle tracking reveals two populations of heterologous P2X7 receptors within the extra-synaptic membrane of hippocampal neurons, one composed of rapidly diffusing receptors and the other stabilised within nanoclusters (Shrivastava et al. 2013). Addition of ATP was found to increase P2X7 receptor diffusion and further stabilise P2X7 receptors within the nanoclusters (Shrivastava et al. 2013). Thus, emerging technologies may reveal new insights into the movement of P2X7 receptors to and from, as well as within the plasma membrane.

12 Concluding Remarks

This chapter focused on the P2X7 receptor itself including its gene and gene products, activation and inhibition, splice and polymorphic variants, transcriptional regulation, post-translational modifications, interaction partners, cellular localisation and trafficking. In part, this chapter highlights gaps in knowledge regarding these salient points of P2X7 receptor biology. Whilst much progress has been made in recent years to understand the role of this receptor in biology more work is required. Recent progress including the first crystal structures of the giant panda P2X7 receptors will advance understanding of its structural biology, but crystal structures of any P2X7 receptor in its open state or of a human P2X7 receptor awaits. Recent developments of humanised P2X7 receptor and other transgenic mice will also facilitate understanding of the biology of this receptor, and will open new opportunities for the pre-clinical testing of small molecule antagonists and biologics. Finally, despite considerable gains in humans and mice, and to a lesser extent rats and dogs, the role of the P2X7 receptor in other mammals and most species remains relatively unexplored. Comparison of this receptor between species will also assist in greater understanding of the P2X7 receptor.

Compliance with Ethical Standards

Conflicts of Interest The author declares that he has no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

References

Acuna-Castillo C, Coddou C, Bull P, Brito J, Huidobro-Toro JP (2007) Differential role of extracellular histidines in copper, zinc, magnesium and proton modulation of the P2X7 purinergic receptor. J Neurochem 101(1):17–26

Adamczyk M, Griffiths R, Dewitt S, Knauper V, Aeschlimann D (2015) P2X7 receptor activation regulates rapid unconventional export of transglutaminase-2. J Cell Sci 128(24):4615–4628

Adinolfi E, Kim M, Young MT, Di Virgilio F, Surprenant A (2003) Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. J Biol Chem 278(39):37344–37351

Adinolfi E, Pizzirani C, Idzko M, Panther E, Norgauer J, Di Virgilio F, Ferrari D (2005) P2X7 receptor: death or life? Purinergic Signal 1(3):219–227

Adinolfi E, Cirillo M, Woltersdorf R, Falzoni S, Chiozzi P, Pellegatti P, Callegari MG, Sandona D, Markwardt F, Schmalzing G, Di Virgilio F (2010) Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. FASEB J 24 (9):3393–3404

Adriouch S, Dox C, Welge V, Seman M, Koch-Nolte F, Haag F (2002) Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X7 receptor. J Immunol 169(8):4108–4112

Adriouch S, Dubberke G, Diessenbacher P, Rassendren F, Seman M, Haag F, Koch-Nolte F (2005) Probing the

expression and function of the P2X7 purinoceptor with antibodies raised by genetic immunization. Cell Immunol 236(1–2):72–77

Adriouch S, Bannas P, Schwarz N, Fliegert R, Guse AH, Seman M, Haag F, Koch-Nolte F (2008) ADP-ribosylation at R125 gates the P2X7 ion channel by presenting a covalent ligand to its nucleotide binding site. FASEB J 22(3):861–869

Adriouch S, Scheuplein F, Bahring R, Seman M, Boyer O, Koch-Nolte F, Haag F (2009) Characterisation of the R276A gain-of-function mutation in the ectodomain of murine P2X7. Purinergic Signal 5(2):151–161

Agrawal A, Gartland A (2015) P2X7 receptors: role in bone cell formation and function. J Mol Endocrinol 54 (2):R75–R88

Ahmadi M, Nowroozi A, Shahlaei M (2015) Constructing an atomic-resolution model of human P2X7 receptor followed by pharmacophore modeling to identify potential inhibitors. J Mol Graph Model 61:243–261

Allsopp RC, Evans RJ (2015) Contribution of the juxtatransmembrane intracellular regions to the time course and permeation of ATP-gated P2X7 receptor ion channels. J Biol Chem 290(23):14556–14566

Allsopp RC, Dayl S, Schmid R, Evans RJ (2017) Unique residues in the ATP gated human P2X7 receptor define a novel allosteric binding pocket for the selective antagonist AZ10606120. Sci Rep 7(1):725

Alves LA, de Melo Reis RA, de Souza CA, de Freitas MS, Teixeira PC, Neto Moreira Ferreira D, Xavier RF (2014) The P2X7 receptor: shifting from a low- to a high-conductance channel – an enigmatic phenomenon? Biochim Biophys Acta 1838(10):2578–2587

Amoroso F, Falzoni S, Adinolfi E, Ferrari D, Di Virgilio F (2012) The P2X7 receptor is a key modulator of aerobic glycolysis. Cell Death Dis 3:e370

Amstrup J, Novak I (2003) P2X7 receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca2+ influx. Biochem J 374 (Pt 1):51–61

Anderson CM, Nedergaard M (2006) Emerging challenges of assigning P2X7 receptor function and immunoreactivity in neurons. Trends Neurosci 29 (5):257–262

Antonio LS, Stewart AP, Xu XJ, Varanda WA, Murrell-Lagnado RD, Edwardson JM (2011) P2X4 receptors interact with both P2X2 and P2X7 receptors in the form of homotrimers. Br J Pharmacol 163 (5):1069–1077

Apolloni S, Amadio S, Parisi C, Matteucci A, Potenza RL, Armida M, Popoli P, D'Ambrosi N, Volonte C (2014) Spinal cord pathology is ameliorated by P2X7 antagonism in a SOD1-mutant mouse model of amyotrophic lateral sclerosis. Dis Model Mech 7(9):1101–1109

Aprile-Garcia F, Metzger MW, Paez-Pereda M, Stadler H, Acuna M, Liberman AC, Senin SA, Gerez J, Hoijman E, Refojo D, Mitkovski M, Panhuysen M, Stuhmer W, Holsboer F, Deussing JM, Arzt E (2016) Co-Expression of Wild-Type P2X7R with Gln460Arg Variant Alters Receptor Function. PLoS One 11(3):e0151862

Assandri R, Mazzanti M (1997) Ionic permeability on isolated mouse liver nuclei: influence of ATP and Ca2+. J Membr Biol 157(3):301–309

Atkinson L, Milligan CJ, Buckley NJ, Deuchars J (2002) An ATP-gated ion channel at the cell nucleus. Nature 420(6911):42

Babelova A, Moreth K, Tsalastra-Greul W, Zeng-Brouwers J, Eickelberg O, Young MF, Bruckner P, Pfeilschifter J, Schaefer RM, Grone HJ, Schaefer L (2009) Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. J Biol Chem 284(36):24035–24048

Backlund L, Lavebratt C, Frisen L, Nikamo P, Hukic Sudic D, Traskman-Bendz L, Landen M, Edman G, Vawter MP, Osby U, Schalling M (2012) P2RX7: expression responds to sleep deprivation and associates with rapid cycling in bipolar disorder type 1. PLoS One 7(8):e43057

Bannas P, Adriouch S, Kahl S, Braasch F, Haag F, Koch-Nolte F (2005) Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts. Blood 105 (9):3663–3670

Barbera-Cremades M, Baroja-Mazo A, Gomez AI, Machado F, Di Virgilio F, Pelegrin P (2012) P2X7 receptor-stimulation causes fever via PGE2 and IL-1beta release. FASEB J 26(7):2951–2962

Barden JA, Sluyter R, Gu BJ, Wiley JS (2003) Specific detection of non-functional human P2X7 receptors in HEK293 cells and B-lymphocytes. FEBS Lett 538 (1–3):159–162

Baroni M, Pizzirani C, Pinotti M, Ferrari D, Adinolfi E, Calzavarini S, Caruso P, Bernardi F, Di Virgilio F (2007) Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles. FASEB J 21(8):1926–1933

Barth K, Weinhold K, Guenther A, Young MT, Schnittler H, Kasper M (2007) Caveolin-1 influences P2X7 receptor expression and localization in mouse lung alveolar epithelial cells. FEBS J 274 (12):3021–3033

Barth K, Weinhold K, Guenther A, Linge A, Gereke M, Kasper M (2008) Characterization of the molecular interaction between caveolin-1 and the P2X receptors 4 and 7 in E10 mouse lung alveolar epithelial cells. Int J Biochem Cell Biol 40(10):2230–2239

Bartlett R, Stokes L, Sluyter R (2014) The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. Pharmacol Rev 66 (3):638–675

Bartlett R, Sluyter V, Watson D, Sluyter R, Yerbury JJ (2017) P2X7 antagonism using Brilliant Blue G reduces body weight loss and prolongs survival in female SOD1G93A amyotrophic lateral sclerosis mice. PeerJ 5:e3064

Basso AM, Bratcher NA, Harris RR, Jarvis MF, Decker MW, Rueter LE (2009) Behavioral profile of P2X7

receptor knockout mice in animal models of depression and anxiety: relevance for neuropsychiatric disorders. Behav Brain Res 198(1):83–90

Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 183(2):787–791

Beaucage KL, Xiao A, Pollmann SI, Grol MW, Beach RJ, Holdsworth DW, Sims SM, Darling MR, Dixon SJ (2014) Loss of P2X7 nucleotide receptor function leads to abnormal fat distribution in mice. Purinergic Signal 10(2):291–304

Beigi RD, Kertesy SB, Aquilina G, Dubyak GR (2003) Oxidized ATP (oATP) attenuates proinflammatory signaling via P2 receptor-independent mechanisms. Br J Pharmacol 140(3):507–519

Bhaskaracharya A, Dao-Ung P, Jalilian I, Spildrejorde M, Skarratt KK, Fuller SJ, Sluyter R, Stokes L (2014) Probenecid blocks human P2X7 receptor-induced dye uptake via a pannexin-1 independent mechanism. PLoS One 9(3):e93058

Bhattacharya A, Biber K (2016) The microglial ATP-gated ion channel P2X7 as a CNS drug target. Glia 64(10):1772–1787

Bhattacharya A, Wang Q, Ao H, Shoblock JR, Lord B, Aluisio L, Fraser I, Nepomuceno D, Neff RA, Welty N, Lovenberg TW, Bonaventure P, Wickenden AD, Letavic MA (2013) Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567. Br J Pharmacol 170 (3):624–640

Bianchi BR, Lynch KJ, Touma E, Niforatos W, Burgard EC, Alexander KM, Park HS, Yu H, Metzger R, Kowaluk E, Jarvis MF, van Biesen T (1999) Pharmacological characterization of recombinant human and rat P2X receptor subtypes. Eur J Pharmacol 376 (1–2):127–138

Bilodeau MS, Arguin G, Gendron FP (2015) C/EBPbeta regulates P2X7 receptor expression in response to glucose challenge in intestinal epithelial cells. Biochem Cell Biol 93(1):38–46

Biswas D, Qureshi OS, Lee WY, Croudace JE, Mura M, Lammas DA (2008) ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages. BMC Immunol 9:35

Bo X, Jiang LH, Wilson HL, Kim M, Burnstock G, Surprenant A, North RA (2003) Pharmacological and biophysical properties of the human P2X5 receptor. Mol Pharmacol 63(6):1407–1416

Boldrini L, Giordano M, Ali G, Melfi F, Romano G, Lucchi M, Fontanini G (2015) P2X7 mRNA expression in non-small cell lung cancer: microRNA regulation and prognostic value. Oncol Lett 9(1):449–453

Booth JW, Tam FW, Unwin RJ (2012) P2 purinoceptors: renal pathophysiology and therapeutic potential. Clin Nephrol 78(2):154–163

Boue-Grabot E, Archambault V, Seguela P (2000) A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X2 ATP-gated channels. J Biol Chem 275 (14):10190–10195

Boumechache M, Masin M, Edwardson JM, Gorecki DC, Murrell-Lagnado R (2009) Analysis of assembly and trafficking of native P2X4 and P2X7 receptor complexes in rodent immune cells. J Biol Chem 284 (20):13446–13454

Boyce AK, Kim MS, Wicki-Stordeur LE, Swayne LA (2015) ATP stimulates pannexin 1 internalization to endosomal compartments. Biochem J 470(3):319–330

Bradley HJ, Liu X, Collins V, Owide J, Goli GR, Smith M, Surprenant A, White SJ, Jiang LH (2010) Identification of an intracellular microdomain of the P2X7 receptor that is crucial in basolateral membrane targeting in epithelial cells. FEBS Lett 584 (23):4740–4744

Bradley HJ, Browne LE, Yang W, Jiang LH (2011a) Pharmacological properties of the rhesus macaque monkey P2X7 receptor. Br J Pharmacol 164 (2b):743–754

Bradley HJ, Baldwin JM, Goli GR, Johnson B, Zou J, Sivaprasadarao A, Baldwin SA, Jiang LH (2011b) Residues 155 and 348 contribute to the determination of P2X7 receptor function via distinct mechanisms revealed by single-nucleotide polymorphisms. J Biol Chem 286(10):8176–8187

Browne LE, Compan V, Bragg L, North RA (2013) P2X7 receptor channels allow direct permeation of nanometer-sized dyes. J Neurosci 33(8):3557–3566

Buell GN, Talabot F, Gos A, Lorenz J, Lai E, Morris MA, Antonarakis SE (1998a) Gene structure and chromosomal localization of the human P2X7 receptor. Receptors Channels 5(6):347–354

Buell G, Chessell IP, Michel AD, Collo G, Salazzo M, Herren S, Gretener D, Grahames C, Kaur R, Kosco-Vilbois MH, Humphrey PP (1998b) Blockade of human P2X7 receptor function with a monoclonal antibody. Blood 92(10):3521–3528

Burnstock G (2015) Intracellular expression of purinoceptors. Purinergic Signal 11(3):275–276

Burnstock G, Kennedy C (2011) P2X receptors in health and disease. Adv Pharmacol 61:333–372

Burnstock G, Ralevic V (2014) Purinergic signaling and blood vessels in health and disease. Pharmacol Rev 66 (1):102–192

Cabrini G, Falzoni S, Forchap SL, Pellegatti P, Balboni A, Agostini P, Cuneo A, Castoldi G, Baricordi OR, Di Virgilio F (2005) A His-155 to Tyr polymorphism confers gain-of-function to the human P2X7 receptor of human leukemic lymphocytes. J Immunol 175 (1):82–89

Caseley EA, Muench SP, Roger S, Mao HJ, Baldwin SA, Jiang LH (2014) Non-synonymous single nucleotide

polymorphisms in the P2X receptor genes: association with diseases, impact on receptor functions and potential use as diagnosis biomarkers. Int J Mol Sci 15 (8):13344–13371

Cesaro A, Brest P, Hofman V, Hebuterne X, Wildman S, Ferrua B, Marchetti S, Doglio A, Vouret-Craviari V, Galland F, Naquet P, Mograbi B, Unwin R, Hofman P (2010) Amplification loop of the inflammatory process is induced by P2X7R activation in intestinal epithelial cells in response to neutrophil transepithelial migration. Am J Physiol Gastrointest Liver Physiol 299(1):G32–G42

Chakfe Y, Seguin R, Antel JP, Morissette C, Malo D, Henderson D, Seguela P (2002) ADP and AMP induce interleukin-1beta release from microglial cells through activation of ATP-primed P2X7 receptor channels. J Neurosci 22(8):3061–3069

Chaumont S, Jiang LH, Penna A, North RA, Rassendren F (2004) Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. J Biol Chem 279(28):29628–29638

Cheewatrakoolpong B, Gilchrest H, Anthes JC, Greenfeder S (2005) Identification and characterization of splice variants of the human P2X7 ATP channel. Biochem Biophys Res Commun 332(1):17–27

Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, Isakson BE, Bayliss DA, Ravichandran KS (2010) Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature 467(7317):863–867

Chen Q, Jin Y, Zhang K, Li H, Chen W, Meng G, Fang X (2014) Alarmin HNP-1 promotes pyroptosis and IL-1beta release through different roles of NLRP3 inflammasome via P2X7 in LPS-primed macrophages. Innate Immun 20(3):290–300

Chessell IP, Simon J, Hibell AD, Michel AD, Barnard EA, Humphrey PP (1998) Cloning and functional characterisation of the mouse P2X7 receptor. FEBS Lett 439(1–2):26–30

Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN (2005) Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114(3):386–396

Chong JH, Zheng GG, Ma YY, Zhang HY, Nie K, Lin YM, Wu KF (2010) The hyposensitive N187D P2X7 mutant promotes malignant progression in nude mice. J Biol Chem 285(46):36179–36187

Cockcroft S, Gomperts BD (1979) ATP induces nucleotide permeability in rat mast cells. Nature 279 (5713):541–542

Cockcroft S, Gomperts BD (1980) The ATP4- receptor of rat mast cells. Biochem J 188(3):789–798

Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North RA, Buell G (1997) Tissue distribution of the P2X7 receptor. Neuropharmacology 36(9):1277–1283

Compan V, Ulmann L, Stelmashenko O, Chemin J, Chaumont S, Rassendren F (2012) P2X2 and P2X5 subunits define a new heteromeric receptor with P2X7-like properties. J Neurosci 32(12):4284–4296

Connon CJ, Young RD, Kidd EJ (2003) P2X7 receptors are redistributed on human monocytes after pore formation in response to prolonged agonist exposure. Pharmacology 67(3):163–168

Costa-Junior HM, Sarmento Vieira F, Coutinho-Silva R (2011) C terminus of the P2X7 receptor: treasure hunting. Purinergic Signal 7(1):7–19

Dagvadorj J, Shimada K, Chen S, Jones HD, Tumurkhuu G, Zhang W, Wawrowsky KA, Crother TR, Arditi M (2015) Lipopolysaccharide induces alveolar macrophage necrosis via CD14 and the P2X7 receptor leading to interleukin-1alpha release. Immunity 42(4):640–653

Danquah W, Meyer-Schwesinger C, Rissiek B, Pinto C, Serracant-Prat A, Amadi M, Iacenda D, Knop JH, Hammel A, Bergmann P, Schwarz N, Assuncao J, Rotthier W, Haag F, Tolosa E, Bannas P, Boue-Grabot E, Magnus T, Laeremans T, Stortelers C, Koch-Nolte F (2016) Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. Sci Transl Med 8(366):366ra162

De Marchi E, Orioli E, Dal Ben D, Adinolfi E (2016) P2X7 eceptor as a therapeutic target. Adv Protein Chem Struct Biol 104:39–79

Deniz E, Erman B (2017) Long noncoding RNA (lincRNA), a new paradigm in gene expression control. Funct Integr Genomics 17(2–3):135–143

Denlinger LC, Fisette PL, Sommer JA, Watters JJ, Prabhu U, Dubyak GR, Proctor RA, Bertics PJ (2001) Cutting edge: the nucleotide receptor P2X7 contains multiple protein- and lipid-interaction motifs including a potential binding site for bacterial lipopolysaccharide. J Immunol 167(4):1871–1876

Denlinger LC, Sommer JA, Parker K, Gudipaty L, Fisette PL, Watters JW, Proctor RA, Dubyak GR, Bertics PJ (2003) Mutation of a dibasic amino acid motif within the C terminus of the P2X7 nucleotide receptor results in trafficking defects and impaired function. J Immunol 171(3):1304–1311

Denlinger LC, Coursin DB, Schell K, Angelini G, Green DN, Guadarrama AG, Halsey J, Prabhu U, Hogan KJ, Bertics PJ (2006) Human P2X7 pore function predicts allele linkage disequilibrium. Clin Chem 52 (6):995–1004

Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF (2009a) Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. Br J Pharmacol 157(7):1203–1214

Donnelly-Roberts DL, Namovic MT, Surber B, Vaidyanathan SX, Perez-Medrano A, Wang Y, Carroll WA, Jarvis MF (2009b) [3H]A-804598 ([3H]2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine) is a novel, potent, and selective antagonist radioligand for P2X7 receptors. Neuropharmacology 56(1):223–229

Dubyak GR (2009) Both sides now: multiple interactions of ATP with pannexin-1 hemichannels. Focus on "A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP". Am J Physiol Cell Physiol 296(2):C235–C241

Dubyak GR (2012) P2X7 receptor regulation of non-classical secretion from immune effector cells. Cell Microbiol 14(11):1697–1706

Elssner A, Duncan M, Gavrilin M, Wewers MD (2004) A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. J Immunol 172(8):4987–4994

Engel T, Brennan GP, Sanz-Rodriguez A, Alves M, Beamer E, Watters O, Henshall DC, Jimenez-Mateos EM (2017) A calcium-sensitive feed-forward loop regulating the expression of the ATP-gated purinergic P2X7 receptor via specificity protein 1 and microRNA-22. Biochim Biophys Acta 1864 (2):255–266

Eser A, Colombel JF, Rutgeerts P, Vermeire S, Vogelsang H, Braddock M, Persson T, Reinisch W (2015) Safety and efficacy of an oral inhibitor of the purinergic receptor P2X7 in adult patients with moderately to severely active Crohn's disease: a randomized placebo-controlled, double-blind, phase IIa study. Inflamm Bowel Dis 21(10):2247–2253

Evans RJ, Lewis C, Buell G, Valera S, North RA, Surprenant A (1995) Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors). Mol Pharmacol 48 (2):178–183

Fairbairn IP, Stober CB, Kumararatne DS, Lammas DA (2001) ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X7-dependent process inducing bacterial death by phagosome-lysosome fusion. J Immunol 167(6):3300–3307

Fang KM, Yang CS, Sun SH, Tzeng SF (2009) Microglial phagocytosis attenuated by short-term exposure to exogenous ATP through P2X receptor action. J Neurochem 111(5):1225–1237

Faulks M, Kuit TA, Sophocleous RA, Curtis BL, Curtis SJ, Jurak LM, Sluyter R (2016) P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes. World J Hematol 5(4):88–93

Feng YH, Wang L, Wang Q, Li X, Zeng R, Gorodeski GI (2005) ATP stimulates GRK-3 phosphorylation and beta-arrestin-2-dependent internalization of P2X7 receptor. Am J Physiol Cell Physiol 288(6):C1342–C1356

Feng YH, Li X, Wang L, Zhou L, Gorodeski GI (2006) A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. J Biol Chem 281(25):17228–17237

Feng JF, Gao XF, Pu YY, Burnstock G, Xiang Z, He C (2015) P2X7 receptors and Fyn kinase mediate ATP-induced oligodendrocyte progenitor cell migration. Purinergic Signal 11(3):361–369

Ferrari D, Pizzirani C, Adinolfi E, Forchap S, Sitta B, Turchet L, Falzoni S, Minelli M, Baricordi R, Di Virgilio F (2004) The antibiotic polymyxin B modulates P2X7 receptor function. J Immunol 173 (7):4652–4660

Ferrari D, Bianchi N, Eltzschig HK, Gambari R (2016) MicroRNAs Modulate the Purinergic Signaling Network. Trends Mol Med 22(10):905–918

Fonfria E, Clay WC, Levy DS, Goodwin JA, Roman S, Smith GD, Condreay JP, Michel AD (2008) Cloning and pharmacological characterization of the guinea pig P2X7 receptor orthologue. Br J Pharmacol 153 (3):544–556

Franceschini A, Capece M, Chiozzi P, Falzoni S, Sanz JM, Sarti AC, Bonora M, Pinton P, Di Virgilio F (2015) The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. FASEB J 29 (6):2450–2461

Franco MC, Ye Y, Refakis CA, Feldman JL, Stokes AL, Basso M, Melero Fernandez de Mera RM, Sparrow NA, Calingasan NY, Kiaei M, Rhoads TW, Ma TC, Grumet M, Barnes S, Beal MF, Beckman JS, Mehl R, Estevez AG (2013) Nitration of Hsp90 induces cell death. Proc Natl Acad Sci U S A 110(12):E1102–E1111

Fuller SJ, Stokes L, Skarratt KK, Gu BJ, Wiley JS (2009) Genetics of the P2X7 receptor and human disease. Purinergic Signal 5(2):257–262

Furlan-Freguia C, Marchese P, Gruber A, Ruggeri ZM, Ruf W (2011) P2X7 receptor signaling contributes to tissue factor-dependent thrombosis in mice. J Clin Invest 121(7):2932–2944

Gangadharan V, Nohe A, Caplan J, Czymmek K, Duncan RL (2015) Caveolin-1 regulates P2X7 receptor signaling in osteoblasts. Am J Physiol Cell Physiol 308(1): C41–C50

Garcia-Huerta P, Diaz-Hernandez M, Delicado EG, Pimentel-Santillana M, Miras-Portugal MT, Gomez-Villafuertes R (2012) The specificity protein factor Sp1 mediates transcriptional regulation of P2X7 receptors in the nervous system. J Biol Chem 287 (53):44628–44644

Garcia-Marcos M, Pochet S, Tandel S, Fontanils U, Astigarraga E, Fernandez-Gonzalez JA, Kumps A, Marino A, Dehaye JP (2006a) Characterization and comparison of raft-like membranes isolated by two different methods from rat submandibular gland cells. Biochim Biophys Acta 1758(6):796–806

Garcia-Marcos M, Perez-Andres E, Tandel S, Fontanils U, Kumps A, Kabre E, Gomez-Munoz A, Marino A, Dehaye JP, Pochet S (2006b) Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. J Lipid Res 47(4):705–714

Garcia-Marcos M, Dehaye JP, Marino A (2009) Membrane compartments and purinergic signalling: the role of plasma membrane microdomains in the modulation of P2XR-mediated signalling. FEBS J 276 (2):330–340

Geraghty NJ, Watson D, Adhikary SR, Sluyter R (2016) P2X7 receptor in skin biology and diseases. World J Dermatol 5(2):72–83

Gilbert SM, Gidley Baird A, Glazer S, Barden JA, Glazer A, Teh LC, King J (2017) A Phase 1 clinical trial demonstrates nfP2X7 targeted antibodies provide a novel, safe and tolerable topical therapy for BCC. Br J Dermatol doi: 10.1111/bjd.15364

Giuliani AL, Sarti AC, Falzoni S, Di Virgilio F (2017) The P2X7 Receptor-Interleukin-1 Liaison. Front Pharmacol 8:123

Glaser T, de Oliveira SL, Cheffer A, Beco R, Martins P, Fornazari M, Lameu C, Junior HM, Coutinho-Silva R, Ulrich H (2014) Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. PLoS One 9(5):e96281

Gomez-Villafuertes R, Garcia-Huerta P, Diaz-Hernandez JI, Miras-Portugal MT (2015) PI3K/Akt signaling pathway triggers P2X7 receptor expression as a pro-survival factor of neuroblastoma cells under limiting growth conditions. Sci Rep 5:18417

Gonnord P, Delarasse C, Auger R, Benihoud K, Prigent M, Cuif MH, Lamaze C, Kanellopoulos JM (2009) Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. FASEB J 23(3):795–805

Gordon JL (1986) Extracellular ATP: effects, sources and fate. Biochem J 233(2):309–319

Grol MW, Zelner I, Dixon SJ (2012) P2X7-mediated calcium influx triggers a sustained, PI3K-dependent increase in metabolic acid production by osteoblast-like cells. Am J Physiol Endocrinol Metab 302(5): E561–E575

Groschel-Stewart U, Bardini M, Robson T, Burnstock G (1999) Localisation of P2X5 and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. Cell Tissue Res 296(3):599–605

Gu BJ, Wiley JS (2006) Rapid ATP-induced release of matrix metalloproteinase 9 is mediated by the P2X7 receptor. Blood 107(12):4946–4953

Gu BJ, Zhang WY, Bendall LJ, Chessell IP, Buell GN, Wiley JS (2000) Expression of P2X7 purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X7 receptors. Am J Physiol Cell Physiol 279(4):C1189–C1197

Gu BJ, Zhang W, Worthington RA, Sluyter R, Dao-Ung P, Petrou S, Barden JA, Wiley JS (2001) A Glu-496 to Ala polymorphism leads to loss of function of the human P2X7 receptor. J Biol Chem 276 (14):11135–11142

Gu BJ, Rathsam C, Stokes L, McGeachie AB, Wiley JS (2009) Extracellular ATP dissociates nonmuscle myosin from P2X7 complex: this dissociation regulates P2X7 pore formation. Am J Physiol Cell Physiol 297 (2):C430–C439

Gu BJ, Saunders BM, Jursik C, Wiley JS (2010) The P2X7-nonmuscle myosin membrane complex regulates phagocytosis of nonopsonized particles and bacteria by a pathway attenuated by extracellular ATP. Blood 115(8):1621–1631

Gu BJ, Saunders BM, Petrou S, Wiley JS (2011) P2X7 is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. J Immunol 187 (5):2365–2375

Gu BJ, Baird PN, Vessey KA, Skarratt KK, Fletcher EL, Fuller SJ, Richardson AJ, Guymer RH, Wiley JS (2013) A rare functional haplotype of the P2RX4 and P2RX7 genes leads to loss of innate phagocytosis and confers increased risk of age-related macular degeneration. FASEB J 27(4):1479–1487

Gu BJ, Field J, Dutertre S, Ou A, Kilpatrick TJ, Lechner-Scott J, Scott R, Lea R, Taylor BV, Stankovich J, Butzkueven H, Gresle M, Laws SM, Petrou S, Hoffjan S, Akkad DA, Graham CA, Hawkins S, Glaser A, Bedri SK, Hillert J, Matute C, Antiguedad A, Wiley JS (2015a) A rare P2X7 variant Arg307Gln with absent pore formation function protects against neuroinflammation in multiple sclerosis. Hum Mol Genet 24(19):5644–5654

Gu BJ, Lovelace MD, Weible MW, 2nd, Allen DG, Eamegdool SS, Chan-Ling T, Wiley JS (2015b) P2X7 is an archaic scavenger receptor recognizing apoptotic neuroblasts in early human neurogenesis. Recept Clin Investig 2:e699

Gudipaty L, Humphreys BD, Buell G, Dubyak GR (2001) Regulation of P2X7 nucleotide receptor function in human monocytes by extracellular ions and receptor density. Am J Physiol Cell Physiol 280(4):C943–C953

Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD (2007) Evidence for functional P2X4/P2X7 heteromeric receptors. Mol Pharmacol 72 (6):1447–1456

Guzman-Aranguez A, Perez de Lara MJ, Pintor J (2017) Hyperosmotic stress induces ATP release and changes in P2X7 receptor levels in human corneal and conjunctival epithelial cells. Purinergic Signal 13(2):249–258

Haanes KA, Schwab A, Novak I (2012) The P2X7 receptor supports both life and death in fibrogenic pancreatic stellate cells. PLoS One 7(12):e51164

Hanley PJ, Kronlage M, Kirschning C, del Rey A, Di Virgilio F, Leipziger J, Chessell IP, Sargin S, Filippov MA, Lindemann O, Mohr S, Konigs V, Schillers H, Bahler M, Schwab A (2012) Transient P2X7 receptor activation triggers macrophage death independent of Toll-like receptors 2 and 4, caspase-1, and pannexin-1 proteins. J Biol Chem 287(13):10650–10663

Hashimoto-Hill S, Friesen L, Kim M, Kim CH (2017) Contraction of intestinal effector T cells by retinoic acid-induced purinergic receptor P2X7. Mucosal Immunol 10(4):912–923

Hattori M, Gouaux E (2012) Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature 485(7397):207–212

He YQ, Chen J, Lu XJ, Shi YH (2013) Characterization of P2X7R and its function in the macrophages of ayu, Plecoglossus altivelis. PLoS One 8(2):e57505

Hedden L, Benes CH, Soltoff SP (2011) P2X7 receptor antagonists display agonist-like effects on cell signaling proteins. Biochim Biophys Acta 1810(5):532–542

Heiss K, Janner N, Mahnss B, Schumacher V, Koch-Nolte F, Haag F, Mittrucker HW (2008) High sensitivity of intestinal CD8+ T cells to nucleotides indicates P2X7 as a regulator for intestinal T cell responses. J Immunol 181(6):3861–3869

Helliwell RM, ShioukHuey CO, Dhuna K, Molero JC, Ye JM, Xue CC, Stokes L (2015) Selected ginsenosides of the protopanaxdiol series are novel positive allosteric modulators of P2X7 receptors. Br J Pharmacol 172 (13):3326–3340

Hibell AD, Thompson KM, Xing M, Humphrey PP, Michel AD (2001) Complexities of measuring antagonist potency at P2X7 receptor orthologs. J Pharmacol Exp Ther 296(3):947–957

Hong S, Schwarz N, Brass A, Seman M, Haag F, Koch-Nolte F, Schilling WP, Dubyak GR (2009) Differential regulation of P2X7 receptor activation by extracellular nicotinamide adenine dinucleotide and ecto-ADP-ribosyltransferases in murine macrophages and T cells. J Immunol 183(1):578–592

Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP, Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF (2006) A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. J Pharmacol Exp Ther 319(3):1376–1385

Huang S, Chen Y, Wu W, Ouyang N, Chen J, Li H, Liu X, Su F, Lin L, Yao Y (2013) miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. PLoS One 8(12):e80707

Hung SC, Choi CH, Said-Sadier N, Johnson L, Atanasova KR, Sellami H, Yilmaz O, Ojcius DM (2013) P2X4 assembles with P2X7 and pannexin-1 in gingival epithelial cells and modulates ATP-induced reactive oxygen species production and inflammasome activation. PLoS One 8(7):e70210

Husted LB, Harslof T, Stenkjaer L, Carstens M, Jorgensen NR, Langdahl BL (2013) Functional polymorphisms in the P2X7 receptor gene are associated with osteoporosis. Osteoporos Int 24(3):949–959

Ide S, Nishizawa D, Fukuda K, Kasai S, Hasegawa J, Hayashida M, Minami M, Ikeda K (2014) Haplotypes of P2RX7 gene polymorphisms are associated with both cold pain sensitivity and analgesic effect of fentanyl. Mol Pain 10:75

Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, Scemes E (2008) P2X7 receptor-Pannexin1 complex: pharmacology and signaling. Am J Physiol Cell Physiol 295(3):C752–C760

Jacob F, Perez Novo C, Bachert C, Van Crombruggen K (2013) Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses. Purinergic Signal 9 (3):285–306

Jiang LH, Mackenzie AB, North RA, Surprenant A (2000) Brilliant blue G selectively blocks ATP-gated rat P2X7 receptors. Mol Pharmacol 58(1):82–88

Jiang LH, Rassendren F, Mackenzie A, Zhang YH, Surprenant A, North RA (2005) N-methyl-D-glucamine and propidium dyes utilize different permeation pathways at rat P2X7 receptors. Am J Physiol Cell Physiol 289(5):C1295–C1302

Jiang H, Zhu AG, Mamczur M, Falck JR, Lerea KM, McGiff JC (2007) Stimulation of rat erythrocyte P2X7 receptor induces the release of epoxyeicosatrienoic acids. Br J Pharmacol 151(7):1033–1040

Jiang LH, Baldwin JM, Roger S, Baldwin SA (2013) Insights into the molecular mechanisms underlying mammalian P2X7 receptor functions and contributions in diseases, revealed by structural modeling and single nucleotide polymorphisms. Front Pharmacol 4:55

Jiang W, Lv H, Wang H, Wang D, Sun S, Jia Q, Wang P, Song B, Ni L (2015a) Activation of the NLRP3/caspase-1 inflammasome in human dental pulp tissue and human dental pulp fibroblasts. Cell Tissue Res 361(2):541–555

Jiang T, Hoekstra J, Heng X, Kang W, Ding J, Liu J, Chen S, Zhang J (2015b) P2X7 receptor is critical in alpha-synuclein--mediated microglial NADPH oxidase activation. Neurobiol Aging 36(7):2304–2318

Jiang LH, Hao Y, Mousawi F, Peng H, Yang X (2017) Expression of P2 purinergic receptors in mesenchymal stem cells and their roles in extracellular nucleotide regulation of cell functions. J Cell Physiol 232 (2):287–297

Jimenez-Mateos EM, Arribas-Blazquez M, Sanz-Rodriguez A, Concannon C, Olivos-Ore LA, Reschke CR, Mooney CM, Mooney C, Lugara E, Morgan J, Langa E, Jimenez-Pacheco A, Silva LF, Mesuret G, Boison D, Miras-Portugal MT, Letavic M, Artalejo AR, Bhattacharya A, Diaz-Hernandez M, Henshall DC, Engel T (2015) microRNA targeting of the P2X7 purinoceptor opposes a contralateral epileptogenic focus in the hippocampus. Sci Rep 5:17486

Jindrichova M, Kuzyk P, Li S, Stojilkovic SS, Zemkova H (2012) Conserved ectodomain cysteines are essential for rat P2X7 receptor trafficking. Purinergic Signal 8 (2):317–325

Jorgensen NR, Husted LB, Skarratt KK, Stokes L, Tofteng CL, Kvist T, Jensen JE, Eiken P, Brixen K, Fuller S, Clifton-Bligh R, Gartland A, Schwarz P, Langdahl BL, Wiley JS (2012) Single-nucleotide polymorphisms in the P2X7 receptor gene are associated with post-menopausal bone loss and vertebral fractures. Eur J Hum Genet 20(6):675–681

Kanjanamekanant K, Luckprom P, Pavasant P (2014) P2X7 receptor-Pannexin1 interaction mediates stress-induced interleukin-1 beta expression in human

periodontal ligament cells. J Periodontal Res 49 (5):595–602

Karasawa A, Kawate T (2016) Structural basis for subtype-specific inhibition of the P2X7 receptor. elife 5:e22153

Kasuya G, Fujiwara Y, Takemoto M, Dohmae N, Nakada-Nakura Y, Ishitani R, Hattori M, Nureki O (2016) Structural insights into divalent cation modulations of ATP-gated P2X receptor channels. Cell Rep 14 (4):932–944

Kawano A, Tsukimoto M, Mori D, Noguchi T, Harada H, Takenouchi T, Kitani H, Kojima S (2012a) Regulation of P2X7-dependent inflammatory functions by P2X4 receptor in mouse macrophages. Biochem Biophys Res Commun 420(1):102–107

Kawano A, Tsukimoto M, Noguchi T, Hotta N, Harada H, Takenouchi T, Kitani H, Kojima S (2012b) Involvement of P2X4 receptor in P2X7 receptor-dependent cell death of mouse macrophages. Biochem Biophys Res Commun 419(2):374–380

Kawate T, Michel JC, Birdsong WT, Gouaux E (2009) Crystal structure of the ATP-gated P2X4 ion channel in the closed state. Nature 460(7255):592–598

Keller R (1966) Tissue mast cells in immune reactions. Monogr Allergy 2:1–144

Keystone EC, Wang MM, Layton M, Hollis S, McInnes IB (2012) Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine. Ann Rheum Dis 71 (10):1630–1635

Khadra A, Tomic M, Yan Z, Zemkova H, Sherman A, Stojilkovic SS (2013) Dual gating mechanism and function of P2X7 receptor channels. Biophys J 104 (12):2612–2621

Khakh BS, Bao XR, Labarca C, Lester HA (1999) Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. Nat Neurosci 2 (4):322–330

Kido Y, Kawahara C, Terai Y, Ohishi A, Kobayashi S, Hayakawa M, Kamatsuka Y, Nishida K, Nagasawa K (2014) Regulation of activity of P2X7 receptor by its splice variants in cultured mouse astrocytes. Glia 62 (3):440–451

Kim M, Jiang LH, Wilson HL, North RA, Surprenant A (2001) Proteomic and functional evidence for a P2X7 receptor signalling complex. EMBO J 20 (22):6347–6358

Klapperstuck M, Buttner C, Nickel P, Schmalzing G, Lambrecht G, Markwardt F (2000) Antagonism by the suramin analogue NF279 on human P2X1 and P2X7 receptors. Eur J Pharmacol 387(3):245–252

Kucenas S, Li Z, Cox JA, Egan TM, Voigt MM (2003) Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience 121 (4):935–945

Kuehnel MP, Rybin V, Anand PK, Anes E, Griffiths G (2009a) Lipids regulate P2X7-receptor-dependent actin assembly by phagosomes via ADP translocation and ATP synthesis in the phagosome lumen. J Cell Sci 122(Pt 4):499–504

Kuehnel MP, Reiss M, Anand PK, Treede I, Holzer D, Hoffmann E, Klapperstueck M, Steinberg TH, Markwardt F, Griffiths G (2009b) Sphingosine-1-phosphate receptors stimulate macrophage plasma-membrane actin assembly via ADP release, ATP synthesis and P2X7R activation. J Cell Sci 122 (Pt 4):505–512

Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW (2014) Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? Pflugers Arch 466(3):407–414

Kurashima Y, Amiya T, Nochi T, Fujisawa K, Haraguchi T, Iba H, Tsutsui H, Sato S, Nakajima S, Iijima H, Kubo M, Kunisawa J, Kiyono H (2012) Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. Nat Commun 3:1034

Kurashima Y, Amiya T, Fujisawa K, Shibata N, Suzuki Y, Kogure Y, Hashimoto E, Otsuka A, Kabashima K, Sato S, Sato T, Kubo M, Akira S, Miyake K, Kunisawa J, Kiyono H (2014) The enzyme Cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis. Immunity 40(4):530–541

Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS (1997) ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. Immunity 7(3):433–444

Lee HY, Bardini M, Burnstock G (2000) Distribution of P2X receptors in the urinary bladder and the ureter of the rat. J Urol 163(6):2002–2007

Lenertz LY, Wang Z, Guadarrama A, Hill LM, Gavala ML, Bertics PJ (2010) Mutation of putative N-linked glycosylation sites on the human nucleotide receptor P2X7 reveals a key residue important for receptor function. Biochemistry 49(22):4611–4619

Lenertz LY, Gavala ML, Zhu Y, Bertics PJ (2011) Transcriptional control mechanisms associated with the nucleotide receptor P2X7, a critical regulator of immunologic, osteogenic, and neurologic functions. Immunol Res 50(1):22–38

Li S, Tomic M, Stojilkovic SS (2011) Characterization of novel Pannexin 1 isoforms from rat pituitary cells and their association with ATP-gated P2X channels. Gen Comp Endocrinol 174(2):202–210

Li M, Silberberg SD, Swartz KJ (2013) Subtype-specific control of P2X receptor channel signaling by ATP and Mg2+. Proc Natl Acad Sci U S A 110(36):E3455–E3463

Li S, Li X, Coddou C, Geng X, Wei J, Sun J (2014) Molecular characterization and expression analysis of ATP-gated P2X7 receptor involved in Japanese

flounder (Paralichthys olivaceus) innate immune response. PLoS One 9(5):e96625

Li G, Zou L, Xie W, Wen S, Xie Q, Gao Y, Xu C, Xu H, Liu S, Wang S, Xue Y, Wu B, Lv Q, Ying M, Zhang X, Liang S (2016) The effects of NONRATT021972 lncRNA siRNA on PC12 neuronal injury mediated by P2X7 receptor after exposure to oxygen-glucose deprivation. Purinergic Signal 12 (3):479–487

Liang L, Schwiebert EM (2005) Large pore formation uniquely associated with P2X7 purinergic receptor channels. Focus on "Are second messengers crucial for opening the pore associated with P2X7 receptor?". Am J Physiol Cell Physiol 288(2):C240–C242

Liang X, Samways DS, Wolf K, Bowles EA, Richards JP, Bruno J, Dutertre S, DiPaolo RJ, Egan TM (2015) Quantifying Ca2+ current and permeability in ATP-gated P2X7 receptors. J Biol Chem 290 (12):7930–7942

Lin C, Ren S, Zhang L, Jin H, Sun J, Zuo Y (2012) Extracellular ATP induces CD44 shedding from macrophage-like P388D1 cells via the P2X7 receptor. Hematol Oncol 30(2):70–75

Liu X, Surprenant A, Mao HJ, Roger S, Xia R, Bradley H, Jiang LH (2008) Identification of key residues coordinating functional inhibition of P2X7 receptors by zinc and copper. Mol Pharmacol 73(1):252–259

Liu X, Ma W, Surprenant A, Jiang LH (2009) Identification of the amino acid residues in the extracellular domain of rat P2X7 receptor involved in functional inhibition by acidic pH. Br J Pharmacol 156 (1):135–142

Liu Y, Xiao Y, Li Z (2011) P2X7 receptor positively regulates MyD88-dependent NF-kappaB activation. Cytokine 55(2):229–236

Liu S, Zou L, Xie J, Xie W, Wen S, Xie Q, Gao Y, Li G, Zhang C, Xu C, Xu H, Wu B, Lv Q, Zhang X, Wang S, Xue Y, Liang S (2016) LncRNA NONRATT021972 siRNA regulates neuropathic pain behaviors in type 2 diabetic rats through the P2X7 receptor in dorsal root ganglia. Mol Brain 9:44

Locovei S, Scemes E, Qiu F, Spray DC, Dahl G (2007) Pannexin1 is part of the pore forming unit of the P2X7 receptor death complex. FEBS Lett 581(3):483–488

Lopez-Castejon G, Young MT, Meseguer J, Surprenant A, Mulero V (2007) Characterization of ATP-gated P2X7 receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin-1 beta. Mol Immunol 44(6):1286–1299

Lopez-Castejon G, Theaker J, Pelegrin P, Clifton AD, Braddock M, Surprenant A (2010) P2X7 receptor-mediated release of cathepsins from macrophages is a cytokine-independent mechanism potentially involved in joint diseases. J Immunol 185 (4):2611–2619

Lorden G, Sanjuan-Garcia I, de Pablo N, Meana C, Alvarez-Miguel I, Perez-Garcia MT, Pelegrin P, Balsinde J, Balboa MA (2017) Lipin-2 regulates NLRP3 inflammasome by affecting P2X7 receptor activation. J Exp Med 214(2):511–528

Lovelace MD, Gu BJ, Eamegdool SS, Weible MW 2nd, Wiley JS, Allen DG, Chan-Ling T (2015) P2X7 receptors mediate innate phagocytosis by human neural precursor cells and neuroblasts. Stem Cells 33 (2):526–541

Mansoor SE, Lu W, Oosterheert W, Shekhar M, Tajkhorshid E, Gouaux E (2016) X-ray structures define human P2X3 receptor gating cycle and antagonist action. Nature 538(7623):66–71

Masin M, Young C, Lim K, Barnes SJ, Xu XJ, Marschall V, Brutkowski W, Mooney ER, Gorecki DC, Murrell-Lagnado R (2012) Expression, assembly and function of novel C-terminal truncated variants of the mouse P2X7 receptor: re-evaluation of P2X7 knockouts. Br J Pharmacol 165(4):978–993

Matute C (2011) Glutamate and ATP signalling in white matter pathology. J Anat 219(1):53–64

Mazzanti M, Innocenti B, Rigatelli M (1994) ATP-dependent ionic permeability on nuclear envelope in in situ nuclei of Xenopus oocytes. FASEB J 8 (2):231–236

Mehta VB, Hart J, Wewers MD (2001) ATP-stimulated release of interleukin (IL)-1beta and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. J Biol Chem 276(6):3820–3826

Menzies J, Paul A, Kennedy C (2003) P2X7 subunit-like immunoreactivity in the nucleus of visceral smooth muscle cells of the guinea pig. Auton Neurosci 106 (2):103–109

Metzger MW, Walser SM, Aprile-Garcia F, Dedic N, Chen A, Holsboer F, Arzt E, Wurst W, Deussing JM (2017) Genetically dissecting P2rx7 expression within the central nervous system using conditional humanized mice. Purinergic Signal 13(2):153–170

Michel AD, Clay WC, Ng SW, Roman S, Thompson K, Condreay JP, Hall M, Holbrook J, Livermore D, Senger S (2008a) Identification of regions of the P2X7 receptor that contribute to human and rat species differences in antagonist effects. Br J Pharmacol 155 (5):738–751

Michel AD, Chambers LJ, Walter DS (2008b) Negative and positive allosteric modulators of the P2X7 receptor. Br J Pharmacol 153(4):737–750

Michel AD, Ng SW, Roman S, Clay WC, Dean DK, Walter DS (2009) Mechanism of action of species-selective P2X7 receptor antagonists. Br J Pharmacol 156(8):1312–1325

Migita K, Ozaki T, Shimoyama S, Yamada J, Nikaido Y, Furukawa T, Shiba Y, Egan TM, Ueno S (2016) HSP90 regulation of P2X7 receptor function requires an intact cytoplasmic C-terminus. Mol Pharmacol 90 (2):116–126

Miller CM, Boulter NR, Fuller SJ, Zakrzewski AM, Lees MP, Saunders BM, Wiley JS, Smith NC (2011) The role of the P2X7 receptor in infectious diseases. PLoS Pathog 7(11):e1002212

Minkiewicz J, de Rivero Vaccari JP, Keane RW (2013) Human astrocytes express a novel NLRP2 inflammasome. Glia 61(7):1113–1121

Moore SF, MacKenzie AB (2007) Murine macrophage P2X7 receptors support rapid prothrombotic responses. Cell Signal 19(4):855–866

Moura G, Lucena SV, Lima MA, Nascimento FD, Gesteira TF, Nader HB, Paredes-Gamero EJ, Tersariol I (2015a) Post-translational allosteric activation of the P2X7 receptor through glycosaminoglycan chains of CD44 proteoglycans. Cell Death Dis 1:15005

Moura GE, Lucena SV, Lima MA, Nascimento FD, Gesteira TF, Nader HB, Paredes-Gamero EJ, Tersariol IL (2015b) P2X7 receptor activity regulation: the role of CD44 proteoglycan GAG chains. Cell Death Dis 6: e1997

Nicke A (2008) Homotrimeric complexes are the dominant assembly state of native P2X7 subunits. Biochem Biophys Res Commun 377(3):803–808

Nicke A, Kuan YH, Masin M, Rettinger J, Marquez-Klaka B, Bender O, Gorecki DC, Murrell-Lagnado RD, Soto F (2009) A functional P2X7 splice variant with an alternative transmembrane domain 1 escapes gene inactivation in P2X7 knock-out mice. J Biol Chem 284(38):25813–25822

Norenberg W, Hempel C, Urban N, Sobottka H, Illes P, Schaefer M (2011) Clemastine potentiates the human P2X7 receptor by sensitizing it to lower ATP concentrations. J Biol Chem 286(13):11067–11081

Norenberg W, Sobottka H, Hempel C, Plotz T, Fischer W, Schmalzing G, Schaefer M (2012) Positive allosteric modulation by ivermectin of human but not murine P2X7 receptors. Br J Pharmacol 167(1):48–66

Norris PC, Gosselin D, Reichart D, Glass CK, Dennis EA (2014) Phospholipase A2 regulates eicosanoid class switching during inflammasome activation. Proc Natl Acad Sci U S A 111(35):12746–12751

North RA (2016) P2X receptors. Philos Trans R Soc Lond Ser B Biol Sci 371(1700):20150427

Novak I, Jans IM, Wohlfahrt L (2010) Effect of P2X7 receptor knockout on exocrine secretion of pancreas, salivary glands and lacrimal glands. J Physiol 588 (Pt 18):3615–3627

Orellano EA, Rivera OJ, Chevres M, Chorna NE, Gonzalez FA (2010) Inhibition of neuronal cell death after retinoic acid-induced down-regulation of P2X7 nucleotide receptor expression. Mol Cell Biochem 337 (1–2):83–99

Ousingsawat J, Wanitchakool P, Kmit A, Romao AM, Jantarajit W, Schreiber R, Kunzelmann K (2015) Anoctamin 6 mediates effects essential for innate immunity downstream of P2X7 receptors in macrophages. Nat Commun 6:6245

Park JH, Kim YC (2017) P2X7 receptor antagonists: a patent review (2010–2015). Expert Opin Ther Pat 27 (3):257–267

Parker JC, Snow RL (1972) Influence of external ATP on permeability and metabolism of dog red blood cells. Am J Phys 223(4):888–893

Paukert M, Hidayat S, Grunder S (2002) The P2X7 receptor from Xenopus laevis: formation of a large pore in Xenopus oocytes. FEBS Lett 513(2–3):253–258

Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 25 (21):5071–5082

Pelegrin P, Surprenant A (2007) Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway. J Biol Chem 282(4):2386–2394

Peng W, Cotrina ML, Han X, Yu H, Bekar L, Blum L, Takano T, Tian GF, Goldman SA, Nedergaard M (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci U S A 106 (30):12489–12493

Pereira VS, Casarotto PC, Hiroaki-Sato VA, Sartim AG, Guimaraes FS, Joca SR (2013) Antidepressant- and anticompulsive-like effects of purinergic receptor blockade: involvement of nitric oxide. Eur Neuropsychopharmacol 23(12):1769–1778

Perez-Flores G, Levesque SA, Pacheco J, Vaca L, Lacroix S, Perez-Cornejo P, Arreola J (2015) The P2X7/P2X4 interaction shapes the purinergic response in murine macrophages. Biochem Biophys Res Commun 467(3):484–490

Perruzza L, Gargari G, Proietti M, Fosso B, D'Erchia AM, Faliti CE, Rezzonico-Jost T, Scribano D, Mauri L, Colombo D, Pellegrini G, Moregola A, Mooser C, Pesole G, Nicoletti M, Norata GD, Geuking MB, McCoy KD, Guglielmetti S, Grassi F (2017) T follicular helper cells promote a beneficial gut ecosystem for host metabolic homeostasis by sensing microbiota-derived extracellular ATP. Cell Rep 18 (11):2566–2575

Pfleger C, Ebeling G, Blasche R, Patton M, Patel HH, Kasper M, Barth K (2012) Detection of caveolin-3/caveolin-1/P2X7R complexes in mice atrial cardiomyocytes in vivo and in vitro. Histochem Cell Biol 138(2):231–241

Pochet S, Tandel S, Querriere S, Tre-Hardy M, Garcia-Marcos M, De Lorenzi M, Vandenbranden M, Marino A, Devleeschouwer M, Dehaye JP (2006) Modulation by LL-37 of the responses of salivary glands to purinergic agonists. Mol Pharmacol 69 (6):2037–2046

Poornima V, Madhupriya M, Kootar S, Sujatha G, Kumar A, Bera AK (2012) P2X7 receptor-pannexin 1 hemichannel association: effect of extracellular calcium on membrane permeabilization. J Mol Neurosci 46(3):585–594

Pupovac A, Sluyter R (2016) Roles of extracellular nucleotides and P2 receptors in ectodomain shedding. Cell Mol Life Sci 73(22):4159–4173

Pupovac A, Geraghty NJ, Watson D, Sluyter R (2015) Activation of the P2X7 receptor induces the rapid shedding of CD23 from human and murine B cells. Immunol Cell Biol 93(1):77–85

Qiu F, Dahl G (2009) A permeant regulating its permeation pore: inhibition of pannexin 1 channels by ATP. Am J Physiol Cell Physiol 296(2):C250–C255

Qu Y, Misaghi S, Newton K, Gilmour LL, Louie S, Cupp JE, Dubyak GR, Hackos D, Dixit VM (2011) Pannexin-1 is required for ATP release during apoptosis but not for inflammasome activation. J Immunol 186(11):6553–6561

Rahman OA, Sasvari-Szekely M, Szekely A, Faludi G, Guttman A, Nemoda Z (2010) Analysis of a polymorphic microRNA target site in the purinergic receptor P2RX7 gene. Electrophoresis 31(11):1790–1795

Rassendren F, Buell GN, Virginio C, Collo G, North RA, Surprenant A (1997) The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. J Biol Chem 272(9):5482–5486

Rissiek B, Haag F, Boyer O, Koch-Nolte F, Adriouch S (2015) P2X7 on mouse T cells: one channel, many functions. Front Immunol 6:204

Robinson LE, Shridar M, Smith P, Murrell-Lagnado RD (2014) Plasma membrane cholesterol as a regulator of human and rodent P2X7 receptor activation and sensitization. J Biol Chem 289(46):31983–31994

Roger S, Pelegrin P, Surprenant A (2008) Facilitation of P2X7 receptor currents and membrane blebbing via constitutive and dynamic calmodulin binding. J Neurosci 28(25):6393–6401

Roger S, Mei ZZ, Baldwin JM, Dong L, Bradley H, Baldwin SA, Surprenant A, Jiang LH (2010a) Single nucleotide polymorphisms that were identified in affective mood disorders affect ATP-activated P2X7 receptor functions. J Psychiatr Res 44(6):347–355

Roger S, Gillet L, Baroja-Mazo A, Surprenant A, Pelegrin P (2010b) C-terminal calmodulin-binding motif differentially controls human and rat P2X7 receptor current facilitation. J Biol Chem 285(23):17514–17524

Roger S, Jelassi B, Couillin I, Pelegrin P, Besson P, Jiang LH (2015) Understanding the roles of the P2X7 receptor in solid tumour progression and therapeutic perspectives. Biochim Biophys Acta 1848(10 Pt B):2584–2602

Roman S, Cusdin FS, Fonfria E, Goodwin JA, Reeves J, Lappin SC, Chambers L, Walter DS, Clay WC, Michel AD (2009) Cloning and pharmacological characterization of the dog P2X7 receptor. Br J Pharmacol 158(6):1513–1526

Rorive G, Kleinzeller A (1972) The effect of ATP and Ca 2+ on the cell volume in isolated kidney tubules. Biochim Biophys Acta 274(1):226–239

Rossi L, Salvestrini V, Ferrari D, Di Virgilio F, Lemoli RM (2012) The sixth sense: hematopoietic stem cells detect danger through purinergic signaling. Blood 120 (12):2365–2375

Royle SJ, Bobanovic LK, Murrell-Lagnado RD (2002) Identification of a non-canonical tyrosine-based endocytic motif in an ionotropic receptor. J Biol Chem 277(38):35378–35385

Rozengurt E, Heppel LA (1975) A specific effect of external ATP on the permeability of transformed 3T3 cells. Biochem Biophys Res Commun 67 (4):1581–1588

Sakaki H, Fujiwaki T, Tsukimoto M, Kawano A, Harada H, Kojima S (2013) P2X4 receptor regulates P2X7 receptor-dependent IL-1beta and IL-18 release in mouse bone marrow-derived dendritic cells. Biochem Biophys Res Commun 432(3):406–411

Sanchez-Nogueiro J, Marin-Garcia P, Bustillo D, Olivos-Ore LA, Miras-Portugal MT, Artalejo AR (2014) Subcellular distribution and early signalling events of P2X7 receptors from mouse cerebellar granule neurons. Eur J Pharmacol 744:190–202

Sanderson J, Dartt DA, Trinkaus-Randall V, Pintor J, Civan MM, Delamere NA, Fletcher EL, Salt TE, Grosche A, Mitchell CH (2014) Purines in the eye: recent evidence for the physiological and pathological role of purines in the RPE, retinal neurons, astrocytes, Muller cells, lens, trabecular meshwork, cornea and lacrimal gland. Exp Eye Res 127:270–279

Sandilos JK, Chiu YH, Chekeni FB, Armstrong AJ, Walk SF, Ravichandran KS, Bayliss DA (2012) Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal autoinhibitory region. J Biol Chem 287 (14):11303–11311

Sanz JM, Chiozzi P, Di Virgilio F (1998) Tenidap enhances P2Z/P2X7 receptor signalling in macrophages. Eur J Pharmacol 355(2–3):235–244

Sathanoori R, Sward K, Olde B, Erlinge D (2015) The ATP receptors P2X7 and P2X4 modulate high glucose and palmitate-induced inflammatory responses in endothelial cells. PLoS One 10(5):e0125111

Schilling E, Hauschildt S (2012) Extracellular ATP induces P2X7-dependent nicotinamide phosphoribosyltransferase release in LPS-activated human monocytes. Innate Immun 18(5):738–744

Schwarz N, Drouot L, Nicke A, Fliegert R, Boyer O, Guse AH, Haag F, Adriouch S, Koch-Nolte F (2012) Alternative splicing of the N-terminal cytosolic and transmembrane domains of P2X7 controls gating of the ion channel by ADP-ribosylation. PLoS One 7(7):e41269

Seman M, Adriouch S, Scheuplein F, Krebs C, Freese D, Glowacki G, Deterre P, Haag F, Koch-Nolte F (2003) NAD-induced T cell death: ADP-ribosylation of cell surface proteins by ART2 activates the cytolytic P2X7 purinoceptor. Immunity 19(4):571–582

Seyffert C, Schmalzing G, Markwardt F (2004) Dissecting individual current components of co-expressed human P2X1 and P2X7 receptors. Curr Top Med Chem 4(16):1719–1730

Sha WG, Shen L, Zhou L, Xu DY, Lu GY (2015) Down-regulation of miR-186 contributes to podocytes apoptosis in membranous nephropathy. Biomed Pharmacother 75:179–184

Shiozaki Y, Sato M, Kimura M, Sato T, Tazaki M, Shibukawa Y (2017) Ionotropic P2X ATP receptor channels mediate purinergic signaling in mouse odontoblasts. Front Physiol 8:3

Shoji KF, Saez PJ, Harcha PA, Aguila HL, Saez JC (2014) Pannexin1 channels act downstream of P2X 7 receptors in ATP-induced murine T-cell death. Channels (Austin) 8(2):142–156

Shrivastava AN, Rodriguez PC, Triller A, Renner M (2013) Dynamic micro-organization of P2X7 receptors revealed by PALM based single particle tracking. Front Cell Neurosci 7:232

Silverman WR, de Rivero Vaccari JP, Locovei S, Qiu F, Carlsson SK, Scemes E, Keane RW, Dahl G (2009) The pannexin 1 channel activates the inflammasome in neurons and astrocytes. J Biol Chem 284 (27):18143–18151

Skarratt KK, Fuller SJ, Sluyter R, Dao-Ung LP, Gu BJ, Wiley JS (2005) A 5′ intronic splice site polymorphism leads to a null allele of the P2X7 gene in 1-2% of the Caucasian population. FEBS Lett 579 (12):2675–2678

Slater M, Danieletto S, Barden JA (2005) Expression of the apoptotic calcium channel P2X7 in the glandular epithelium. J Mol Histol 36(3):159–165

Sluyter R (2015) P2X and P2Y receptor signaling in red blood cells. Front Mol Biosci 2:60

Sluyter R, Stokes L (2011) Significance of P2X7 receptor variants to human health and disease. Recent Pat DNA Gene Seq 5(1):41–54

Sluyter R, Stokes L (2014) Polymorphic variants of the P2X7 receptor in the domestic dog. JJ Vet Sci Res 1 (1):008

Sluyter R, Barden JA, Wiley JS (2001) Detection of P2X purinergic receptors on human B lymphocytes. Cell Tissue Res 304(2):231–236

Smart ML, Panchal RG, Bowser DN, Williams DA, Petrou S (2002) Pore formation is not associated with macroscopic redistribution of P2X7 receptors. Am J Physiol Cell Physiol 283(1):C77–C84

Smart ML, Gu B, Panchal RG, Wiley J, Cromer B, Williams DA, Petrou S (2003) P2X7 receptor cell surface expression and cytolytic pore formation are regulated by a distal C-terminal region. J Biol Chem 278(10):8853–8860

Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA (2001) Altered cytokine production in mice lacking P2X7 receptors. J Biol Chem 276(1):125–132

Sommer A, Fries A, Cornelsen I, Speck N, Koch-Nolte F, Gimpl G, Andra J, Bhakdi S, Reiss K (2012) Melittin modulates keratinocyte function through P2 receptor-dependent ADAM activation. J Biol Chem 287 (28):23678–23689

Sophocleous RA, Mullany PR, Winter KM, Marks DC, Sluyter R (2015) Propensity of red blood cells to undergo P2X7 receptor-mediated phosphatidylserine exposure does not alter during in vivo or ex vivo aging. Transfusion 55(8):1946–1954

Sorge RE, Trang T, Dorfman R, Smith SB, Beggs S, Ritchie J, Austin JS, Zaykin DV, Vander Meulen H, Costigan M, Herbert TA, Yarkoni-Abitbul M, Tichauer D, Livneh J, Gershon E, Zheng M, Tan K, John SL, Slade GD, Jordan J, Woolf CJ, Peltz G, Maixner W, Diatchenko L, Seltzer Z, Salter MW, Mogil JS (2012) Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. Nat Med 18(4):595–599

Sperlagh B, Illes P (2014) P2X7 receptor: an emerging target in central nervous system diseases. Trends Pharmacol Sci 35(10):537–547

Spildrejorde M, Bartlett R, Stokes L, Jalilian I, Peranec M, Sluyter V, Curtis BL, Skarratt KK, Skora A, Bakhsh T, Seavers A, McArthur JD, Dowton M, Sluyter R (2014) R270C polymorphism leads to loss of function of the canine P2X7 receptor. Physiol Genomics 46(14):512–522

Stock TC, Bloom BJ, Wei N, Ishaq S, Park W, Wang X, Gupta P, Mebus CA (2012) Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. J Rheumatol 39(4):720–727

Stokes L, Jiang LH, Alcaraz L, Bent J, Bowers K, Fagura M, Furber M, Mortimore M, Lawson M, Theaker J, Laurent C, Braddock M, Surprenant A (2006) Characterization of a selective and potent antagonist of human P2X7 receptors, AZ11645373. Br J Pharmacol 149(7):880–887

Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS (2010) Two haplotypes of the P2X7 receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. FASEB J 24(8):2916–2927

Stokes L, Spencer SJ, Jenkins TA (2015) Understanding the role of P2X7 in affective disorders-are glial cells the major players? Front Cell Neurosci 9:258

Stolz M, Klapperstuck M, Kendzierski T, Detro-Dassen S, Panning A, Schmalzing G, Markwardt F (2015) Homodimeric anoctamin-1, but not homodimeric anoctamin-6, is activated by calcium increases mediated by the P2Y1 and P2X7 receptors. Pflugers Arch 467(10):2121–2140

Sun C, Chu J, Singh S, Salter RD (2010) Identification and characterization of a novel variant of the human P2X7 receptor resulting in gain of function. Purinergic Signal 6(1):31–45

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272(5262):735–738

Syberg S, Schwarz P, Petersen S, Steinberg TH, Jensen JE, Teilmann J, Jorgensen NR (2012a) Association between P2X7 receptor polymorphisms and bone status in mice. J Osteoporos 2012:637986

Syberg S, Petersen S, Beck Jensen JE, Gartland A, Teilmann J, Chessell I, Steinberg TH, Schwarz P, Jorgensen NR (2012b) Genetic background strongly influences the bone phenotype of P2X7 receptor knockout mice. J Osteoporos 2012:391097

Tang Y, Wang Y, Park KM, Hu Q, Teoh JP, Broskova Z, Ranganathan P, Jayakumar C, Li J, Su H, Tang Y,

Ramesh G, Kim IM (2015) MicroRNA-150 protects the mouse heart from ischaemic injury by regulating cell death. Cardiovasc Res 106(3):387–397

Taylor SR, Gonzalez-Begne M, Sojka DK, Richardson JC, Sheardown SA, Harrison SM, Pusey CD, Tam FW, Elliott JI (2009) Lymphocytes from P2X7-deficient mice exhibit enhanced P2X7 responses. J Leukoc Biol 85(6):978–986

Tewari M, Seth P (2015) Emerging role of P2X7 receptors in CNS health and disease. Ageing Res Rev 24 (Pt B):328–342

Todd JN, Poon W, Lyssenko V, Groop L, Nichols B, Wilmot M, Robson S, Enjyoji K, Herman MA, Hu C, Zhang R, Jia W, Ma R, Florez JC, Friedman DJ (2015) Variation in glucose homeostasis traits associated with P2RX7 polymorphisms in mice and humans. J Clin Endocrinol Metab 100(5):E688–E696

Tomasinsig L, Pizzirani C, Skerlavaj B, Pellegatti P, Gulinelli S, Tossi A, Di Virgilio F, Zanetti M (2008) The human cathelicidin LL-37 modulates the activities of the P2X7 receptor in a structure-dependent manner. J Biol Chem 283 (45):30471–30481

Torres GE, Egan TM, Voigt MM (1999) Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. J Biol Chem 274(10):6653–6659

Trams EG (1974) Evidence for ATP action on the cell surface. Nature 252(5483):480–482

Truve K, Dickinson P, Xiong A, York D, Jayashankar K, Pielberg G, Koltookian M, Muren E, Fuxelius HH, Weishaupt H, Swartling FJ (2016) Utilizing the dog genome in the search for novel candidate genes involved in glioma development-genome wide association mapping followed by targeted massive parallel sequencing identifies a strongly associated locus. PLoS Genet 12(5):e1006000

Tu G, Zou L, Liu S, Wu B, Lv Q, Wang S, Xue Y, Zhang C, Yi Z, Zhang X, Li G, Liang S (2016) Long noncoding NONRATT021972 siRNA normalized abnormal sympathetic activity mediated by the upregulation of P2X7 receptor in superior cervical ganglia after myocardial ischemia. Purinergic Signal 12(3):521–535

Virginio C, MacKenzie A, Rassendren FA, North RA, Surprenant A (1999) Pore dilation of neuronal P2X receptor channels. Nat Neurosci 2(4):315–321

Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, Li P, Xu Q, Liu QS, Goldman SA, Nedergaard M (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. Nat Med 10(8):821–827

Wang J, Huo K, Ma L, Tang L, Li D, Huang X, Yuan Y, Li C, Wang W, Guan W, Chen H, Jin C, Wei J, Zhang W, Yang Y, Liu Q, Zhou Y, Zhang C, Wu Z, Xu W, Zhang Y, Liu T, Yu D, Zhang Y, Chen L, Zhu D, Zhong X, Kang L, Gan X, Yu X, Ma Q, Yan J, Zhou L, Liu Z, Zhu Y, Zhou T, He F, Yang X (2011) Toward an understanding of the protein interaction network of the human liver. Mol Syst Biol 7:536

Wei L, Caseley E, Li D, Jiang LH (2016) ATP-induced P2X receptor-dependent large pore formation: how much do we know? Front Pharmacol 7:5

Weinhold K, Krause-Buchholz U, Rodel G, Kasper M, Barth K (2010) Interaction and interrelation of P2X7 and P2X4 receptor complexes in mouse lung epithelial cells. Cell Mol Life Sci 67(15):2631–2642

Weng T, Mishra A, Guo Y, Wang Y, Su L, Huang C, Zhao C, Xiao X, Liu L (2012) Regulation of lung surfactant secretion by microRNA-150. Biochem Biophys Res Commun 422(4):586–589

Wesselius A, Bours MJ, Henriksen Z, Syberg S, Petersen S, Schwarz P, Jorgensen NR, van Helden S, Dagnelie PC (2013) Association of P2X7 receptor polymorphisms with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients. Osteoporos Int 24(4):1235–1246

Wickert LE, Blanchette JB, Waldschmidt NV, Bertics PJ, Denu JM, Denlinger LC, Lenertz LY (2013) The C-terminus of human nucleotide receptor P2X7 is critical for receptor oligomerization and N-linked glycosylation. PLoS One 8(5):e63789

Wiley JS, Dubyak GR (1989) Extracellular adenosine triphosphate increases cation permeability of chronic lymphocytic leukemic lymphocytes. Blood 73 (5):1316–1323

Wiley JS, Gu BJ (2012) A new role for the P2X7 receptor: a scavenger receptor for bacteria and apoptotic cells in the absence of serum and extracellular ATP. Purinergic Signal 8(3):579–586

Wiley JS, Chen R, Wiley MJ, Jamieson GP (1992) The ATP4- receptor-operated ion channel of human lymphocytes: inhibition of ion fluxes by amiloride analogs and by extracellular sodium ions. Arch Biochem Biophys 292(2):411–418

Wiley JS, Gargett CE, Zhang W, Snook MB, Jamieson GP (1998) Partial agonists and antagonists reveal a second permeability state of human lymphocyte P2Z/P2X7 channel. Am J Phys 275(5 Pt 1):C1224–C1231

Wiley JS, Dao-Ung LP, Li C, Shemon AN, Gu BJ, Smart ML, Fuller SJ, Barden JA, Petrou S, Sluyter R (2003) An Ile-568 to Asn polymorphism prevents normal trafficking and function of the human P2X7 receptor. J Biol Chem 278(19):17108–17113

Wilson HL, Wilson SA, Surprenant A, North RA (2002) Epithelial membrane proteins induce membrane blebbing and interact with the P2X7 receptor C terminus. J Biol Chem 277(37):34017–34023

Woods LT, Camden JM, Batek JM, Petris MJ, Erb L, Weisman GA (2012) P2X7 receptor activation induces inflammatory responses in salivary gland epithelium. Am J Physiol Cell Physiol 303(7):C790–C801

Wu C, Ma MH, Brown KR, Geisler M, Li L, Tzeng E, Jia CY, Jurisica I, Li SS (2007) Systematic identification of SH3 domain-mediated human protein-protein

interactions by peptide array target screening. Proteomics 7(11):1775–1785

Wu PY, Lin YC, Chang CL, Lu HT, Chin CH, Hsu TT, Chu D, Sun SH (2009) Functional decreases in P2X7 receptors are associated with retinoic acid-induced neuronal differentiation of Neuro-2a neuroblastoma cells. Cell Signal 21(6):881–891

Wu B, Zhang C, Zou L, Ma Y, Huang K, Lv Q, Zhang X, Wang S, Xue Y, Yi Z, Jia T, Zhao S, Liu S, Xu H, Li G, Liang S (2016) LncRNA uc.48+ siRNA improved diabetic sympathetic neuropathy in type 2 diabetic rats mediated by P2X7 receptor in SCG. Auton Neurosci 197:14–18

Xu XJ, Boumechache M, Robinson LE, Marschall V, Gorecki DC, Masin M, Murrell-Lagnado RD (2012) Splice variants of the P2X7 receptor reveal differential agonist dependence and functional coupling with pannexin-1. J Cell Sci 125(Pt 16):3776–3789

Xu H, He L, Liu C, Tang L, Xu Y, Xiong M, Yang M, Fan Y, Hu F, Liu X, Ding L, Gao Y, Xu C, Li G, Liu S, Wu B, Zou L, Liang S (2016) LncRNA NONRATT021972 siRNA attenuates P2X7 receptor expression and inflammatory cytokine production induced by combined high glucose and free fatty acids in PC12 cells. Purinergic Signal 12(2):259–268

Yamamoto M, Kamatsuka Y, Ohishi A, Nishida K, Nagasawa K (2013) P2X7 receptors regulate engulfing activity of non-stimulated resting astrocytes. Biochem Biophys Res Commun 439(1):90–95

Yan Z, Li S, Liang Z, Tomic M, Stojilkovic SS (2008) The P2X7 receptor channel pore dilates under physiological ion conditions. J Gen Physiol 132(5):563–573

Yang D, He Y, Munoz-Planillo R, Liu Q, Nunez G (2015) Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. Immunity 43(5):923–932

Young MT, Pelegrin P, Surprenant A (2006) Identification of Thr283 as a key determinant of P2X7 receptor function. Br J Pharmacol 149(3):261–268

Young MT, Pelegrin P, Surprenant A (2007) Amino acid residues in the P2X7 receptor that mediate differential sensitivity to ATP and BzATP. Mol Pharmacol 71 (1):92–100

Young CN, Brutkowski W, Lien CF, Arkle S, Lochmuller H, Zablocki K, Gorecki DC (2012) P2X7 purinoceptor alterations in dystrophic mdx mouse muscles: relationship to pathology and potential target for treatment. J Cell Mol Med 16 (5):1026–1037

Young CN, Sinadinos A, Lefebvre A, Chan P, Arkle S, Vaudry D, Gorecki DC (2015) A novel mechanism of autophagic cell death in dystrophic muscle regulated by P2RX7 receptor large-pore formation and HSP90. Autophagy 11(1):113–130

Zheng L, Zhang X, Yang F, Zhu J, Zhou P, Yu F, Hou L, Xiao L, He Q, Wang B (2014) Regulation of the P2X7R by microRNA-216b in human breast cancer. Biochem Biophys Res Commun 452(1):197–204

Zhou L, Qi X, Potashkin JA, Abdul-Karim FW, Gorodeski GI (2008) MicroRNAs miR-186 and miR-150 down-regulate expression of the pro-apoptotic purinergic P2X7 receptor by activation of instability sites at the 3′-untranslated region of the gene that decrease steady-state levels of the transcript. J Biol Chem 283(42):28274–28286

Zhou L, Luo L, Qi X, Li X, Gorodeski GI (2009) Regulation of P2X7 gene transcription. Purinergic Signal 5 (3):409–426

Zou L, Tu G, Xie W, Wen S, Xie Q, Liu S, Li G, Gao Y, Xu H, Wang S, Xue Y, Wu B, Lv Q, Ying M, Zhang X, Liang S (2016) LncRNA NONRATT021972 involved the pathophysiologic processes mediated by P2X7 receptors in stellate ganglia after myocardial ischemic injury. Purinergic Signal 12(1):127–137

Adv Exp Med Biol - Protein Reviews (2017) 19:55–69
DOI 10.1007/5584_2017_55

Published online: 22 June 2017

P2X Receptor Activation

Toshimitsu Kawate

Abstract

Extracellular ATP-gated P2X receptors are trimeric non-selective cation channels important for many physiological events including immune response and neural transmission. These receptors belong to a unique class of ligand-gated ion channels composed of only six transmembrane helices and a relatively small extracellular domain that harbors three ATP-binding pockets. The crystal structures of P2X receptors, including the recent P2X3 structures representing three different stages of the gating cycle, have provided a compelling structural foundation for understanding how this class of ligand-gated ion channels function. These structures, in combination with numerous functional studies ranging from classic mutagenesis and electrophysiology to modern optogenetic pharmacology, have uncovered unique molecular mechanisms of P2X receptor function. This review article summarizes the current knowledge in P2X receptor activation, especially focusing on the mechanisms underlying ATP-binding, conformational changes in the extracellular domain, and channel gating and desensitization.

Keywords

Activation • ATP-binding • Channel gating • Desensitization • Extracellular flexing • P2X receptor • Structure

Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPγS	Adenosine-5′-(γ-thio)-triphosphate
DF	dorsal fin
hP2X2	human P2X2 receptor
hP2X4	human P2X4 receptor
LB	lower body
LF	left flipper
MTS	methanethiosulfonate

T. Kawate (✉)
Department of Molecular Medicine, Cornell University, Ithaca, NY 14853, USA
e-mail: toshi.kawate@cornell.edu

RF	right flipper
rP2X2	rat P2X2 receptor
rP2X4	rat P2X4 receptor
SCAM	substituted cysteine accessibility method
TM	transmembrane
UB	upper body
VCF	voltage clamp fluorometry
zfP2X4	zebrafish P2X4 receptor
α,β-meATP	(α,β-methylene)adenosine 5′-triphosphate

1 Introduction

A great deal of mechanistic insights into P2X receptor activation has been uncovered since the molecular cloning of this unique class of ligand-gated ion channels in 1994 (Valera et al. 1994; Brake et al. 1994). In particular, crystal structures of the P2X4 receptor in its apo-closed (Kawate et al. 2009) and ATP-bound (Hattori and Gouaux 2012) states have provided a strong foundation for dissecting the activation mechanism (Fig. 1a). These crystal structures established that each of the three P2X receptor subunits resembles the shape of a dolphin, comprising multiple sub-domains commonly referred to as the head, upper body (UB), lower body (LB), dorsal fin (DF), right flipper (RF), left flipper (LF), and fluke (Fig. 1b). Guided by these crystal structures, a wide spectrum of functional and computational studies have been carried out to investigate conformational changes associated with P2X receptor activation. Interested readers are referred to the excellent reviews (Young 2010; Browne et al. 2010; Coddou et al. 2011; Hausmann et al. 2015; Habermacher et al. 2016a; Wang and Yu 2016).

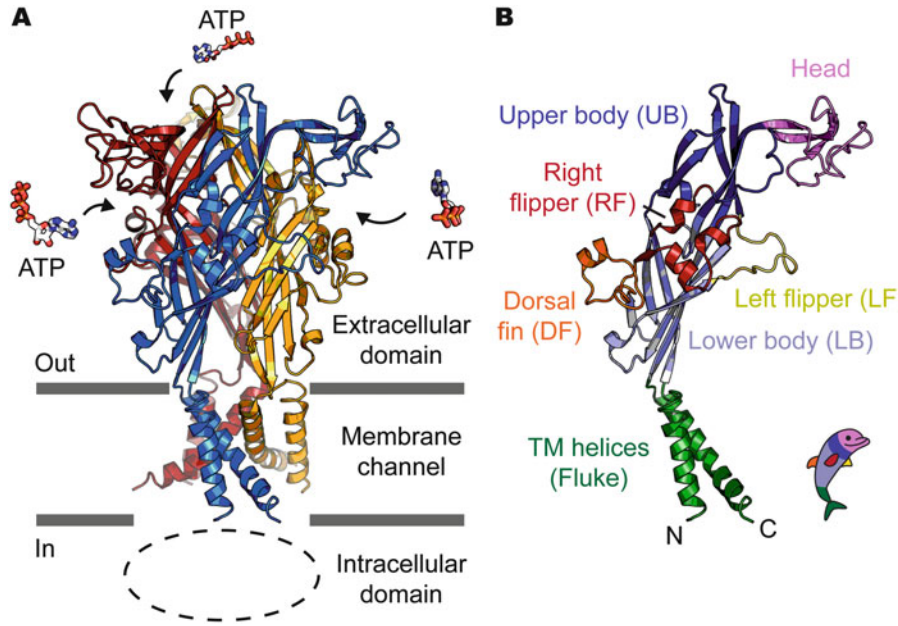

Fig. 1 Crystal structure of the P2X4 receptor. (**a**) Cartoon representation exhibiting the trimeric architecture composed of the extracellular domain and the membrane channel. Intracellular domain is missing in this structure (*dashed line*). Each of the three ATP molecules (not scaled) binds to a binding jaw formed between each of the two subunits in the extracellular domain. *Grey bars* indicate the predicted membrane boundary. Each subunit is depicted in a different color. (**b**) Cartoon representation of the protomer structure resembling the shape of a dolphin. For better comparison, each domain is colored consistent with the previous studies (Kawate et al. 2009; Hattori and Gouaux 2012). The apo-closed conformation of the zebrafish P2X4 receptor (zfP2X4; PDB ID: 4WD0) was used. All the figures in this review article were created using the PyMOL Molecular Graphics System, Version 1.8 (Schrödinger 2015)

In 2016, our knowledge in P2X structure was redoubled thanks to a number of new crystal structures determined for other subtypes. These new structures include an invertebrate P2X receptor (AmP2X) in complex with ATP and a divalent cation (Kasuya et al. 2016), the P2X3 receptor in its apo-closed, open, desensitized, and competitive-antagonist bound forms (Mansoor et al. 2016), and the P2X7 receptor in its allosteric-antagonist bound forms (Karasawa and Kawate 2016). These new crystal structures not only confirm the central activation mechanisms such as ATP-binding and movement of the extracellular region, but also postulate a novel channel gating mechanism involving the previously unseen intracellular domains. In addition, comparison of the crystal structures among different subtypes sheds light on poorly understood subtype-specific mechanisms. This review aims to integrate these exciting new discoveries and gives an overview of the mechanism of P2X receptor activation with focus on ATP binding, extracellular-domain flexing, and channel gating.

2 ATP Binding

Under physiological conditions, P2X receptors are activated most commonly by ATP (North and Surprenant 2000; Kaczmarek-Hajek et al. 2012). Strictly speaking, ATP exists in two different forms in a typical extracellular space: a free ion form (ATP^{4-}) or a complex with Mg^{2+} ($MgATP^{2-}$) (O'Sullivan and Perrin 1964). By meticulously controlling the concentrations of these two different forms of ATP, Li et al. astutely demonstrated that ATP^{4-} is a universal agonist for P2X receptors whereas $MgATP^{2-}$ can activate only P2X1 or P2X3 subtypes (Li et al. 2013). Though the underlying mechanism is not well understood, the subtype-specific control by Mg^{2+} is an excellent way of diversifying signaling though P2X receptors. Besides ATP, naturally existing diadenosine polyphosphates also activate several P2X subtypes (Pintor et al. 2000), however, ATP metabolites, such as ADP, AMP, or adenosine fail to open P2X channels. Notably, ATP hydrolysis is not required for P2X receptor activation, as metabolically stable α,β-meATP or nonhydrolyzable ATPγS can agonize these receptors (Kaczmarek-Hajek et al. 2012).

How does ATP turn on P2X receptors? Activation of P2X receptors is initiated by binding of ATP into the inter-subunit cleft surrounded by the head, UB, LB, LF, and DF domains, commonly referred to as the "binding jaw" (Fig. 2a, b). The crystal structure of the ATP-bound P2X4 revealed that the bound ATP adopts an unusual U-shaped structure (Hattori and Gouaux 2012), where the phosphates are folded towards the adenosine group (Fig. 3b). While a similar U-shaped ATP structure has been discovered in class II aminoacyl transfer RNA synthetases (Cavarelli et al. 1994), a stretched conformation is more common in other ATP-binding proteins (Mateja et al. 2015; Hopfner 2016) (Fig. 3a). How is the U-shaped ATP established in the binding pocket? Molecular simulations based on the apo-closed P2X4 structure suggest that there are at least two other modes of ATP-binding that are energetically favored (Huang et al. 2014). However, molecular dynamics (MD) simulations show that the constantly wiggling head domain prefers the U-shaped ATP over the other forms. In addition, Fryatt et al. recently demonstrated using voltage clamp fluorometry (VCF) and MD simulations that this U-shaped structure is stabilized by a hydrogen bond between the 2′ hydroxyl group on the ribose ring and the γ-phosphate, and that this hydrogen bond is crucial for efficacious action of ATP (Fryatt et al. 2016). Therefore, a combination of the inherent mobility of the ATP-binding pocket and the intramolecular forces within the ATP molecule establishes the U-shaped conformation. The recent crystal structures of the ATP-bound AmP2X, P2X3, and P2X7 receptors substantiated that binding of the U-shaped ATP is not specific to the P2X4 receptor but common to all members of the P2X receptor family.

The bound ATP molecule is coordinated by the highly conserved residues K70, K72, T189, N296, R298, and K316 (zfP2X4 numbering) through hydrogen bonding and ionic interactions

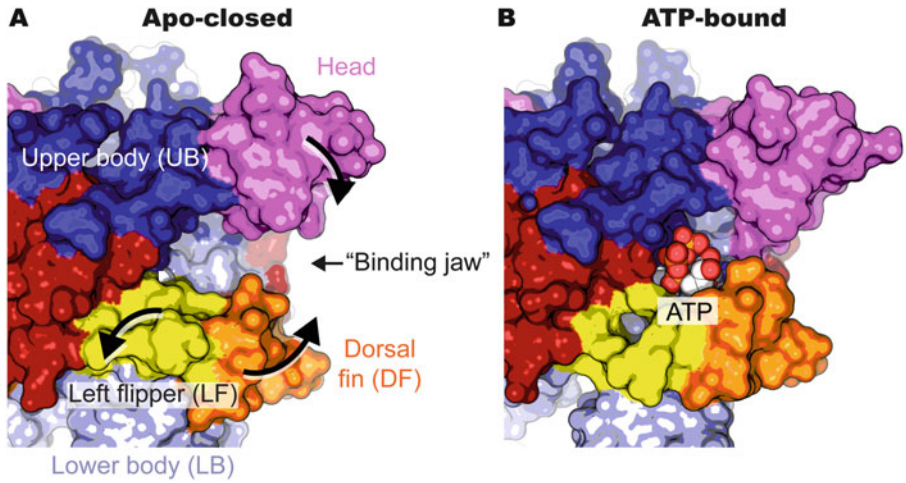

Fig. 2 ATP-binding closes the binding jaw. Surface representations of zfP2X4 in its *apo-closed* (**a**; PDB ID: 4WD0) or in its *ATP-bound* (**b**; PDB ID: 4WD1) conformations. The *binding jaw* is surrounded by the head, upper body (*UB*), lower body (*LB*), left flipper (*LF*), and dorsal fin (*DF*) domains. The ATP molecule is depicted as a sphere. Each domain is colored according to Fig. 1b

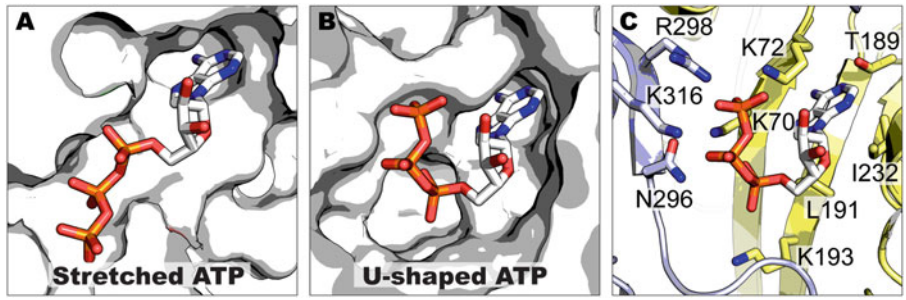

Fig. 3 Bound ATP molecule forms a U-shape in the P2X receptor binding pocket. (**a**) *Stretched ATP* conformation in Get3 (guided-entry of TA proteins; PDB ID: 4XVU). The ATP molecule is depicted as a stick and the binding pocket is shown as a white surface. (**b**) *U-shaped ATP* molecule in zfP2X4 receptor (PDB ID: 4WD1). (**c**) ATP-binding residues in zfP2X4 (PDB ID: 4WD1). ATP and binding residues are depicted as a stick. *Blue* and *yellow* colors represent two of the three subunits and the secondary structures are shown as a cartoon

(Chataigneau et al. 2013) (Fig. 3c). Extensive site-directed mutagenesis and electrophysiological recordings confirmed that all of these residues are crucial for ATP binding (Jiang et al. 2000; Ennion et al. 2000; Wilkinson et al. 2006; Yan et al. 2006; Fischer et al. 2007; Roberts et al. 2008; Bodnar et al. 2011; Allsopp et al. 2011). In addition to those six residues, two other highly conserved phenylalanine residues (F188 and F297; zfP2X4) were shown to play important roles in ATP binding (Roberts and Evans 2007; Zemkova et al. 2007; Roberts et al. 2009). While these residues do not directly interact with the ATP molecule, they probably stabilize the ATP-binding pocket through interactions with residues behind the binding pocket (Hattori and Gouaux 2012). The adenosine group of the bound ATP interacts with residues L191, L217, I232 (zfP2X4) through hydrophobic interaction (Hattori and Gouaux 2012) (Fig. 3c). Though amino acids at these positions are not well conserved, the corresponding residues in the other P2X subtypes are mostly non-polar, providing a similar hydrophobic surface for accommodating the adenosine group. Interestingly, P2X7 receptors lack four amino acid

residues around L217 (Surprenant et al. 1996). This difference may account for the reason why ATP is less potent on P2X7 receptors. Unfortunately, the resolution of the recent ATP/antagonist-bound P2X7 structure was too poor to identify specific interactions in the ATP-binding pocket (Karasawa and Kawate 2016). Further investigation is required for clarifying subtype specific ATP coordination.

ATP-binding triggers a series of conformational changes that lead to the closure of the binding jaw. Crystal structure of the ATP-bound P2X4 receptor shows that, upon ATP binding, the head domain moves down towards the DF domain, the DF domain swings upwards, and the LF domain is pushed away from the DF domain (Hattori and Gouaux 2012) (Fig. 2). Importantly, this closing movement of the binding jaw is likely a central mechanism among the P2X receptor family, as the crystal structures of AmP2X, P2X3, and P2X7 receptors all consistently show similar conformational changes triggered by ATP-binding. Closing of the binding jaw is supported by a number of functional studies using cysteine mutants that form disulfides or metal bridges (Jiang et al. 2012a; Kowalski et al. 2014; Zhao et al. 2014), VCF (Lorinczi et al. 2012), cysteine accessibility analysis (Roberts et al. 2012), and *in silico* simulation studies (Jiang et al. 2012a; Du et al. 2012). Interestingly, normal mode analysis and MD simulations suggest that the head domain fluctuates and constantly gets closer to the DF even in the absence of ATP (Pierdominici-Sottile et al. 2016). This continuous motion is consistent with the spontaneous disulfide-bond formation of an artificially introduced cysteine pair that are too far apart to interact with each other in the closed conformation (Kowalski et al. 2014). However, the intrinsic motion of the head domain is insufficient for channel opening, as no spontaneous channel activity is observed in the absence of ATP (Cao et al. 2007). What prevents the P2X receptor from undergoing conformational changes necessary for channel opening? Hausmann et al. demonstrated that the conserved residues R290 and E167 (rP2X2 numbering) form a salt bridge in the closed conformation, which serves as one of the energy barriers for channel opening (Hausmann et al. 2013). ATP breaks this salt bridge through a stronger interaction with the guanidine group of R290, which relaxes the interaction between the UB domain and the loop connecting the head domain and the right flipper (RF) domain. In addition, a detailed MD study suggests that a number of potential hydrogen-bonding pairs exists between different domains around the ATP-binding pocket, which likely stabilizes the closed conformation (Pierdominici-Sottile et al. 2016). These studies suggest that disruption of intersubunit interactions by the bound-ATP molecule is crucial for completely closing the binding jaw.

The trimeric P2X receptor harbors three ATP-binding pockets. How many ATP molecules are required to open the P2X receptor channel? ATP dose-response curves obtained from electrophysiological recordings using smooth muscle cells showed that Hill coefficients were in the range of 1–3 (Friel 1988; Ugur et al. 1997; Bean 1992). Likewise, analysis of heterologously expressed P2X receptors revealed a similar range of Hill coefficients (Brake et al. 1994; Chen et al. 1995; Seguela et al. 1996; Soto et al. 1996; Torres et al. 1998; Le et al. 1999; Haines et al. 1999), suggesting that ATP-binding is positively cooperative and more than one ATP molecule is required for P2X receptor activation. Supporting this idea, single channel recordings and kinetic analysis of P2X2 receptors as well as mathematical modeling of P2X7 receptors suggested that two ATP molecules are necessary and sufficient to open P2X channels (Ding and Sachs 1999; Yan et al. 2010; Khadra et al. 2012). Furthermore, mutating a residue critical for ATP-binding (K69) in one of the three binding pockets maintained essentially identical channel opening properties of P2X2 receptors, whereas mutations in two of the three binding pockets destroyed channel activity (Stelmashenko et al. 2012). All of these studies strongly indicate that binding of one ATP molecule is insufficient but binding of two or more ATP molecules is necessary for channel opening. However, a recent study using MD simulation and VCF

demonstrated that an ATP molecule still binds to the pocket harboring the K69A mutation (rP2X2), while the ATP binding at this site may not contribute to the channel opening (Fryatt et al. 2016). This study proposes an interesting possibility that three ATP molecules bind to the P2X receptor under a normal condition, even though only two ATP molecules are required for channel opening.

Positive cooperativity in ATP-binding postulates the existence of an intermediate closed state where an ATP molecule occupies one of the three binding sites and induces conformational rearrangements that facilitate the second ATP-binding. Using an ultra-rapid perfusion system, Moffatt and Hume measured the timing of the P2X2 receptor mediated currents upon a very brief (<0.2 ms) ATP application (Moffatt and Hume 2007). By carefully analyzing the activation and deactivation kinetics, they found that the channels open at least 0.8 ms after exposure to ATP and that a significant fraction of ATP molecules dissociated from fully-liganded P2X2 receptors without opening channels. Kinetic modeling incorporating these observations indicated that the P2X2 receptor must undergo an ATP-bound closed state. More recently, multiple groups demonstrated using affinity labeling techniques that the binding of an agonist renders P2X receptors more sensitive to ATP and responsive to other nucleotides (Jiang et al. 2011, 2012b; Bhargava et al. 2012; Browne and North 2013). While the underlying mechanism remains unclear, the first ATP-binding likely promotes the closure of one binding jaw, which may stabilize the spontaneous movements of the neighboring subunits in favor for subsequent ATP-binding. The affinity labeling technique is an intriguing tool for trapping the P2X receptor in an intermediate closed state for structural studies.

3 Extracellular Domain Flexing

Closing of the ATP binding jaw pulls the DF upward and pushes the neighboring LF downward, forcing these two domains to move away from each other (Fig. 4). Because both the LF and DF are linked to the β-strand-rich LB of the respective subunit, a fenestration formed between two LBs widens by ~5–10 Å. This flexing motion of the LBs triggers the subsequent rearrangement in the connected transmembrane domain, which eventually leads to the opening of the membrane channel. Like the conformational changes around the ATP-binding pocket, this orchestrated movement of the extracellular domain is likely conserved among the P2X receptor family, as the crystal structures of P2X3, P2X4, and P2X7 all exhibit essentially the same conformational changes upon ATP binding. Furthermore, a number of functional studies on different P2X subtypes consistently support the flexing motion of the extracellular domain during activation (Allsopp et al. 2011; Rokic et al. 2013, 2014; Gao et al. 2015). Particularly, systematic immobilization of the extracellular domain using inter-subunit disulfides highlighted that separation of the neighboring LBs, including uncoupling of conserved salt bridges (Hausmann et al. 2013; Jiang et al. 2010), is required for P2X receptor activation (Zhao et al. 2014; Roberts et al. 2012; Stelmashenko et al. 2014; Stephan et al. 2016; Caseley et al. 2017).

In contrast to the flexible LB domain, the UB domain remains relatively static during P2X receptor activation (Hattori and Gouaux 2012; Mansoor et al. 2016). This rigid UB domain likely serves as a hinge necessary for swiveling the LB domains. In particular, the highly conserved salt bridge between R309 and D85 (rP2X4) and the cation-π interaction between R309 and W164 (rP2X4) play critical roles in stabilizing the rigid body structure (Zhao et al. 2016). In addition, disrupting the interactions among the three UB domains facing the central threefold axis by artificially introduced disulfide bridges (e.g. P89C/S97C in rP2X2) diminishes P2X receptor channel activity (Stelmashenko et al. 2014). This result indicates that the isolation of UB domains away from each other hampers channel opening. Furthermore, the crystal structure of AmP2X demonstrated that Zn^{2+}, which potentiates ATP-dependent channel

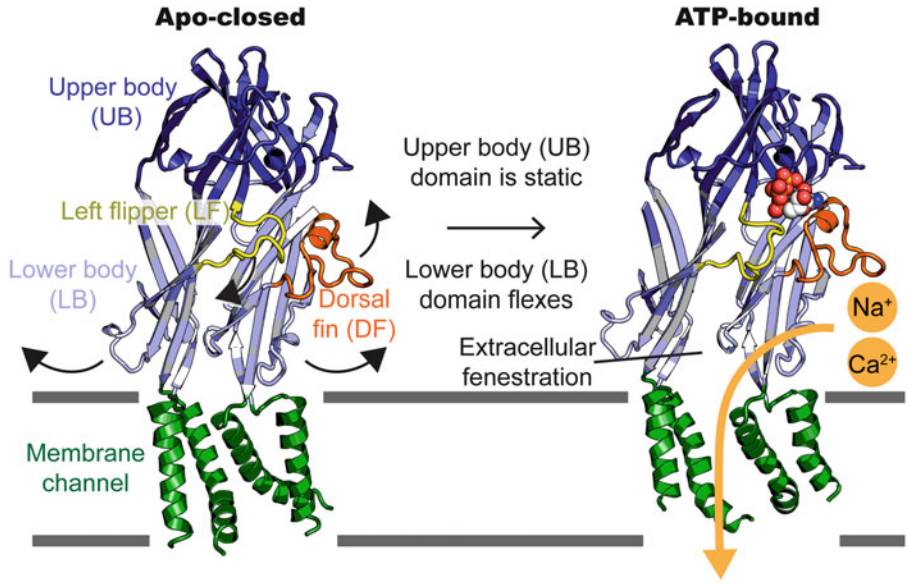

Fig. 4 Extracellular domain flexes during activation of a P2X channel. Cartoon representations of the *apo-closed* (PDB ID: 4DW0) and the *ATP-bound* (PDB ID: 4DW1) P2X4 receptor highlight that the *upper body* domain remains relatively static while the *lower body* domain flexes during channel activation. The extracellular fenestration widens upon the flexing of the lower body domain, which allows extracellular cations such as Na^+ and Ca^{2+} to access the membrane pore (*orange arrow*). The ATP molecule is depicted as a sphere. For clarity, only select domains from two of the three subunits are shown in this figure

activity of P2X4, is coordinated by three E95 residues (rP2X4) that face the UB interface along the molecular threefold axis (Kasuya et al. 2016). Construing from this structure, Zn^{2+} binding likely brings the three UB domains together at the receptor center, which facilitates the flexing of LB domains. These data support that the UB domains need to be tightly packed against each other during P2X receptor activation.

Flexing of LB domains widens the extracellular fenestrations formed at each subunit-subunit interface around the extracellular vestibule (lateral pathway), allowing permeant ions to access the membrane pore (Fig. 4). While an alternative ion pathway through the receptor center along the molecular threefold axis (central pathway) was proposed based on the first crystal structure that revealed unusually large internal vestibules (Kawate et al. 2009), a line of evidences contests this potential ion access pathway. First, electrostatic interactions favor cations to take the lateral pathway whereas the energy barrier impedes cations to permeate the central pathway (Kawate et al. 2011). Second, modification of introduced cysteine residues around the lateral pathway (E56 and D58 in hP2X4 and I328 in rP2X2) with MTS reagents reduces the channel activity, while none of the introduced cysteine residues along the central pathway presented current reduction by MTS reagents (Kawate et al. 2011; Samways et al. 2011). Third, inter-subunit disulfides, which constrain movements upon ATP-binding, hindered the channel activity when introduced at the lateral pathway but had no effect or even facilitated the activity when introduced at the central pathway (Roberts et al. 2012; Stelmashenko et al. 2014; Kawate et al. 2011). And fourth, the crystal structures of P2X3, P2X4, and AmP2X receptors showed that the central pathway remains constricted after ATP-binding (Hattori and Gouaux 2012; Kasuya et al. 2016; Mansoor et al. 2016). These data collectively suggest that permeant ions access the membrane pore through the lateral fenestrations.

The recently determined crystal structures of the P2X7 receptor unveiled a subtype specific conformational change in the extracellular domain (Karasawa and Kawate 2016). Unlike P2X3 or P2X4 receptors, the "turret" like structure located at the top of the central pathway of P2X7 is at least ~4 Å wide. By just looking at this structure, one could argue that ions may permeate the central pathway in the P2X7 receptor. However, Q98 and G99 located just below the turret tightly constrict the central pathway, preventing ions from reaching the central vestibule that leads to the membrane pore. Furthermore, the turret seems to become narrower upon ATP binding, as accessibility of the Y295C mutant significantly decreases during channel opening. This narrowing motion of the turret is in contrast to the relatively static (slight widening if any) turret in P2X3 and P2X4. Another unique structural rearrangement was discovered using small molecules that specifically inhibit P2X7 receptors. The crystal structures of antagonist-bound P2X7 receptors revealed a novel allosteric drug-binding pocket, which is located between neighboring UB domains juxtaposed with the ATP-binding site. Cysteine scanning mutagenesis and accessibility studies demonstrated that this drug-binding pocket also narrows upon ATP-binding and that this structural rearrangement is necessary for the channel opening. Interestingly, the equivalent regions in P2X3 and P2X4 are too small (with or without ATP) for the P2X7 specific inhibitors to enter, providing a mechanistic basis for why these drugs specifically inhibit the P2X7 receptor. Considering that both the turret and the drug-binding pocket need to narrow for P2X7 activation, the extracellular domain of P2X7 in the open state may resemble those of P2X3 and P2X4.

4 Channel Gating

P2X receptors are among the simplest ion channels, with only three transmembrane helices (one from each subunit), constituting the pore and another three helices surrounding them (Alves et al. 2014). Flexing of the LB triggered by ATP-binding promotes the rotation of both the pore-lining second transmembrane helix (TM2) and the surrounding transmembrane helix (TM1) by ~15° counterclockwise, rendering the upper part of the TM region ~10 Å wider than that in the closed conformation (Hattori and Gouaux 2012; Mansoor et al. 2016; Habermacher et al. 2016b) (Fig. 5). Accessibility of the pore-facing residues on TM2 in this region, therefore, increases during channel activation (Li et al. 2008; Pippel et al. 2017). Importantly, widening of the upper TM region is necessary and sufficient for transition from the closed state to the open channel. Introduction of an intrasubunit disulfide bridge between V48 in TM1 and I328 in TM2 (rP2X2), which restricts the widening movement of the upper TM region, diminishes ATP-dependent channel activity that can be recovered by reducing the disulfide bridge (Stelmashenko et al. 2014; Jiang et al. 2001, 2003; Liang et al. 2013; Rothwell et al. 2014). Conversely, cis-trans isomerization of an artificially introduced photoswitchable ligand at the upper TM region (e.g. I328 or P329 in rP2X2) evokes robust channel opening in the absence of ATP (Browne et al. 2014; Lemoine et al. 2013). These data highlight that opening of P2X channels are tightly coupled with widening of the upper TM regions, which is achieved by flexing the LB domain upon ATP binding.

To open the P2X channel, the most constricted region in the closed conformation (i.e. gate) needs to become wider than the size of permeant ions. The gate is located at the intersection of the three TM2s, which cross to each other about halfway in the lipid bilayer, forming an hourglass shape (Kawate et al. 2009; Mansoor et al. 2016; Karasawa and Kawate 2016) (Fig. 5). Consistent with the closed-state crystal structures, electrophysiological studies using the substituted cysteine accessibility method (SCAM) identified potential gate residues on TM2 (Li et al. 2008; Pippel et al. 2017; Rassendren et al. 1997; Egan et al. 1998; Kracun et al. 2010). In particular, residues T336 and T339 (rP2X2) seem to play central roles as the gate residues, as supported by studies analyzing ion selectivity and spontaneous activity of the

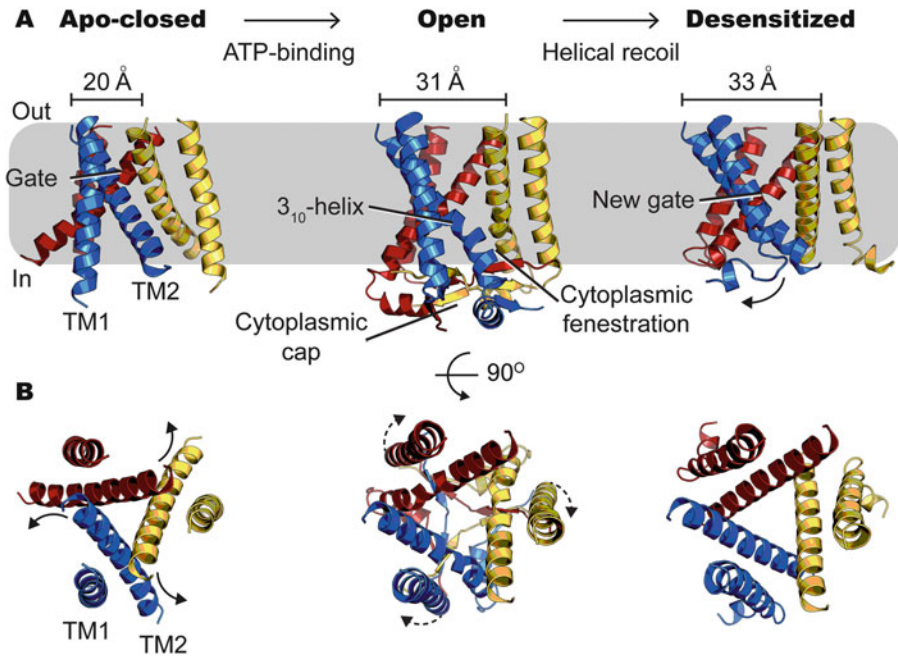

Fig. 5 P2X channel gating and desensitization. Cartoon representations of the P2X3 receptor transmembrane helices in the *apo-closed* (PDB ID: 5SVJ), *open* (PDB ID: 5SVK), or *desensitized* (PDB ID: 5SVL) conformations viewed from the side (**a**) or from the top (**b**). The TM2-TM2 distance at the membrane boundary on the extracellular side is indicated for each state. Each subunit is depicted in a different color and the *grey area* represents the predicted transmembrane region

mutant at these residues (Cao et al. 2007; 2009; Migita et al. 2001; Browne et al. 2011). How does widening of the upper TM region open the channel gate? Simple rotation of the TM helices around the threefold axis running through the center, obviously, does not promote gate expansion. By measuring accessibility changes for systematically introduced cysteine residues in both TM helices, and by analyzing Cd^{2+} coordination mediated by a cysteine mutant at V343 (rP2X2) located just below the gate, Li et al. demonstrated that TM2 helices vertically straighten during activation, with respect to the steeply angled positions in the closed state viewed from the side (Li et al. 2010). This experiment indicates that the lower part of TM2 rotates to a lesser extent. Further studies using metal bridges and MD simulation demonstrated that the lower TM region rearranges during channel opening and creates both intersubunit and intrasubunit contacts among the TM helices (Heymann et al. 2013). These extra interactions hold the lower parts of TM1 and TM2 together, which counteracts the rotation of the upper TM region. In combination with the overall counterclockwise rotation, the vertically straightening TM2 helices enable the expansion of the channel gate in an iris-like motion. This movement is consistent with the tryptophan scanning mutagenesis (Silberberg et al. 2005, 2007) and the recent study using photoswitchable molecular tweezers implemented both vertically and horizontally in the pore of the rat P2X2 receptor (Habermacher et al. 2016b).

Once ATP escapes from the binding pocket, the LB domain quickly relaxes back to its resting conformation, which forces the upper TM region to narrow, leading to the channel closure. While this channel deactivation process is simply the reverse of channel activation, the desensitization process cannot be construed easily. How do P2X channels start closing in the presence of ATP, which should keep the upper TM region wide? Comparison of the slowly desensitizing P2X2

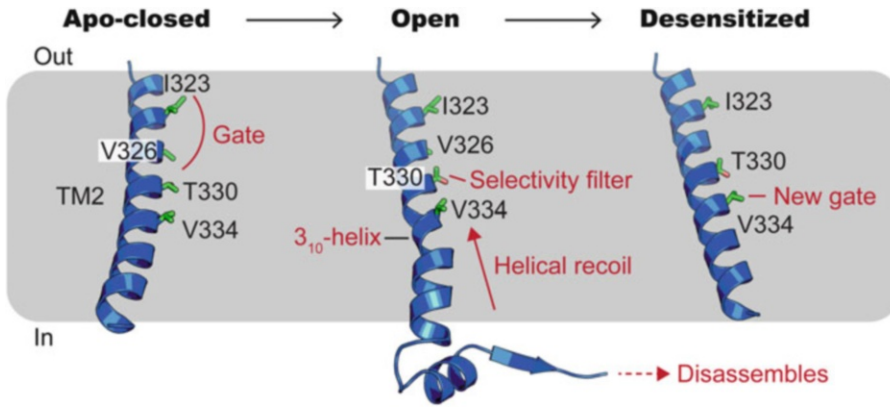

Fig. 6 Conformational change of the pore-lining TM2 helix during the gating cycle. Cartoon representations of the P2X3 receptor TM2 helix in the *apo-closed* (PDB ID: 5SVJ), *open* (PDB ID: 5SVK), or *desensitized* (PDB ID: 5SVL) conformations viewed from the side. Important gating residues are shown as a stick. *Grey area* represents the predicted transmembrane region

receptor with its naturally existing splice variant that shows relatively faster desensitization prompted that the C-terminus (residues 370–472 in rP2X2) includes a region important for slowing the rate of channel desensitization (Brandle et al. 1997; Simon et al. 1997; Smith et al. 1999). Further studies using C-terminally modified P2X2 receptors suggested that the residues between V370 and P376 are particularly important for the slow desensitization kinetics (Koshimizu et al. 1998, 1999; Fountain and North 2006). Interestingly, however, the C-terminally truncated P2X2 receptor still desensitizes slower than the rapidly desensitizing P2X1 or P2X3 receptors. To investigate the contribution of each domain of the P2X1 receptor in desensitization, the Evans group analyzed the desensitization kinetics of chimeras between P2X1 and P2X2 (Allsopp and Evans 2011). When the N-terminus of P2X1 was replaced with that of P2X2, the channel desensitized much more slowly. The reverse chimera, on the other hand, showed rapid desensitization. They also demonstrated that the TM regions and the ATP-binding extracellular domain are essentially irrelevant for desensitization. Indeed, the light-gated P2X receptor using photoswitchable ligand, which renders the upper TM region wide, desensitizes normally, supporting that movement of the extracellular domain plays minimal roles in desensitization (Habermacher et al. 2016b; Browne et al. 2014). Altogether, these studies strongly suggest that the intracellular N- and C-termini promote channel closure even in the presence of ATP.

How do the intracellular domains govern the desensitization process? The recent crystal structures of the P2X3 receptor determined by the Gouaux group unveiled the much-needed first structural insights into the roles of the intracellular domains in channel gating (Mansoor et al. 2016). While the closed structure of the P2X3 receptor resembles that of the P2X4 receptor in general, the ATP-bound open structure revealed striking new features. First, each of the three N- and C-terminus interacts with each other, forming a previously unseen triangular structure termed "cytoplasmic cap" (Fig. 5). This cap structure bundles the six TM helices together, providing extra support for holding the rearranged TM helices in the open conformation. Second, the TM2 transforms from a regular α-helix into a 3_{10}-helix between G333 and G335, which stretches this pore-lining helix by ~4 Å. Third, the extracellular gate residues I323 and V326 move upward by ~6 Å and swing away from the pore (Figs. 5 and 6). At the same time, the cytoplasmic gate residue T330 becomes the narrowest point of the pore, potentially serving as a selectivity filter. During the transition from this

open state to the desensitized conformation, TM2 helix in the desensitized state recoils back to the normal α-helix and rotates counterclockwise for another ~9°. Notably, the cytoplasmic cap was not observed in the desensitized structure. Furthermore, the α-helix at the C-terminus (N349-K357) swings away from the central threefold axis. These observations postulate a novel desensitization mechanism termed, "helical recoil model". In this model, the cytoplasmic cap unfolds or disassembles while the extracellular domain still accommodates ATP molecules in its binding pockets. This triggers the stretched TM2 helices to shrink back to its normal length, which makes V334 move up by a few angstroms and rotate towards the pore, forming a new gate that constricts the ion permeation pathway (Fig. 6). This elegant model fits well with the previous studies demonstrating that desensitization kinetics dramatically changes when the counterpart residues forming the cytoplasmic cap are mutated (Koshimizu et al. 1998; Allsopp and Evans 2011; Hausmann et al. 2014).

5 Conclusion

Like other ligand-gated ion channels, P2X receptors convey extracellular messages into the cell through the gating cycle comprising at least three distinctive stages, namely, closed, open, and desensitized states. Extensive studies using electrophysiology, X-ray crystallography, and molecular simulations have elucidated the fundamental mechanisms underlying transition from the closed to open state for this class of ligand-gated ion channels. The recent crystal structures of the P2X3 receptor postulate a completely new channel opening mechanism involving the intracellular domains and shed light on the poorly understood transition from the open to the desensitized state. In addition, the first crystal structures of P2X7 uncovered a subtype specific conformational change in its extracellular domain, providing a structural foundation for dissecting the mechanisms for this unique subtype. There are, however, many unanswered questions. For example, is the cytoplasmic cap common to all other P2X subtypes? How does the cytoplasmic cap assemble and disassemble? What are the molecular basis for subtype specific Mg^{2+} dependency, ATP sensitivity, or large pore formation? Considering the current progress being made in the mechanistic research for this important class of ligand-gated ion channels, we should start to discover the answers to these questions in the near future.

Acknowledgement I thank Kevin Michalski, Phillip Nguyen, and Akira Karasawa for helpful discussions. I am also grateful to Cindy Westmiller for helpful comments on the article. T.K. is supported by the National Institutes of Health (GM114379).

Conflicts of Interest The author declares that I have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Allsopp RC, Evans RJ (2011) The intracellular amino terminus plays a dominant role in desensitization of ATP-gated P2X receptor ion channels. J Biol Chem 286(52):44691–44701

Allsopp RC, El Ajouz S, Schmid R, Evans RJ (2011) Cysteine scanning mutagenesis (residues Glu52-Gly96) of the human P2X1 receptor for ATP: mapping agonist binding and channel gating. J Biol Chem 286 (33):29207–29217

Alves LA, da Silva JH, Ferreira DN, Fidalgo-Neto AA, Teixeira PC, de Souza CA, Caffarena ER, de Freitas MS (2014) Structural and molecular modeling features of P2X receptors. Int J Mol Sci 15 (3):4531–4549

Bean BP (1992) Pharmacology and electrophysiology of ATP-activated ion channels. Trends Pharmacol Sci 13 (3):87–90

Bhargava Y, Rettinger J, Mourot A (2012) Allosteric nature of P2X receptor activation probed by photoaffinity labelling. Br J Pharmacol 167 (6):1301–1310

Bodnar M, Wang H, Riedel T, Hintze S, Kato E, Fallah G, Groger-Arndt H, Giniatullin R, Grohmann M, Hausmann R, Schmalzing G, Illes P, Rubini P (2011) Amino acid residues constituting the agonist binding site of the human P2X3 receptor. J Biol Chem 286 (4):2739–2749

Brake AJ, Wagenbach MJ, Julius D (1994) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 371(6497):519–523

Brandle U, Spielmanns P, Osteroth R, Sim J, Surprenant A, Buell G, Ruppersberg JP, Plinkert PK, Zenner HP, Glowatzki E (1997) Desensitization of the P2X(2) receptor controlled by alternative splicing. FEBS Lett 404(2–3):294–298

Browne LE, North RA (2013) P2X receptor intermediate activation states have altered nucleotide selectivity. J Neurosci 33(37):14801–14808

Browne LE, Jiang LH, North RA (2010) New structure enlivens interest in P2X receptors. Trends Pharmacol Sci 31(5):229–237

Browne LE, Cao L, Broomhead HE, Bragg L, Wilkinson WJ, North RA (2011) P2X receptor channels show threefold symmetry in ionic charge selectivity and unitary conductance. Nat Neurosci 14(1):17–18

Browne LE, Nunes JP, Sim JA, Chudasama V, Bragg L, Caddick S, North RA (2014) Optical control of trimeric P2X receptors and acid-sensing ion channels. Proc Natl Acad Sci U S A 111(1):521–526

Cao L, Young MT, Broomhead HE, Fountain SJ, North RA (2007) Thr339-to-serine substitution in rat P2X2 receptor second transmembrane domain causes constitutive opening and indicates a gating role for Lys308. J Neurosci 27(47):12916–12923

Cao L, Broomhead HE, Young MT, North RA (2009) Polar residues in the second transmembrane domain of the rat P2X2 receptor that affect spontaneous gating, unitary conductance, and rectification. J Neurosci 29(45):14257–14264

Caseley EA, Muench SP, Jiang LH (2017) Conformational changes during human P2X7 receptor activation examined by structural modelling and cysteine-based cross-linking studies. Purinergic Signal 13 (1):135–141

Cavarelli J, Eriani G, Rees B, Ruff M, Boeglin M, Mitschler A, Martin F, Gangloff J, Thierry JC, Moras D (1994) The active site of yeast aspartyl-tRNA synthetase: structural and functional aspects of the aminoacylation reaction. EMBO J 13(2):327–337

Chataigneau T, Lemoine D, Grutter T (2013) Exploring the ATP-binding site of P2X receptors. Front Cell Neurosci 7:273

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377 (6548):428–431

Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, Stojilkovic SS (2011) Activation and regulation of purinergic P2X receptor channels. Pharmacol Rev 63(3):641–683

Ding S, Sachs F (1999) Single channel properties of P2X2 purinoceptors. J Gen Physiol 113(5):695–720

Du J, Dong H, Zhou HX (2012) Gating mechanism of a P2X4 receptor developed from normal mode analysis and molecular dynamics simulations. Proc Natl Acad Sci U S A 109(11):4140–4145

Egan TM, Haines WR, Voigt MM (1998) A domain contributing to the ion channel of ATP-gated P2X2 receptors identified by the substituted cysteine accessibility method. J Neurosci 18(7):2350–2359

Ennion S, Hagan S, Evans RJ (2000) The role of positively charged amino acids in ATP recognition by human P2X1 receptors. J Biol Chem 275(45):35656

Fischer W, Zadori Z, Kullnick Y, Groger-Arndt H, Franke H, Wirkner K, Illes P, Mager PP (2007) Conserved lysin and arginin residues in the extracellular loop of P2X(3) receptors are involved in agonist binding. Eur J Pharmacol 576(1–3):7–17

Fountain SJ, North RA (2006) A C-terminal lysine that controls human P2X4 receptor desensitization. J Biol Chem 281(22):15044–15049

Friel DD (1988) An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. J Physiol 401:361–380

Fryatt AG, Dayl S, Cullis PM, Schmid R, Evans RJ (2016) Mechanistic insights from resolving ligand-dependent kinetics of conformational changes at ATP-gated P2X1R ion channels. Sci Rep 6:32918

Gao C, Yu Q, Xu H, Zhang L, Liu J, Jie Y, Ma W, Samways DS, Li Z (2015) Roles of the lateral fenestration residues of the P2X(4) receptor that contribute to the channel function and the deactivation effect of ivermectin. Purinergic Signal 11(2):229–238

Habermacher C, Dunning K, Chataigneau T, Grutter T (2016a) Molecular structure and function of P2X receptors. Neuropharmacology 104:18–30

Habermacher C, Martz A, Calimet N, Lemoine D, Peverini L, Specht A, Cecchini M, Grutter T (2016b) Photo-switchable tweezers illuminate pore-opening motions of an ATP-gated P2X ion channel. elife 5: e11050

Haines WR, Torres GE, Voigt MM, Egan TM (1999) Properties of the novel ATP-gated ionotropic receptor composed of the P2X(1) and P2X(5) isoforms. Mol Pharmacol 56(4):720–727

Hattori M, Gouaux E (2012) Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature 485(7397):207–212

Hausmann R, Gunther J, Kless A, Kuhlmann D, Kassack MU, Bahrenberg G, Markwardt F, Schmalzing G (2013) Salt bridge switching from Arg290/Glu167 to Arg290/ATP promotes the closed-to-open transition of the P2X2 receptor. Mol Pharmacol 83(1):73–84

Hausmann R, Bahrenberg G, Kuhlmann D, Schumacher M, Braam U, Bieler D, Schlusche I, Schmalzing G (2014) A hydrophobic residue in position 15 of the rP2X3 receptor slows desensitization and reveals properties beneficial for pharmacological analysis and high-throughput screening. Neuropharmacology 79:603–615

Hausmann R, Kless A, Schmalzing G (2015) Key sites for P2X receptor function and multimerization: overview of mutagenesis studies on a structural basis. Curr Med Chem 22(7):799–818

Heymann G, Dai J, Li M, Silberberg SD, Zhou HX, Swartz KJ (2013) Inter- and intrasubunit interactions between transmembrane helices in the open state of P2X receptor channels. Proc Natl Acad Sci U S A 110 (42):E4045–E4054

Hopfner KP (2016) Invited review: architectures and mechanisms of ATP binding cassette proteins. Biopolymers 105(8):492–504

Huang LD, Fan YZ, Tian Y, Yang Y, Liu Y, Wang J, Zhao WS, Zhou WC, Cheng XY, Cao P, Lu XY, Yu Y (2014) Inherent dynamics of head domain correlates with ATP-recognition of P2X4 receptors: insights gained from molecular simulations. PLoS One 9(5): e97528

Jiang LH, Rassendren F, Surprenant A, North RA (2000) Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. J Biol Chem 275(44):34190–34196

Jiang LH, Rassendren F, Spelta V, Surprenant A, North RA (2001) Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X(2) receptor. J Biol Chem 276 (18):14902–14908

Jiang LH, Kim M, Spelta V, Bo X, Surprenant A, North RA (2003) Subunit arrangement in P2X receptors. J Neurosci 23(26):8903–8910

Jiang R, Martz A, Gonin S, Taly A, de Carvalho LP, Grutter T (2010) A putative extracellular salt bridge at the subunit interface contributes to the ion channel function of the ATP-gated P2X2 receptor. J Biol Chem 285(21):15805–15815

Jiang R, Lemoine D, Martz A, Taly A, Gonin S, Prado de Carvalho L, Specht A, Grutter T (2011) Agonist trapped in ATP-binding sites of the P2X2 receptor. Proc Natl Acad Sci U S A 108(22):9066–9071

Jiang R, Taly A, Lemoine D, Martz A, Cunrath O, Grutter T (2012a) Tightening of the ATP-binding sites induces the opening of P2X receptor channels. EMBO J 31(9):2134–2143

Jiang R, Taly A, Lemoine D, Martz A, Specht A, Grutter T (2012b) Intermediate closed channel state (s) precede(s) activation in the ATP-gated P2X2 receptor. Channels (Austin) 6(5):398–402

Kaczmarek-Hajek K, Lorinczi E, Hausmann R, Nicke A (2012) Molecular and functional properties of P2X receptors–recent progress and persisting challenges. Purinergic Signal 8(3):375–417

Karasawa A, Kawate T (2016) Structural basis for subtype-specific inhibition of the P2X7 receptor. eLife 5

Kasuya G, Fujiwara Y, Takemoto M, Dohmae N, Nakada-Nakura Y, Ishitani R, Hattori M, Nureki O (2016) Structural insights into divalent cation modulations of ATP-gated P2X receptor channels. Cell Rep 14 (4):932–944

Kawate T, Michel JC, Birdsong WT, Gouaux E (2009) Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. Nature 460(7255):592–598

Kawate T, Robertson JL, Li M, Silberberg SD, Swartz KJ (2011) Ion access pathway to the transmembrane pore in P2X receptor channels. J Gen Physiol 137 (6):579–590

Khadra A, Yan Z, Coddou C, Tomic M, Sherman A, Stojilkovic SS (2012) Gating properties of the P2X2a and P2X2b receptor channels: experiments and mathematical modeling. J Gen Physiol 139(5):333–348

Koshimizu T, Tomic M, Koshimizu M, Stojilkovic SS (1998) Identification of amino acid residues contributing to desensitization of the P2X2 receptor channel. J Biol Chem 273(21):12853–12857

Koshimizu T, Koshimizu M, Stojilkovic SS (1999) Contributions of the C-terminal domain to the control of P2X receptor desensitization. J Biol Chem 274 (53):37651–37657

Kowalski M, Hausmann R, Dopychai A, Grohmann M, Franke H, Nieber K, Schmalzing G, Illes P, Riedel T (2014) Conformational flexibility of the agonist binding jaw of the human P2X3 receptor is a prerequisite for channel opening. Br J Pharmacol 171 (22):5093–5112

Kracun S, Chaptal V, Abramson J, Khakh BS (2010) Gated access to the pore of a P2X receptor: structural implications for closed-open transitions. J Biol Chem 285(13):10110–10121

Le KT, Boue-Grabot E, Archambault V, Seguela P (1999) Functional and biochemical evidence for heteromeric ATP-gated channels composed of P2X1 and P2X5 subunits. J Biol Chem 274(22):15415–15419

Lemoine D, Habermacher C, Martz A, Mery PF, Bouquier N, Diverchy F, Taly A, Rassendren F, Specht A, Grutter T (2013) Optical control of an ion channel gate. Proc Natl Acad Sci U S A 110 (51):20813–20818

Li M, Chang TH, Silberberg SD, Swartz KJ (2008) Gating the pore of P2X receptor channels. Nat Neurosci 11 (8):883–887

Li M, Kawate T, Silberberg SD, Swartz KJ (2010) Pore-opening mechanism in trimeric P2X receptor channels. Nat Commun 1:44

Li M, Silberberg SD, Swartz KJ (2013) Subtype-specific control of P2X receptor channel signaling by ATP and Mg^{2+}. Proc Natl Acad Sci U S A 110(36):E3455–E3463

Liang X, Xu H, Li C, Yin S, Xu T, Liu J, Li Z (2013) Functional identification of close proximity amino acid side chains within the transmembrane-spanning helixes of the P2X2 receptor. PLoS One 8(8):e70629

Lorinczi E, Bhargava Y, Marino SF, Taly A, Kaczmarek-Hajek K, Barrantes-Freer A, Dutertre S, Grutter T, Rettinger J, Nicke A (2012) Involvement of the cysteine-rich head domain in activation and desensitization of the P2X1 receptor. Proc Natl Acad Sci U S A 109(28):11396–11401

Mansoor SE, Lu W, Oosterheert W, Shekhar M, Tajkhorshid E, Gouaux E (2016) X-ray structures define human P2X3 receptor gating cycle and antagonist action. Nature 538(7623):66–71

Mateja A, Paduch M, Chang HY, Szydlowska A, Kossiakoff AA, Hegde RS, Keenan RJ (2015) Protein targeting. Structure of the Get3 targeting factor in complex with its membrane protein cargo. Science 347(6226):1152–1155

Migita K, Haines WR, Voigt MM, Egan TM (2001) Polar residues of the second transmembrane domain influence cation permeability of the ATP-gated P2X (2) receptor. J Biol Chem 276(33):30934–30941

Moffatt L, Hume RI (2007) Responses of rat P2X2 receptors to ultrashort pulses of ATP provide insights into ATP binding and channel gating. J Gen Physiol 130(2):183–201

North RA, Surprenant A (2000) Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40:563–580

O'Sullivan WJ, Perrin DD (1964) The stability constants of metal-adenine nucleotide complexes. Biochemistry 3:18–26

Pierdominici-Sottile G, Moffatt L, Palma J (2016) The dynamic behavior of the P2X4 ion channel in the closed conformation. Biophys J 111(12):2642–2650

Pintor J, Diaz-Hernandez M, Gualix J, Gomez-Villafuertes R, Hernando F, Miras-Portugal MT (2000) Diadenosine polyphosphate receptors. from rat and guinea-pig brain to human nervous system. Pharmacol Ther 87(2–3):103–115

Pippel A, Stolz M, Woltersdorf R, Kless A, Schmalzing G, Markwardt F (2017) Localization of the gate and selectivity filter of the full-length P2X7 receptor. Proc Natl Acad Sci U S A 114(11):E2156–E2165

Rassendren F, Buell G, Newbolt A, North RA, Surprenant A (1997) Identification of amino acid residues contributing to the pore of a P2X receptor. EMBO J 16(12):3446–3454

Roberts JA, Evans RJ (2007) Cysteine substitution mutants give structural insight and identify ATP binding and activation sites at P2X receptors. J Neurosci 27(15):4072–4082

Roberts JA, Digby HR, Kara M, El Ajouz S, Sutcliffe MJ, Evans RJ (2008) Cysteine substitution mutagenesis and the effects of methanethiosulfonate reagents at P2X2 and P2X4 receptors support a core common mode of ATP action at P2X receptors. J Biol Chem 283(29):20126–20136

Roberts JA, Valente M, Allsopp RC, Watt D, Evans RJ (2009) Contribution of the region Glu181 to Val200 of the extracellular loop of the human P2X1 receptor to agonist binding and gating revealed using cysteine scanning mutagenesis. J Neurochem 109 (4):1042–1052

Roberts JA, Allsopp RC, El Ajouz S, Vial C, Schmid R, Young MT, Evans RJ (2012) Agonist binding evokes extensive conformational changes in the extracellular domain of the ATP-gated human P2X1 receptor ion channel. Proc Natl Acad Sci U S A 109 (12):4663–4667

Rokic MB, Stojilkovic SS, Vavra V, Kuzyk P, Tvrdonova V, Zemkova H (2013) Multiple roles of the extracellular vestibule amino acid residues in the function of the rat P2X4 receptor. PLoS One 8(3): e59411

Rokic MB, Stojilkovic SS, Zemkova H (2014) Structural and functional properties of the rat P2X4 purinoreceptor extracellular vestibule during gating. Front Cell Neurosci 8:3

Rothwell SW, Stansfeld PJ, Bragg L, Verkhratsky A, North RA (2014) Direct gating of ATP-activated ion channels (P2X2 receptors) by lipophilic attachment at the outer end of the second transmembrane domain. J Biol Chem 289(2):618–626

Samways DS, Khakh BS, Dutertre S, Egan TM (2011) Preferential use of unobstructed lateral portals as the access route to the pore of human ATP-gated ion channels (P2X receptors). Proc Natl Acad Sci U S A 108(33):13800–13805

Schrödinger LLC (2015) The PyMOL molecular graphics system, Version 1.8

Seguela P, Haghighi A, Soghomonian JJ, Cooper E (1996) A novel neuronal P2x ATP receptor ion channel with widespread distribution in the brain. J Neurosci 16 (2):448–455

Silberberg SD, Chang TH, Swartz KJ (2005) Secondary structure and gating rearrangements of transmembrane segments in rat P2X4 receptor channels. J Gen Physiol 125(4):347–359

Silberberg SD, Li M, Swartz KJ (2007) Ivermectin interaction with transmembrane helices reveals widespread rearrangements during opening of P2X receptor channels. Neuron 54(2):263–274

Simon J, Kidd EJ, Smith FM, Chessell IP, Murrell-Lagnado R, Humphrey PP, Barnard EA (1997) Localization and functional expression of splice variants of the P2X2 receptor. Mol Pharmacol 52(2):237–248

Smith FM, Humphrey PP, Murrell-Lagnado RD (1999) Identification of amino acids within the P2X2 receptor C-terminus that regulate desensitization. J Physiol 520 (Pt 1):91–99

Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, Stuhmer W (1996) P2X4: an ATP-activated ionotropic receptor cloned from rat brain. Proc Natl Acad Sci U S A 93(8):3684–3688

Stelmashenko O, Lalo U, Yang Y, Bragg L, North RA, Compan V (2012) Activation of trimeric P2X2 receptors by fewer than three ATP molecules. Mol Pharmacol 82(4):760–766

Stelmashenko O, Compan V, Browne LE, North RA (2014) Ectodomain movements of an ATP-gated ion channel (P2X2 receptor) probed by disulfide locking. J Biol Chem 289(14):9909–9917

Stephan G, Kowalski-Jahn M, Zens C, Schmalzing G, Illes P, Hausmann R (2016) Inter-subunit disulfide locking of the human P2X3 receptor elucidates ectodomain movements associated with channel gating. Purinergic Signal 12(2):221–233

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272(5262):735–738

Torres GE, Haines WR, Egan TM, Voigt MM (1998) Co-expression of P2X1 and P2X5 receptor subunits reveals a novel ATP-gated ion channel. Mol Pharmacol 54(6):989–993

Ugur M, Drummond RM, Zou H, Sheng P, Singer JJ, Walsh JV Jr (1997) An ATP-gated cation channel with some P2Z-like characteristics in gastric smooth muscle cells of toad. J Physiol 498(Pt 2):427–442

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G (1994) A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. Nature 371(6497):516–519

Wang J, Yu Y (2016) Insights into the channel gating of P2X receptors from structures, dynamics and small molecules. Acta Pharmacol Sin 37(1):44–55

Wilkinson WJ, Jiang LH, Surprenant A, North RA (2006) Role of ectodomain lysines in the subunits of the heteromeric P2X2/3 receptor. Mol Pharmacol 70 (4):1159–1163

Yan Z, Liang Z, Obsil T, Stojilkovic SS (2006) Participation of the Lys313-Ile333 sequence of the purinergic P2X4 receptor in agonist binding and transduction of signals to the channel gate. J Biol Chem 281 (43):32649–32659

Yan Z, Khadra A, Li S, Tomic M, Sherman A, Stojilkovic SS (2010) Experimental characterization and mathematical modeling of P2X7 receptor channel gating. J Neurosci 30(42):14213–14224

Young MT (2010) P2X receptors: dawn of the post-structure era. Trends Biochem Sci 35(2):83–90

Zemkova H, Yan Z, Liang Z, Jelinkova I, Tomic M, Stojilkovic SS (2007) Role of aromatic and charged ectodomain residues in the P2X(4) receptor functions. J Neurochem 102(4):1139–1150

Zhao WS, Wang J, Ma XJ, Yang Y, Liu Y, Huang LD, Fan YZ, Cheng XY, Chen HZ, Wang R, Yu Y (2014) Relative motions between left flipper and dorsal fin domains favour P2X4 receptor activation. Nat Commun 5:4189

Zhao WS, Sun MY, Sun LF, Liu Y, Yang Y, Huang LD, Fan YZ, Cheng XY, Cao P, Hu YM, Li L, Tian Y, Wang R, Yu Y (2016) A highly conserved salt bridge stabilizes the kinked conformation of β2,3-sheet essential for channel function of P2X4 receptors. J Biol Chem 291(15):7990–8003

Adv Exp Med Biol - Protein Reviews (2017) 19:71–89
DOI 10.1007/5584_2017_57

Published online: 22 June 2017

$P2Y_1$ Receptors – Properties and Functional Activities

Jolanta Barańska, Rafał Czajkowski, and Paweł Pomorski

Abstract

In this chapter we try to show a comprehensive image of current knowledge of structure, activity and physiological role of the $P2Y_1$ purinergic receptor. The structure, distribution and changes in the expression of this receptor are summarized, as well as the mechanism of its signaling activity by the intracellular calcium mobilization. We try to show the connection between the components of its G protein activation and cellular or physiological effects, starting from changes in protein phosphorylation patterns and ending with such remote effects as receptor-mediated apoptosis. The special emphasis is put on the role of the $P2Y_1$ receptor in cancer cells and neuronal plasticity. We concentrate on the $P2Y_1$ receptor, it is though impossible to completely abstract from other aspects of nucleotide signaling and cross-talk with other nucleotide receptors is here discussed. Especially, the balance between $P2Y_1$ and $P2Y_{12}$ receptors, sharing the same ligand but signaling through different pathways, is presented.

Keywords

Calcium signaling • Cancer cells • Neuronal plasticity • $P2Y_1$ receptors • $P2Y_1$/$P2Y_{12}$ cross-talk

J. Barańska
Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., PL 02-093 Warsaw, Poland
e-mail: j.baranska@nencki.gov.pl

R. Czajkowski
Laboratory of Spatial Memory, Department of Molecular and Cellular Neurobiology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., PL 02-093 Warsaw, Poland
e-mail: r.czajkowski@nencki.gov.pl

P. Pomorski (✉)
Laboratory of Molecular Basis of Cell Motility, Department of Cell Biochemistry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., PL 02-093 Warsaw, Poland
e-mail: p.pomorski@nencki.gov.pl

Abbreviations

AC	Adenylate cyclase
Akt	Protein kinase B/Akt kinase
$[Ca^{2+}]$	Intracellular calcium concentration
CA1	*Cornu Ammonis* area 1
CA3	*Cornu Ammonis* area 3
DAG	Diacylglycerol
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinases 1/2
GABA	γ-Aminobutyric acid
IP_3	Inositol-1,4,5-trisphosphate
LTD	Long term depression
mGluR	Metabotropic glutamate receptor
2MeSADP	2-Methylthio ADP
MRS2365	(N)-Methanocarba 2MeSADP
NMDA	N-Methyl-D-aspartic acid
NR2B	NMDA receptor subtype 2B
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane calcium pump
PPADS	Pyridoxal-phosphate-6-azophenyl-2′,4′disulfonic acid
PTEN	3-Phosphatase and tensin homolog
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule
TM	Transmembrane domain

1 $P2Y_1$ Receptors – Principles

The $P2Y_1$ receptor was the first P2 receptor originally cloned as early as nearly 25 years ago (Webb et al. 1993). The cDNA encoding this receptor was isolated from an embryonic chick whole brain cDNA library by hybridization screening. It was found that the encoded protein consists of 362 amino acids, belongs to the G-protein-coupled receptor class and binds ATP. This protein was then designated as a novel P2Y purinoceptor and given the number one. The $P2Y_1$ designation was further supported by the restricted expression of the corresponding mRNA transcript (Webb et al. 1993). Afterwards, $P2Y_1$ receptors were cloned and characterized in numerous other animal species: *Meleagris gallopavo*, turkey (Filtz et al. 1994); *Bos taurus*, cow (Henderson et al. 1995); rodent (Tokuyama et al. 1995); *Homo sapiens* (Janssens et al. 1996; Léon et al. 1996); *Xenopus laevis* (O'Grady et al. 1996), showing 83–95% amino acids identity (Léon et al. 1996).

The later pharmacological studies on the $P2Y_1$ receptor showed that ADP is more potent natural agonist than ATP itself, while UTP and UDP, CTP and GTP are not effective (Léon et al. 1997). Several artificial agonists were later synthesized and non-hydrolyzing analogs of ADP, 2-methylthio-ADP (2MeSADP) and (N)-methanocarba-2MeSADP (MRS2365) were found to be even more potent, exhibiting EC_{50} values in a low nanomolar range (Chhatriwala et al. 2004). However, it is now firmly established that ADP is a natural agonist not only for $P2Y_1$, but also for $P2Y_{12}$ and $P2Y_{13}$ receptors and both ADP and 2MeSADP activate these receptors subtypes (Fumagalli et al. 2004; Hollopeter et al. 2001). Therefore, the most selective $P2Y_1$ agonist known presently is MRS2365, activating $P2Y_1$ with a high potency, but not activating, or activating with a very low potency, $P2Y_{12}$ and $P2Y_{13}$ receptors (Chhatriwala et al. 2004; Carrasquero et al. 2005; Koch et al. 2015). Nevertheless, since the signal transduction pathway induced by activated $P2Y_1$ differs distinctly from that induced by $P2Y_{12}$ and $P2Y_{13}$ (see Fig. 1A), ADP and 2MeSADP are widely and successfully used for $P2Y_1$ activity assessment. It has been found that the $P2Y_1$ receptor is inhibited by suramine and pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid (PPADS), but the most effective and selective antagonists are MRS2179, MRS2279 and MRS2500 (for review, see (Burnstock and Knight 2004; Abbracchio et al. 2006; Burnstock 2007)).

Similarly to other metabotropic G-protein-coupled receptors, $P2Y_1$ contains seven

hydrophobic transmembrane domains (TM) linked to each other by three extracellular loops and three intracellular loops. Intracellular loops participate in G-protein coupling. NH_2 terminus is extracellular while intracellular terminus contains COOH group (Ralevic and Burnstock 1998). Site-directed mutagenesis of the human $P2Y_1$ receptor has shown a critical role in receptor activation of some positively charged amino acids residues on TM 3, 6 and 7, which are suggested to assist in forming ligand–docking pocket (Ralevic and Burnstock 1998; Jiang et al. 1997). In addition, four cystein residues in the extracellular loops have been proposed to be critical for the proper trafficking of the human $P2Y_1$ receptor to the cell surface (Hoffmann et al. 1999).

From the phylogenetic and structural point of view, P2Y receptor family can be divided into two subgroups with high structural divergence (Abbracchio et al. 2003). The first one contains $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, while the other includes $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ subtypes (Abbracchio et al. 2003). Each subgroup is characterized by specific motifs in TM 6 and TM 7 that might be important for agonist–receptor interactions and receptor activity. All P2Y receptors from both subgroups share the presence in TM 6 of a histidine – X – X – arginine/lysine (**H**-X-X-**R/K**), motif proposed as crucial for receptor activity (symbols of amino acids important for ligand binding are bold-faced). In TM 7, a tyrosine – glutamine/lysine – X – X – arginine (Y-**Q/K**-X-X-**R**) motif for the first P2Y subgroup and a lysine – glutamic acid – X – X – leucine (**K**-E-X-X-**L**) for the second one are considered to participate in the different mode of agonist binding for these two receptor subgroups. In particular, human $P2Y_1$ receptor ($hP2Y_1$) is characterized by a histidine – valine – methionine – lysine (**H**-V-M-**K**) and a tyrosine – glutamine – valine – threonine – arginine (Y-**Q**-V-T-**R**) motifs in TM 6 and TM 7, respectively (Abbracchio et al. 2003).

It is worth adding that *Homo sapiens* $P2Y_1$ receptor contains a sequence of 372 amino acids (Léon et al. 1996) and it's gene is located at human third chromosome long arm (locus 3q24–25) (Abbracchio et al. 2003, 2006). In this chromosome, $P2Y_1$ is a member of a seven receptors cluster together with $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ and three other metabotropic receptors (Abbracchio et al. 2003). Most recently, the X-ray crystal structure of the human $P2Y_1$ receptor has been presented, postulating two separate ligand binding sites in the receptor protein (Zhang et al. 2015).

When stimulated with nucleotides, all P2Y receptors act *via* binding α,β,γ – heterotrimeric G protein at the inner surface of the cell. Two P2Y receptor subgroups mentioned above differ from each other structurally, which leads to their ability to couple with different G protein subfamilies. Thus, P2Y receptors of the first subgroup ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) are coupled with G_q protein and activate phospholipase C β (PLCβ), leading to the increase in inositol-1,4,5-trisphosphate (IP_3) and cytosolic Ca^{2+}, whereas those belonging to the second subgroup ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) are coupled with $G_{i/o}$ and their activation leads to inhibition of adenylate cyclase (AC) and the decrease in cAMP level (Fig. 1A). However, while the $P2Y_1$ receptor is coupled exclusively with the G_q protein subfamily, the others of this subgroup are additionally able to signal *via*: G_o ($P2Y_2$, $P2Y_4$); G_{12} ($P2Y_2$, $P2Y_6$); G_{13} ($P2Y_6$); or G_s ($P2Y_{11}$) inducing the increase in cAMP level (Erb and Weisman 2012; Waldo and Harden 2004). According to Soulet et al. coupling of the platelet ADP–stimulated $P2Y_1$ receptor with G_q activates not only PLCβ, but also small, monomeric G proteins, particularly the Rac small G protein (Soulet et al. 2005), however the mechanism of this effect is unclear. Nevertheless, although RhoA and Rac are crucial proteins for cytoskeleton rearrangements (Kłopocka et al. 2013), their downstream signaling will not be discussed here. The main function of the $P2Y_1$ receptor still seems to be intracellular Ca^{2+} mobilization (Wypych and Barańska 2013; Wypych and Pomorski 2013) and this signaling pathway will be further described in detail (see Sect. 3).

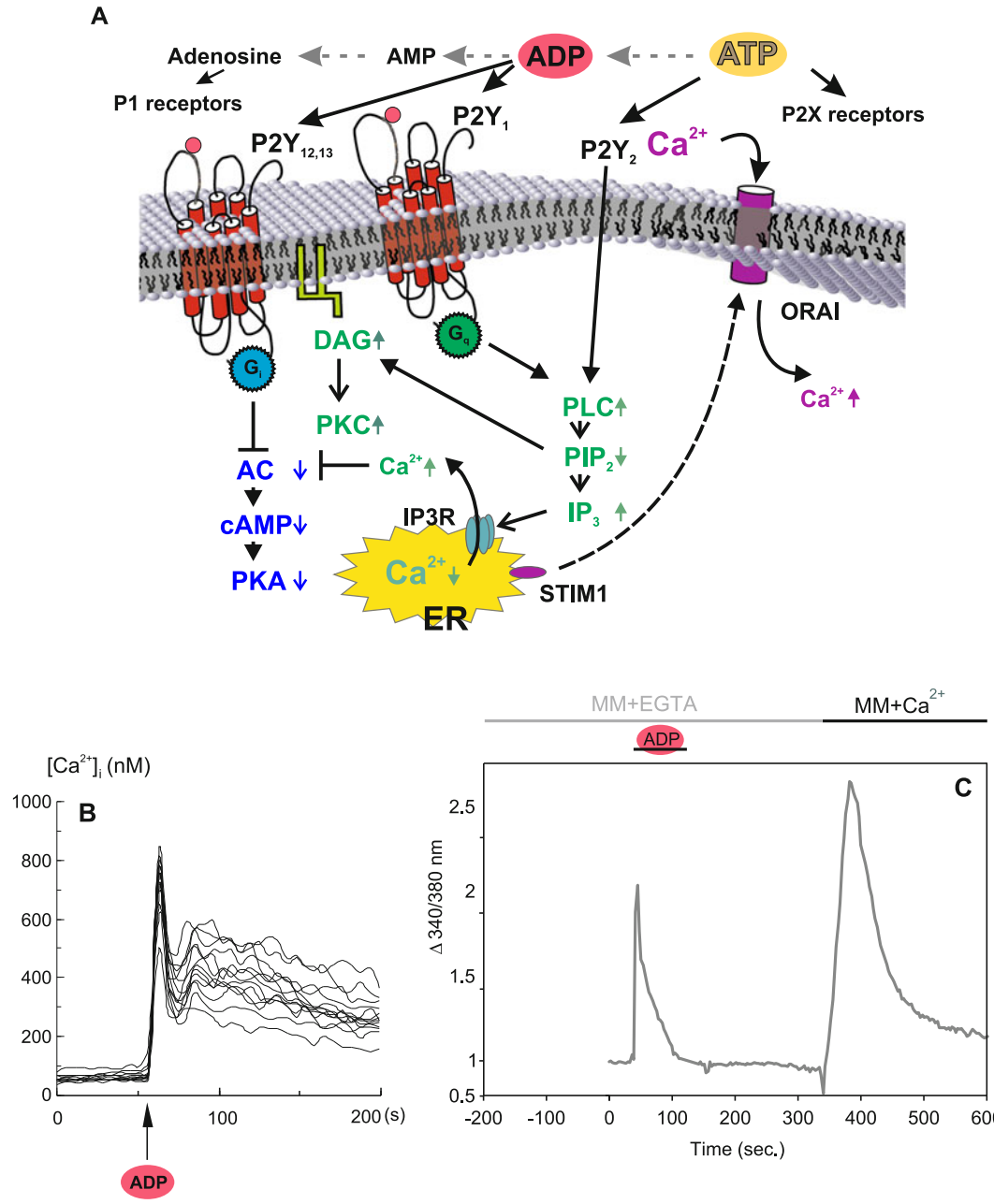

Fig. 1 Schematic diagram of the $P2Y_1$ receptor signaling pathways. (**A**) Effects of extracellular ADP on metabotropic $P2Y_1$ and $P2Y_{12/13}$ receptors. Extracellular ATP is hydrolyzed by ectoenzymes to ADP and further to adenosine (which acts on P1 receptors). ADP stimulates both $P2Y_1$ and $P2Y_{12}$ as well as $P2Y_{13}$ receptors. The $P2Y_1$ receptor activates PLC *via* G_q protein (*green pathways*). PLC converts PIP_2 to IP_3 and DAG, which activates PKC. IP_3 binds to specific receptors (IP3R) on the endoplasmic reticulum (*ER*) membrane and causes release of Ca^{2+} into the cytosol. Calcium inhibits adenylate cyclase (*AC*) activity. The $P2Y_{12}$ and $P2Y_{13}$ receptor coupled with G_i protein inhibits AC activity, decreases intracellular level of cAMP and inhibits protein kinase A (*PKA*) activity (*blue*). Emptying of ER calcium stores activates STIM1 sensor (purple pathway, SOCE mechanism). STIM1 activates ORAI channel in the plasma membrane and initiates calcium influx from the cell environment. The $P2Y_2$ receptor (not shown), activated by ATP (and UTP) signals in the same way as

2 $P2Y_1$ Receptors – Distribution

$P2Y_1$ receptors are widely distributed in animals and abundant in nearly all human and rodent tissues. In situ hybridization, immunochemistry and functional studies have demonstrated that this receptor subtype is expressed in such diverse tissues, as heart, testis, prostate glands, ovary, placenta, neural tissues, blood cells, blood vessels, platelets, or skeletal and smooth muscle (for review see: (Burnstock and Knight 2004), see also a recent special issue of the "Purinergic Signaling" containing review articles on the role of purinergic receptors in visceral organs (Burnstock G ed. 2014)). The special and very important role is played by the $P2Y_1$ receptor in platelets, where it cooperates with $P2Y_{12}$ receptor in thrombosis (Hechler and Gachet 2015). The role of purinergic signaling, including $P2Y_1$ receptors, was most recently described in the cardiovascular system (Burnstock 2017). Thus, $P2Y_1$-mediated responses occur both in excitable and noon-excitable cell types, involving short- and long-term signaling (Burnstock and Knight 2004).

In comparison with other tissues, the human brain exhibits the presence of $P2Y_1$ mRNA in large quantities (Moore et al. 2001). $P2Y_1$ mRNA was most highly expressed in regions of the basal ganglia including the striatum, nucleus accumbens, putamen and caudate nucleus and was detected in the globus pallidus, hippocampus, cerebellum and many regions of the cerebral cortex (Illes and Ribeiro 2004). A $P2Y_1$ polyclonal antibody indicated the receptor localization to neuronal structure of the human cerebral cortex, cerebellar cortex, hippocampus, caudate nucleus, putamen, globus pallidus and midbrain (Moore et al. 2000). Similar high expression and basically similar distribution of this receptor was observed in the rat brain (Morán-Jiménez and Matute 2000). The $P2Y_1$ receptor, highly expressed in central nervous system (CNS), may take a part in many functions and exhibits various effects both in neuronal and glial cells (see Sect. 7). Interesting phenomenon concerning $P2Y_1$ receptors expression and distribution is the fact that in contrast to healthy neuronal cells where the $P2Y_1$ predominate, neuronal cancer cell lines, both of human and rodent origin, do not contain any detectable $P2Y_1$ receptors (Illes and Ribeiro 2004; Sak and Illes 2005). Sak and Illes suggested that the lack of this receptor subtype could be a general property of neuronal malignant cells. It is assumed that this phenomenon could be related to the pathogenesis of neuronal malignancies (Sak and Illes 2005).

In many studies, cell lines have been used as a model to examine *in vitro* receptors expression profile and properties. Such examples are neuronal cell lines of human origin (SH-SY5Y, SK-N-BE, IMR-32 neuroblastomas), rat neuronal cells (PC12 rat pheochromocytoma), or mouse neuroblastoma and rat glioma fusion cells (NG108–15), used as a model to investigate the functions of neuronal receptors (Sak and Illes 2005). On the other hand, rat glioma C6 cells, belonging to the type of non-excitable cells (Barańska et al. 1995), is a commonly used model system for related studies on glial cell biology, both for astrocytes and oligodendrocytes, as well as for glioma cells (Barańska et al. 2004).

Fig. 1 (continued) the $P2Y_1$ receptor. (**B**) Effect of ADP on $[Ca^{2+}]i$ measured in Fura-2 loaded glioma C6 cells. Experiment performed in the presence of extracellular Ca^{2+}. Each trace represents the response of an individual cell. (**C**) Schematic presentation of the averaged data from biphasic experiment showing mechanism of SOCE. Appearance of ADP in calcium free medium (Minimal Medium with 500 μM EGTA, MM+EGTA) activates $P2Y_1$ receptors and leads to the release of Ca^{2+} from ER through IP_3 receptors (the first phase of the signal), then plasma membrane calcium pumps remove Ca^{2+} from cytoplasm while STIM1-ORAI system remains active. After exchange of medium to one containing 2 mM calcium (MM+Ca^{2+}), a sustained increase in $[Ca^{2+}]_i$ results from the influx of calcium ions through open ORAI channels (the second phase of the signal). For details, see text. (Fig. 1B) (Adapted from (Sabala et al. 2001), with permission of John Wiley and Sons)

The presence of the $P2Y_1$ receptor in glioma C6 cells seemed to be doubtful and was the subject of a serious debate in the 1990s. It was generally accepted that this cell line expressed the $P2Y_2$ receptor, responding to ATP (and UTP), activating PLC and affecting IP_3 formation. However, ADP used as an agonist induced only the inhibition of adenylate cyclase (AC) (Boarder and Hourani 1998; Schachter et al. 1996). It was therefore concluded that glioma C6 cells were devoid of the $P2Y_1$ receptor (Claes et al. 2001; Grobben et al. 2001). On the other hand, Webb and others showed that the cloned rat $P2Y_1$ receptor cDNA and that derived from C6 cells were 100% identical in the entire coding region. The authors suggested the same $P2Y_1$ receptor could have different properties in different cell types, i.e. in C6 cells it could inhibit AC without activating PLC (Webb et al. 1996). The question of presence of the $P2Y_1$ receptor in glioma C6 cells was finally clarified due to experiments which showed the ability of ADP- and 2MeSADP-activated cells to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (see Sect. 3). It has been also shown that the presence or absence of these receptor in C6 cells depends on the differences in culture conditions (Barańska et al. 2004; Czajkowski et al. 2004) (see Sects. 4 and 5).

3 $P2Y_1$ Receptors – Signaling Mechanism

As mentioned before, the $P2Y_1$ receptor belongs to the wider category of G-protein coupled receptors, which means that receptor protein forms the functional entity with heterotrimeric GTP bounding protein complex. In case of the $P2Y_1$ receptor it is $G_{\alpha q}$ subclass member. $G_{\alpha q}$ protein activates PLCβ which cleaves phosphatidylinositol-4,5-biphosphate (PIP_2) into the IP_3 and diacylglycerol (DAG). IP_3 diffuses through cytoplasm and binds to the IP_3 receptor on the endoplasmic reticulum (ER), opening calcium channels and releasing Ca^{2+} ions into the cytoplasm. DAG activates protein kinase C (PKC) (Wypych and Pomorski 2013; Putney and Bird 1993) (Fig 1A). $G_{\beta\gamma}$ complex is also a signaling molecule and may regulate plasma membrane channels, mainly G-protein-regulated inwardly rectifying K^+ channels (GIRKs) and P/Q- as well as N-type voltage-gated Ca^{2+} channels in excitable cells. This will be discussed later, since the role of $G_{\beta\gamma}$ complex in the calcium signal formation was postulated during studies on cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors (Van Kolen et al. 2006; Van Kolen and Slegers 2006) (see Sect. 5).

Release of calcium from the ER (the first phase) does not end the signaling event. The emptying of the reticulum leads to the phenomenon known as the capacitative calcium entry (Putney 1990) (or the store operated calcium entry, SOCE). When calcium concentration in the reticulum falls, STIM1 (stromal interaction molecule 1), calcium binding protein abundant in the ER membrane (Roos et al. 2005), starts to oligomerize and forms large complexes, known as *foci*. Those complexes bind plasma membrane protein ORAI1 (Zhang et al. 2005; Calloway et al. 2009), leading to formation of plasma membrane voltage-independent calcium channel and further extracellular calcium flow into the cytoplasm (the second phase of SOCE), known also as the Calcium Release-Activated Current ($CRAC_I$) (Fig. 1A, C). This time influx originates from the extracellular environment. SOCE mechanism works as digital switch (Bird et al. 2009), enhancing signal and preventing permanent emptying of the intracellular calcium stores.

This is not a place to discuss the whole field of calcium signaling, since the topic is extremely wide and interferes with every aspect of cell physiology. It is however, worth to notice, that SOCE rarely leads to cell calcium overstimulation with all its toxic effects (Salińska et al. 2005). Rather contrarily, there are studies showing that SOCE down regulation inhibits cancer cell proliferation, DNA synthesis, cell migration and impairs cell adhesion. Specifically, STIM1 silencing with siRNA leads to cell cycle arrest (Chen et al. 2011; Li et al. 2013), DNA synthesis inhibition (Yoshida et al. 2012), weaker cell contractility (Chen et al. 2013) and focal adhesions degradation (Yang et al. 2009,

2013). Thus, even if calcium signal is widely considered as proapoptotic, SOCE induced by $P2Y_1$ is rather prosurvival and thus will promote cancer development by both proliferation and motility enhancement leading to metastasis promotion.

Figure 1B illustrates typical calcium response compatible with capacitative model of Ca^{2+} influx initiated by ADP (Sabala et al. 2001). ADP induces $[Ca^{2+}]_i$ elevation that starts with an initial peak response (the first phase of the cell response) resulting from a direct effect of IP_3 on the ER Ca^{2+} stores, followed by the second phase – a long, sustained *plateau*, generated by the opening of voltage-independent plasma membrane Ca^2 channels and extracellular Ca^{2+} influx into the cell. These data as well as facts that stimulation of the glioma C6 cells with ADP caused the breakdown of PIP_2 by activated PLC while knockdown of the $P2Y_1$ receptor caused a loss of PLC activity have proved that ADP stimulates here functionally active $P2Y_1$ receptors (Czajkowski et al. 2002).

4 $P2Y_1$ Receptors –Properties and Functional Activity

The main property of the $P2Y_1$ receptor is its ability to induce the intracellular Ca^{2+} mobilization, described in the previous section. The very important functional activity of the $P2Y_1$ receptor established in platelets is just concerned with this signaling pathway. Thus, through $P2Y_1$ generated Ca^{2+} mobilization, $P2Y_1$ is involved in control platelet shape change and in initiation of ADP-induced platelet aggregation. Furthermore, under pathological condition $P2Y_1$ plays a crucial role in different models of thrombosis. In this process $P2Y_1$ positively cooperates with $P2Y_{12}$ receptors, both activated by ADP. $P2Y_1$ contributes also to chronic vascular inflammation and related diseases as e.g. atherosclerosis. The platelet $P2Y_1$/$P2Y_{12}$ cooperation has been extensively studied (for recent review see (Hechler and Gachet 2015)).

In central nervous system the $P2Y_1$ receptor plays a crucial role in the regulation of synaptic plasticity (see Sect. 7). The $P2Y_1$ protein abundance in neurons is important also in peripheral nerves. Well documented is the dependence of pain and temperature sensing on extracellular nucleotides. It was shown, that MRS2500, specific inhibitor of the $P2Y_1$ receptor, blocks sensing formalin-induced pain in rats, used as animal model of inflammatory pain (Barragán-Iglesias et al. 2015). Also models of ischemic pain, based on injection of acidic saline into rat paws, are dependent on the $P2Y_1$ receptor activity and sensitive to MRS2179, inhibitor of this receptor. In the case of ischemic pain it was also shown, that pain sensation is sensitive to inhibition of the TRPV1 protein activity, directly suggesting capacitative calcium signaling as mechanism of $P2Y_1$ receptor participation in pain transmission (Kwon et al. 2014). Similar results were obtained during studies of mouse and human colon pain. Animal studies suggested ADP and UTP as stimulators of visceral nociceptors. $P2Y_1$ and $P2Y_2$ receptors were detected in respectively 80% and 56% retrogradely labeled colonic neurons, suggesting the $P2Y_1$ as one of the crucial receptors responsible for visceral pain during gastrointestinal diseases, such as colon cancer, severe constipations and Crohn's disease (Hockley et al. 2016). Studies on the $P2Y_1$ role in neuropathic pain suggest that this receptor is engaged in the stimuli transmission and is directly connected to importance of this receptor in CNS system function (see Sect. 7). Intrathecal administration of MRS2500, aforementioned inhibitor of the $P2Y_1$ receptor, inhibited pain sensation resulting from the nerve injuries. Peripheral nerve injuries caused also elevation of the $P2Y_1$ receptor level in spinal cord cells, which was inhibited by injections of MRS2500 into spinal canal (Barragán-Iglesias et al. 2016).

The other property of the $P2Y_1$ receptor seems to be its pro-apoptotic activity. This property leads to flexibility of the receptor expression in cancer development (see Sect. 6). Although other P2Y receptors, e.g. $P2Y_2$, may be up- or down-regulated upon pathophysiological conditions

(Burnstock 2007), the $P2Y_1$ receptor has ability to reversible disappearance under physiological conditions, what may lead to incorrect conclusions about its presence in some cell types. This flexibility was well documented in glioma C6 cells. It has been namely shown that the culture condition can cause the presence, or absence of the $P2Y_1$ receptor in these cells. It has been found that the $P2Y_1$ receptor mRNA expression, as well as its protein level and the response to nucleotide agonist, can depend on the presence of serum in the culture medium. The lack of activity described previously might be therefore explained by culturing of C6 cells in a chemically defined serum free medium (Schachter et al. 1996; Grobben et al. 2001). Long-term absence of serum in the medium specifically affected the $P2Y_1$ receptor, decreasing very strongly its mRNA, protein level as well as activity, while was without any effect on the $P2Y_2$ receptor mRNA and protein level (Barańska et al. 2004; Czajkowski et al. 2002, 2004; Krzemiński et al. 2007).

In pathophysiological conditions this receptor flexibility is even better pronounced, as in already mentioned situation where $P2Y_1$ is the predominant receptor in neuronal healthy cells and is not detectable in neuronal cancer cells (Sak and Illes 2005). In platelets, $P2Y_1$ can be overexpressed leading to increased ADP-induced platelets aggregation and arterial thrombosis, or can be downregulated under conditions of injury or inflammation. The latter process is combined with the increase in expression of the A_{2B} adenosine receptor coupled with G_s and inducing increase in cAMP levels and resulting in reduced ADP-dependent aggregation (Hechler and Gachet 2015). Also, during C2C12 myoblasts differentiation into myotubes, the expression of $P2Y_1$ receptors distinctly diminished (Banachewicz et al. 2005). Interestingly, 10% fetal bovine serum withdrawal for 48 h induced cell-cycle arrest and changed glioma C6 cells morphology from fibroblast-like to astrocyte-like appearance. This process did not reflect differentiation into astrocytes or oligodendrocytes, but occurred simultaneously with the strong decrease in the level of $P2Y_1$ receptors. Transfer of the cells into fresh medium containing serum reversed all of these changes, demonstrating their temporary character (Barańska et al. 2004; Krzemiński et al. 2007). Taken together, these data allow to speculate that the $P2Y_1$ receptor may be involved in many important, cellular regulatory functions at the level of protein expression.

Among properties of the $P2Y_1$ receptor one should mention its ability to form oligomers. There is evidence that the human $P2Y_1$ receptors form homodimers (Choi et al. 2005). The $P2Y_1$ receptor forms also hetero-oligomers (Nakata et al. 2003). In HEK293 or 1321NA1 cells, the $P2Y_1$ receptor forms a heterodimeric complex with the $P2Y_{11}$ receptor, which alters the $P2Y_{11}$ receptor function by promoting agonist-induced receptor endocytosis (Ecke et al. 2008). On the other hand, in cotransfected HEK293 cells rat $P2Y_1$ receptor forms heteromeric complex with adenosine A_1 receptor. Such heteromeric complex seems to respond predominantly to the $P2Y_1$ receptor agonists, since $P2Y_1$ and not A_1 receptor appears to be involved in the process of $G_{\alpha q}$ activation and IP_3 formation, suggesting that the cross-talk with G proteins for both receptors complex is unidirectional (Yoshioka et al. 2001). What is more interesting, such $P2Y_1/A_1$ hetero-dimers were also observed *in vivo* with confocal laser microscopy in rat brain – in cortex, hippocampus and cerebellum. Yoshioka and others suggested that $P2Y_1/A_1$ hetero-oligomers display a wide-spread colocalization in the brain, since both receptors can exist in the same neuron (Yoshioka et al. 2002). However, it is generally believed from the studies on solid tumor cells or on transfected cells that the A_1 adenosine receptors exerts pro-survival and anti-apoptotic actions. The increase in its expression could even represent a diagnostic marker for glioblastoma progression (Ceruti and Abbracchio 2013). Since in gliomas, the $P2Y_1$ receptor has just opposite, pro-apoptotic properties, these two – $P2Y_1$ (coupled with G_q) and A_1 adenosine (coupled with G_i, G_q and G_o) receptors interactions greatly increased the diversity of purinergic signaling. It is worth to add that the ability to form homo- and hetero-oligomers has been also found

among other P2Y receptors: $P2Y_4$ and $P2Y_6$ (D'Ambrosi et al. 2007). More recently, the $P2Y_1$ receptor heteromeric association with $P2Y_2$ and $P2Y_4$ receptors was shown in mouse granulocytes. However the association between $P2Y_2$ and $P2Y_4$ receptors was not observed (Ribeiro-Filho et al. 2016).

5 $P2Y_1$ Receptors – Cross-Talk with $P2Y_{12}$ Receptors

$P2Y_1$ and $P2Y_{12}$ are both activated by the same nucleotide, thus the cross-talk between these receptors, even on the level of the accessibility of the agonist, seems to be obvious and was observed in many cells. In glioma C6 cells, long-term (48 h) absence of serum in the culture medium resulted in the decrease in $P2Y_1$ mRNA expression correlated with an increase in $P2Y_{12}$ receptor mRNA expression, while the $P2Y_2$ receptor mRNA level was not changed. The original level of $P2Y_1$ and $P2Y_{12}$ receptors mRNA was restored by supplementation with serum. The similar pattern was observed for protein level (Czajkowski et al. 2004; Krzemiński et al. 2007). However, the silencing of $P2Y_1$ with siRNA did not lead to the increase in $P2Y_{12}$ receptors expression. Thus, the increased $P2Y_{12}$ protein level during serum starvation was not a consequence of the inhibition of $P2Y_1$ receptor expression, but it rather should be a result of the change in cell culture medium, i.e. the lack of some factor present in serum (Wypych and Pomorski 2012). One could speculate that this important serum component might be lysophosphatidic acid.

Lysophosphatidic acid, bound to albumin and carried in the blood stream, interacts with receptor coupled to G_i protein and similarly as $P2Y_{12}$ is negatively-coupled with AC and decreases cAMP synthesis (Jalink et al. 1994; Kranenburg and Moolenaar 2001; Moolenaar 1999). In contrast to neurons, in primary astrocytes and glioma C6 cells, the increase in cAMP concentration inhibits extracellular signal-regulated kinase 1/2 (ERK1/2) cascade and in consequence blocks cell growth and promotes differentiation (for review, see (Stork and Schmitt 2002)) In glioma C6 cells growing in chemically defined serum free medium, agents activating β-adrenergic receptor or membrane-permeable cAMP analogues (dibutyryl cAMP) stimulate AC, increase cAMP synthesis and induce differentiation of C6 cells into type II astrocytes (Messens and Slegers 1992). Claes et al. showed that this cAMP induced differentiation of C6 cells could be inhibited by activation of the $P2Y_{12}$ receptor (Claes et al. 2004). Thus, serum starvation and the increase in intracellular cAMP level produce similar morphological changes as a result of inhibition of cellular proliferation, but – as it was mentioned above – when the level of cAMP is not additionally increased, absence of serum causes only differentiation-like changes. Thus, most probably, during long-term serum starvation, due to the lack of lysophosphatidic acid in extracellular space the level of intracellular cAMP might increase. This cAMP up-regulation might be even slight, but sufficient to give a signal for the increase in $P2Y_{12}$ receptors expression. Such increase acts to maintain the low intracellular cAMP concentration and, due to that, to block cAMP-induced inhibition of cellular proliferation and cell differentiation. In this precise cellular scenery, $P2Y_1$ receptor seems to be an important regulatory molecule, since the strong decrease in its expression during serum starvation, or its lack in some malignant cells (e.g. neuronal cancer cells), allows the $P2Y_{12}$ receptor to be the main player responsible for ADP-evoked signal transduction. On the contrary, in healthy neurons, where the increase in cAMP activates ERK1/2, thus stimulating their growth and survival (Dugan et al. 1999), $P2Y_1$ is the predominating P2Y receptor (Sak and Illes 2005) and this receptor (but not $P2Y_{12}$) is the main component responsible for ADP-induced signaling. Taken together, it seems that in a variety of cell types, in different physiological and pathological conditions, the most important cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors might occur at the level of the regulation of protein expression.

The interactions of ADP with two distinct receptors, $P2Y_1$ and $P2Y_{12}$, may also involve

the action of second messengers. In glioma C6 cells, the $P2Y_1$ receptor activation leads to Ca^{2+} mobilization, which may down-regulate type VI of AC, predominating in gliomas (Chiono et al. 1995). Thus, the increase in $[Ca^{2+}]_I$ induced by $P2Y_1$ may in cooperation with $P2Y_{12}$ negatively regulate cAMP synthesis in favor of the cells growth. On the other hand, Van Kolen and coworkers suggested that the stimulation of the $P2Y_{12}$ receptor may generate, via Gβγ subunits of the G protein, a direct entry of extracellular Ca^{2+} by a $P2Y_{12}$-dependent N-type calcium channel in plasma membrane of glioma C6 cells (Van Kolen et al. 2006; Van Kolen and Slegers 2006). However, according to our studies this hypothesis and existence of such channel seems to be doubtful (Suplat et al. 2007). We have found that Ca^{2+} response evoked by $P2Y_1$ receptors might be indeed potentiated by $P2Y_{12}$, but most probably due to the other mechanism proposed by Hardy et al. in platelets, i.e. due to a lower level of cAMP induced during $P2Y_{12}$ receptors stimulation, leading to the inhibition of the plasma membrane calcium pump (PMCA), which disrupts the removal of calcium from the cytoplasm (Hardy et al. 2004). Thus, the activation of both, $P2Y_1$ and $P2Y_{12}$ receptors by the same agonist may generate the increase in the intracellular Ca^{2+} level as a result of the positive cross-talk between these two receptors – $P2Y_1$ inducing capacitative Ca^{2+} entry and $P2Y_{12}$ playing modulatory role in calcium signaling (Suplat et al. 2007). In glioma C6 cells, ADP, *via* both $P2Y_1$ and $P2Y_{12}$ receptors, also takes part in the activation of ERK1/2 (a known cell proliferation regulator), but this effect is primarily mediated by the $P2Y_{12}$ receptor (Czajkowski et al. 2004; Krzemiński et al. 2007).

There are data, showing that $P2Y_1$ and $P2Y_{12}$ receptors work together in a wide variety of the cell systems. De Simone and coworkers showed that both receptors take their part in LPS and TGF-β dependent activation of microglia as well as regulate microglia motility (De Simone et al. 2010). Similar phenomenon was shown for monocytes where crucial role of the $P2Y_{12}$ and ERK kinases in cell activation was proved (Zhang et al. 2016). On the other hand, immunohistochemical studies of sinus endothelial cells of the rat spleen revealed strong colocalization between $P2Y_1$ and $P2Y_{12}$ receptors in those cells (Uehara and Uehara 2011). Inhibition of $P2Y_{12}$ receptor by AR-C69931MX abolished also ADP evoked contractility in rat intrapulmonary artery showing the influence of $P2Y_{12}$ receptor on $P2Y_1$ (or $P2Y_6$) calcium signaling in smooth muscle cells (Mitchell 2012). There are, however, no systematic studies how common is this cross-talk mechanism among tissues and cell types.

It is worth to add that in cerebellar astrocytes, Carrasquero et al. postulate co-expression and cross-talk between $P2Y_1$ and $P2Y_{13}$ receptors, while $P2Y_{12}$ was not detected. The authors suggested that in these cells $P2Y_{13}$ substitutes for $P2Y_{12}$ and cooperates with $P2Y_1$ similarly as it was observed in platelets (Carrasquero et al. 2005).

6 $P2Y_1$ Receptors and Cancer

The role of purinergic receptors in anticancer activity was described for the first time in 1983 (Rapaport 1983). In this *in vivo* studies, mice inoculated with cancer cells were systematically treated with ATP and ADP, which caused an inhibition of their weight loss and prolonged the survival time. It was also shown that ATP injected daily into mice significantly inhibited cancer growth in animals with CT26 colon tumor (Rapaport and Fontaine 1989; Rapaport 1990). However, since ATP (due to the action of ectonucleotidases) can be degraded to ADP and further to adenosine in animal body, the participation of particular P2 receptor subtypes in this process was unknown. To address this problem *in vitro* studies in different cell lines and different types of cancer were further performed. These *in vitro* studies on agonists and antagonists have in general suggested that among P2Y receptor subtypes, $P2Y_1$ is the one which mediates a decrease in cancer cell number. On the opposite, the activation of $P2Y_2$ and $P2Y_{12}$ receptors generates an increase in cells

population (for review, see (White and Burnstock 2006)).

According to much evidence, $P2Y_1$ receptors may indeed regulate cancer cells growth and death (White and Burnstock 2006). For example, in 1321 N astrocytoma cells expressing recombinant human $P2Y_1$ receptor, an agonist-activation of this receptor induces apoptosis and inhibits proliferation (Sellers et al. 2001; Mamedova et al. 2006). Similar anti-proliferative effect of the $P2Y_1$ receptor was observed in A375 melanoma cells (White et al. 2005). In prostatic carcinoma PC-3 cells the prolonged (8 h) $P2Y_1$ receptor activation by specific agonist (MRS2365) had also pro-apoptotic effect (Wei et al. 2011).

As it was already discussed in the one of the earlier sections of this Chapter, the main functional activity of the $P2Y_1$ receptor is connected with the signaling pathway that leads to the $G_{\alpha q}$ and PLCβ activation, IP_3 formation and intracellular Ca^{2+} mobilization. However, the same signaling pathway is induced by $P2Y_2$ receptors, which in contrast to $P2Y_1$ increase and not decrease the cell growth (White and Burnstock 2006). Since it has been found that the $P2Y_1$ receptor-mediated apoptosis was correlated with ERK1/2 activation, both in 131N1 astrocytoma (Sellers et al. 2001), as well as in prostate cancer cells (Wei et al. 2011), Wei and co-authors suggested a crucial role of ERK1/2 signaling in this process (Wei et al. 2011). On the other hand, the intense proliferation and invasiveness of various types of cancer, including glioma, seems to depend on up-regulation of the phosphatidylinositol 3-kinase/Akt kinase (PI3K/Akt) signaling pathway (Kubiatowski et al. 2001).

PI3K/Akt up-regulation may be explained by a finding that in a wide variety of human cancer cells the gene encoding the tumor suppressor protein (PTEN), a 3-phosphatase that dephosphorylates phosphatidylinositol-3,4,5-phosphate (PIP_3), is absent or mutated (Cantley and Neel 1999). Due to a loss or a very low level of PTEN, the expression of PI3K and PIP_3 is up-regulated, which results in a constitutively high activation of serine-threonine kinase Akt (also called protein kinase B). Over-expression of constitutively active forms of Akt promotes cell proliferation and invasiveness, regulates cell growth and is strongly involved in cell survival mechanisms (Kubiatowski et al. 2001; Cantley and Neel 1999). Furthermore, Akt promotes rescue from apoptosis by direct phosphorylation and inactivation of components of apoptotic machinery, including Bad protein (Sellers et al. 2001).

Studies on glioma C6 cells have shown that both, in the presence and in the absence of serum in the culture medium, the $P2Y_1$ receptor has an inhibitory effect on the PI3K/Akt signaling. In contrast, $P2Y_{12}$ receptors take a part in the activation of PI3K/Akt pathway (Czajkowski et al. 2004; Krzemiński et al. 2007). Similar relationship was presented in platelets, showing inhibitory and stimulatory effects of, respectively, $P2Y_1$ and $P2Y_{12}$ on PI3K signaling (Hardy et al. 2004). Thus, the $P2Y_1$ receptor, which acts as an antagonist of PI3K/Akt signaling may be also, in both, platelets and C6 cells, characterized as the receptor that mediates apoptosis. Although the mode of such pro-apoptotic action of $P2Y_1$ receptors is still not clear, all these data point out a potential therapeutic role of $P2Y_1$ receptors in the regulation of cancer growth and suggest their use in clinical trials. An example of such treatment may be melanoma treated with 2MeSADP and MRS2179, a selective agonist and antagonist, respectively, of $P2Y_1$ receptors (Burnstock 2002).

It is worth to add that the control of cells growth and death is regulated by a crucial balance of activities induced by various signaling pathways generated by different receptors, which particularly in cancer cells may evoked cells self-sufficient mechanisms. Such mechanisms may be involved in, and may explain the inhibitory effect of $P2Y_1$ receptors on PI3K/Akt signaling that leads in glioma C6 cells to a strong decrease in these receptor protein level in the unfavorable condition of serum withdrawal. Also the lack of $P2Y_1$ receptors in neuronal cancer cell lines may be a result of and may be explained by a possible cell self-sufficient mechanisms, acting against the pro-apoptotic and anti-survival $P2Y_1$ activity. Furthermore, according to us, such pro-survival mechanisms may also include the increase of

ERK1/2 activity in the $P2Y_1$ receptor mediated apoptosis, characteristic for prolonged receptor activation. The effect was described in astrocytoma and prostate cancer cells (Sellers et al. 2001; Wei et al. 2011). However, one has to be aware that simple inhibition or induction of single receptor or signaling pathway is not enough for promising therapeutic effect. The cross-talks and compensations described above lead us to conclusion, that only multiple drug approach may be really promising and that a real inhibition of the cancer growth needs simultaneous attack aimed on multiple signaling events.

Furthermore, cancer progression is more complex phenomenon than just multiplication of the cancer cells. Other factors influence tumor growth and one of them is it vascularization. The $P2Y_1$ receptor plays here additional role by induction of epithelial cells proliferation and promoting angiogenesis (Burnstock 2017; Cha et al. 2000).

7 $P2Y_1$ Receptors in Neuronal Plasticity

$P2Y_1$ receptors signaling, apart from its role in tumor survival and metastasis, play an important part in the regulation of brain physiology on multiple levels (Burnstock et al. 2011). The basic cellular mechanism of $P2Y_1$ receptors activation in neurons is similar to that observed in cancer cells. However, there is substantial difference in the expression of these receptors, with $P2Y_1$ being predominant, and $P2Y_{12}$ playing only a minor role (Burnstock and Knight 2004) (see also previous Sections of this Chapter).

Despite relative abundance, the $P2Y_1$ receptor does not appear to participate directly in the process of neurotransmission. Instead, it regulates the process of adaptive changes in neuronal connectivity, known as neuronal plasticity. The precise nature of its involvement in this phenomenon initially remained elusive despite cloning of the receptor in 1993 and subsequent distribution studies (Moore et al. 2000; Morán-Jiménez and Matute 2000). The major shift of paradigm in understanding the role of all P1 and P2Y receptors occurred when the idea of tripartite synapse was introduced, acknowledging the importance of the interplay not only between presynaptic and postsynaptic element but also with adjacent glial cells (Araque et al. 1999). The concept of tripartite synapse created a framework to incorporate growing evidence of the involvement of glial cells (mainly astrocytes, but also microglia and oligodendrocytes) in modulating the process of neurotransmission.

ATP and its metabolites can be stored in the neuronal synaptic vesicles and co-released with other transmitters or independently (Pankratov et al. 2006, 2007; Fields 2011). Astrocytes also possess the ability to release neurotransmitters, neuromodulators, and growth factors. Astrocytic $P2X_7$ receptor and connexin hemichannels (Montero and Orellana 2015) are permeable to ATP, but the release process mostly depends on vesicular transport. The discovery of this mechanism, and subsequent astrocyte- specific genetic manipulations opened the possibility to precisely attribute certain biological effects to gliotransmission (Lalo et al. 2014; Pascual et al. 2005). The $P2Y_1$ receptor plays a pivotal role in these mechanisms. It is worth noticing that the natural agonist of the $P2Y_1$ receptor is ADP, usually resulting from ATP hydrolysis by ectonucleotidases, widely distributed in the brain (Zimmermann 2006).

ATP released from presynaptic terminals acts mainly via the P2X receptors and contributes to fast neurotransmission (Evans et al. 1992; Pankratov et al. 1999). The astrocytic pool, acting via $P2Y_1$ receptor, as well as other subtypes of P2 family, modulates a number of other neurotransmitter systems, both pre- and postsynaptically. The direct presynaptic action of ATP is mainly inhibitory. P2Y receptors, including $P2Y_1$, acting via interaction with $G_{\beta\gamma}$ subunit have the capability of inhibiting the N-type Ca^{2+} channels (see Sect. 3). This effect may cause the decrease in release probability at the synaptic terminal. Indeed, such effect was observed for glutamate and noradrenaline in hippocampal (Mendoza-Fernández et al. 2000; Rodrigues et al. 2005; Koizumi et al. 2003) and cortical neurons (Bennett and Boarder 2000; Guzman

et al. 2005) and for serotonin in cortex (von Kügelgen et al. 1997). The $P2Y_1$ receptor activation may also cause postsynaptic effects, inhibiting NMDA receptor, triggering the internalization of metabotropic glutamate receptors (mGluR1), and enhancing sensitivity of GABA receptors. In all cases its action leads to decreased synaptic transmission (Luthardt et al. 2003; Saitow et al. 2005).

Astrocytes respond to external stimuli by elevating the cytoplasmic Ca^{2+} level, following activation of several families of G protein-coupled receptors. These calcium waves can be transmitted from one cell to another, synchronizing the activity of astrocytic network (Halassa and Haydon 2010; Anderson et al. 2004; Guthrie et al. 1999). While the P2X receptors and gap junctions mediate fast calcium waves, the $P2Y_1$ receptor orchestrates this activity on much longer timescale (Fumagalli et al. 2003) and across longer distances (Bowser and Khakh 2007). It acts via G protein – dependent PLC activity and calcium release from intracellular stores (see Sect. 3). These Ca^{2+} waves often emerge under pathological conditions: ischemia, mechanical injury or during the course of Alzheimer's disease (Moraga-Amaro et al. 2014). The process leads to long-term changes in cell physiology and allows for adaptive changes in response to the abnormal conditions. The downstream processes triggered by glia usually occur on a much slower timescale than fast synaptic transmission, and they often reflect the overall metabolic state of the brain. This in turn leads to complex network patterns of activation. An example of such interactions was observed in the prefrontal cortex, the key brain structure involved in short term and working memory as well as cognitive control of thalamus and the limbic system (Miller and Cohen 2001). One of the mechanisms related to learning in this area is the long term depression (LTD). ATP release leads to the activation of the presynaptic $P2Y_1$ receptor and prolonged Ca^{2+} increase that interferes with LTD formation (Guzman et al. 2010). Synaptic ATP also causes behavioral impairments in prefrontal-dependent tasks, most probably by interfering with dopaminergic signaling in medial prefrontal cortex and nucleus accumbens. The $P2Y_1$ receptor agonist, MRS2365, attenuated prepulse inhibition of the acoustic startle reflex, impaired accuracy in the delayed non-matching to position task and delayed reversal learning (Koch et al. 2010).

The $P2Y_1$ receptor appears to be working in concert with the metabotropic receptor for glutamate, mGluR5. The two receptors share common signaling pathways that link them *via* G_q protein to inhibition of specific ion channels (Filippov et al. 2010). The $P2Y_1$ receptor is responsible for the heterosynaptic depression of synaptic transmission in hippocampal neurons (Zhang et al. 2003). Synaptic glutamate spills over from potentiated synapse to the astrocytes and activates the mGluR5 receptor. This in turn leads to prolonged ATP release to the adjacent, non-active synapses. It acts presynaptically via A1 and P2Y receptors, decreasing release probability and enhancing the net effect at the potentiated synapse (Pascual et al. 2005). Regulation of activity in the *Cornu Ammonis* area 1 (CA1) and 3 (CA3) fields of hippocampus occurs also by modulation of inhibitory neurotransmission. Astrocytic $P2Y_1$ receptors present on interneurons, trigger GABA release and lead to silencing of the network (Bowser and Khakh 2007; Kawamura et al. 2004). Interestingly, in the dentate gyrus, the opposite net effect was observed when the sequence of glutamate and ATP action was reversed (Jourdain et al. 2007). ATP triggered $P2Y_1$ receptor-dependent astrocytic activation and glutamate release. This led to activation of presynaptic NMDA receptor (with NR2B as dominant subunit) and to increase in synaptic release. The ATP action appears not to be limited to functional plasticity. In developing brain, activation of the $P2Y_1$ receptor triggers mechanism of synapse elimination in thalamus. This process also depends on IP_3-dependent capacitative Ca^{2+} signal (Yang et al. 2016). All these phenomena indicate the possible involvement of ATP signaling in the synaptic scaling, a process of regulation of relative synaptic strength during experience or developmental plasticity, in order to preserve overall firing rate and avoid "overdrive" of the system.

The tripartite synapse appears to be extremely complicated and regulated on multiple levels. Microglia is currently emerging as another important player (Tremblay and Majewska 2011). Its activation causes weak ATP release that can be later amplified by astrocytes (Imura et al. 2013). Triggering the astrocytic $P2Y_1$ receptor leads to glutamate release and activation of postsynaptic GluR5 receptors (Pascual et al. 2012).

Taken together, these observations show a role for the $P2Y_1$ receptor in regulating synaptic plasticity on an extended timescale in large neuronal ensembles. This process is orchestrated by the synchrony and extent of glial network involved. Interestingly, the astrocytic and microglia activation often occurs as a response to stressing factors. The $P2Y_1$ receptor antagonism is therefore emerging as a possible intervention in cognitive impairments after injury or disease (Naviaux et al. 2013, 2014). The role of the entire family of P2Y receptors as therapeutic agents in cognitive dysfunctions has been reviewed recently (Guzman and Gerevich 2016).

8 Concluding Remarks

The $P2Y_1$ receptor is widely distributed in animal body. It has been found in nearly all tissues and cultured cell lines examined so far. It has wide spectrum of activities and some of them, e.g. $P2Y_1$ receptor role in platelets were extensively studied for years. In this Chapter we point out to increasing knowledge about this receptor in the nervous system and cancer progression.

One should remember that the $P2Y_1$ may be described as a typical G-protein coupled receptor, signaling only *via* $G_{\alpha q}$ signaling pathway and evoking capacitative calcium signal. In this it differs from such receptors as $P2Y_2$, which may engage different G_α proteins in different molecular environments, so that the signaling of the receptor is regulated by the change of the signaling pathway. Moreover, in contrast to many other receptors (e.g. aforementioned $P2Y_2$ receptor), it seems that the $P2Y_1$ signaling is regulated by the changes in receptor expression and its protein production, strongly dependent on the cell physiological condition, so this is the active protein level that regulates the receptor signaling. This special property of the $P2Y_1$ receptor is clearly visible in cancer cells, where the level of active receptor is strongly diminished if cells are grown in adverse environment (e.g. during prolonged serum deprivation). This disappearance of $P2Y_1$ receptor has strong pro-survival effect, promoting PI3K/Akt signaling activity. The effect was postulated as a possible drug handle for antitumor therapy, however the complex network of cross-talks between nucleotide receptors and common ligand shared with $P2Y_{12}$ receptor may easily compensate any simple approach of using this receptor in cancer treatment. In contrast to cancer cells, $P2Y_1$ receptors are predominant in neurons, playing an important role in the modulation of the synaptic transmission. Thus $P2Y_1$ receptor takes part in the regulation of neuronal plasticity in central nervous system as well as pain transmission in the peripheral nerves. While we know pretty well how $P2Y_1$ and other nucleotide receptors work, the knowledge about interplay between them, their cross-talks, as well as the ability to form complexes (homo- and hetero-oligomers) is still limited and should be further increased, since both nucleotide receptors and extracellular nucleotides are common at least in metazoan organisms.

Conflicts of Interest Authors clearly state that no data present in this manuscript creates any conflict of interest for any of coauthors. The article is an review and do not contain any experimental data which could violate any standards of animal or human participation in studies.

References

Abbracchio MP, Boeynaems J-MM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 24:52–55

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the

P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341

Anderson CM, Bergher JP, Swanson RA (2004) ATP-induced ATP release from astrocytes. J Neurochem 88:246–256

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208–215

Banachewicz W, Supłat D, Krzemiński P, Pomorski P, Barańska J (2005) P2 nucleotide receptors on C2C12 satellite cells. Purinergic Signal 1:249–257

Barańska J, Chaban V, Czarny M, Sabała P (1995) Changes in Ca2+ concentration in phorbol ester and thapsigargin treated glioma C6 cells. The role of protein kinase C in regulation of Ca2+ entry. Cell Calcium 17:207–215

Barańska J, Czajkowski R, Sabała P (2004) Cross-talks between nucleotide receptor-induced signaling pathways in serum-deprived and non-starved glioma C6 cells. Adv Enzym Regul 44:219–232

Barragán-Iglesias P, Mendoza-Garcés L, Pineda-Farias JB, Solano-Olivares V, Rodríguez-Silverio J, Flores-Murrieta FJ, Granados-Soto V, Rocha-González HI (2015) Participation of peripheral P2Y1, P2Y6 and P2Y11 receptors in formalin-induced inflammatory pain in rats. Pharmacol Biochem Behav 128:23–32

Barragán-Iglesias P, Pineda-Farias JB, Bravo-Hernández M, Cervantes-Durán C, Price TJ, Murbartián J, Granados-Soto V (2016) Predominant role of spinal P2Y1 receptors in the development of neuropathic pain in rats. Brain Res 1636:43–51

Bennett GC, Boarder MR (2000) The effect of nucleotides and adenosine on stimulus-evoked glutamate release from rat brain cortical slices. Br J Pharmacol 131:617–623

Bird GS, Hwang S-Y, Smyth JT, Fukushima M, Boyles RR, Putney JW (2009) STIM1 is a calcium sensor specialized for digital signaling. Curr Biol 19:1724–1729

Boarder MR, Hourani SM (1998) The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol Sci 19:99–107

Bowser DN, Khakh BS (2007) Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes. J Gen Physiol 129:485–491

Burnstock G (2002) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med 2:45–53

Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64:1471–1483

Burnstock G (2017) Purinergic signaling in the cardiovascular system. Circ Res 120:207–228

Burnstock G ed. (2014) Special issue: purinergic signalling in visceral organs in health and disease. Purinergic Signal 10:1–231

Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 240:31–304

Burnstock G, Krügel U, Abbracchio MP, Illes P (2011) Purinergic signalling: from normal behaviour to pathological brain function. Prog Neurobiol 95:229–274

Calloway N, Vig M, Kinet J-P, Holowka D, Baird B (2009) Molecular clustering of STIM1 with orai1/CRACM1 at the plasma membrane depends dynamically on depletion of Ca2+ stores and on electrostatic interactions. Mol Biol Cell 20:389–399

Cantley LC, Neel BG (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96:4240–4245

Carrasquero LMG, Delicado EG, Jiménez AI, Pérez-Sen R, Miras-Portugal MT (2005) Cerebellar astrocytes co-express several ADP receptors. Presence of functional P2Y(13)-like receptors. Purinergic Signal 1:153–159

Ceruti S, Abbracchio MP (2013) Adenosine signaling in glioma cells. Adv Exp Med Biol 986:13–30

Cha SH, Hahn TW, Sekine T, Lee KH, Endou H (2000) Purinoceptor-mediated calcium mobilization and cellular proliferation in cultured bovine corneal endothelial cells. Jpn J Pharmacol 82:181–187

Chen Y-F, Chiu W-T, Chen Y-T, Lin P-Y, Huang H-J, Chou C-Y, Chang H-C, Tang M-J, Shen M-R (2011) Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. Proc Natl Acad Sci 108:15225–15230

Chen Y-T, Chen Y-F, Chiu W-T, Wang Y-K, Chang H-C, Shen M-R (2013) The ER Ca^{2+} sensor STIM1 regulates actomyosin contractility of migratory cells. J Cell Sci 126:1260–1267

Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA, Harden TK (2004) Induction of novel agonist selectivity for the ADP-activated P2Y1 receptor versus the ADP-activated P2Y12 and P2Y13 receptors by conformational constraint of an ADP analog. J Pharmacol Exp Ther 311:1038–1043

Chiono M, Mahey R, Tate G, Cooper DM (1995) Capacitative Ca2+ entry exclusively inhibits cAMP synthesis in C6-2B glioma cells. Evidence that physiologically evoked Ca2+ entry regulates Ca(2+)-inhibitable adenylyl cyclase in non-excitable cells. J Biol Chem 270:1149–1155

Choi R, Wong CSS, Simon J, Barnard EA (2005) Agonist-induced homodimerisation of P2Y1 receptors demonstrated by fluorescence energy transfer analysis. Proc Br Pharmacol Soc 3:abst010P. http//www.pa2online.org/abstracts/Vol3Issue2abst001P.pdf

Claes P, Grobben B, Van Kolen K, Roymans D, Slegers H (2001) P2Y(AC)(−)-receptor agonists enhance the proliferation of rat C6 glioma cells through activation of the p42/44 mitogen-activated protein kinase. Br J Pharmacol 134:402–408

Claes P, Van Kolen K, Roymans D, Blero D, Vissenberg K, Erneux C, Verbelen J-P, Esmans EL, Slegers H (2004) Reactive blue 2 inhibition of cyclic AMP-dependent differentiation of rat C6 glioma cells

by purinergic receptor-independent inactivation of phosphatidylinositol 3-kinase. Biochem Pharmacol 67:1489–1498

Czajkowski R, Lei L, Sabała P, Barańska J (2002) ADP-evoked phospholipase C stimulation and adenylyl cyclase inhibition in glioma C6 cells occur through two distinct nucleotide receptors, P2Y(1) and P2Y(12). FEBS Lett 513:179–183

Czajkowski R, Banachewicz W, Ilnytska O, Drobot LB, Baranska J (2004) Differential effects of P2Y1 and P2Y12 nucleotide receptors on ERK1/ERK2 and phosphatidylinositol 3-kinase signalling and cell proliferation in serum-deprived and nonstarved glioma C6 cells. Br J Pharmacol 141:497–507

D'Ambrosi N, Iafrate M, Saba E, Rosa P, Volonté C (2007) Comparative analysis of P2Y4 and P2Y6 receptor architecture in native and transfected neuronal systems. Biochim Biophys Acta Biomembr 1768:1592–1599

De Simone R, Niturad CE, De Nuccio C, Ajmone-Cat MA, Visentin S, Minghetti L (2010) TGF-β and LPS modulate ADP-induced migration of microglial cells through P2Y1 and P2Y12 receptor expression. J Neurochem 115:450–459

Dugan LL, Kim JS, Zhang Y, Bart RD, Sun Y, Holtzman DM, Gutmann DH (1999) Differential effects of cAMP in neurons and astrocytes. Role of B-raf J Biol Chem 274:25842–25848

Ecke D, Hanck T, Tulapurkar ME, Schäfer R, Kassack M, Stricker R, Reiser G (2008) Hetero-oligomerization of the $P2Y_{11}$ receptor with the $P2Y_{1}$ receptor controls the internalization and ligand selectivity of the $P2Y_{11}$ receptor. Biochem J 409:107–116

Erb L, Weisman GA (2012) Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdiscip Rev Membr Transp Signal 1:789–803

Evans RJ, Derkach V, Surprenant A (1992) ATP mediates fast synaptic transmission in mammalian neurons. Nature 357:503–505

Fields RD (2011) Nonsynaptic and nonvesicular ATP release from neurons and relevance to neuron-glia signaling. Semin Cell Dev Biol 22:214–219

Filippov AK, Simon J, Barnard EA, Brown DA (2010) The scaffold protein NHERF2 determines the coupling of P2Y1 nucleotide and mGluR5 glutamate receptor to different ion channels in neurons. J Neurosci 30:11068–11072

Filtz TM, Li Q, Boyer JL, Nicholas RA, Harden TK (1994) Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. Mol Pharmacol 46:8–14

Fumagalli M, Brambilla R, D'Ambrosi N, Volonté C, Matteoli M, Verderio C, Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. Glia 43:218–213

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio MP (2004) Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. Biochem Pharmacol 68:113–124

Grobben B, Claes P, Van Kolen K, Roymans D, Fransen P, Sys SU, Slegers H (2001) Agonists of the P2Y(AC)-receptor activate MAP kinase by a ras-independent pathway in rat C6 glioma. J Neurochem 78:1325–1338

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520–528

Guzman SJ, Gerevich Z (2016) P2Y receptors in synaptic transmission and plasticity: therapeutic potential in cognitive dysfunction. Neural Plast 2016:1–12

Guzman SJ, Gerevich Z, Hengstler JG, Illes P, Kleemann W (2005) P2Y1 receptors inhibit both strength and plasticity of glutamatergic synaptic neurotransmission in the rat prefrontal cortex. Synapse 57:235–238

Guzman SJ, Schmidt H, Franke H, Krügel U, Eilers J, Illes P, Gerevich Z (2010) P2Y1 receptors inhibit long-term depression in the prefrontal cortex. Neuropharmacology 59:406–415

Halassa MM, Haydon PG (2010) Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. Annu Rev Physiol 72:335–355

Hardy AR, Jones ML, Mundell SJ, Poole AW (2004) Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. Blood 104:1745–1752

Hechler B, Gachet C (2015) Purinergic receptors in thrombosis and inflammation. Arterioscler Thromb Vasc Biol 35:2307–2315

Henderson DJ, Elliot DG, Smith GM, Webb TE, Dainty IA (1995) Cloning and characterisation of a bovine P2Y receptor. Biochem Biophys Res Commun 212:648–656

Hockley JRF, Tranter MM, McGuire C, Boundouki G, Cibert-Goton V, Thaha MA, Blackshaw LA, Michael GJ, Baker MD, Knowles CH, Winchester WJ, Bulmer DC (2016) P2Y receptors sensitize mouse and human colonic nociceptors. J Neurosci 36:2364–2376

Hoffmann C, Moro S, Nicholas RA, Harden TK, Jacobson KA (1999) The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. J Biol Chem 274:14639–14647

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207

Illes P, Ribeiro AJ (2004) Molecular physiology of P2 receptors in the central nervous system. Eur J Pharmacol 483:5–17

Imura Y, Morizawa Y, Komatsu R, Shibata K, Shinozaki Y, Kasai H, Moriishi K, Moriyama Y, Koizumi S (2013) Microglia release ATP by exocytosis. Glia 61:1320–1330

Jalink K, Hordijk PL, Moolenaar WH (1994) Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. Biochim Biophys Acta 1198:185–196

Janssens R, Communi D, Pirotton S, Samson M, Parmentier M, Boeynaems JM (1996) Cloning and tissue distribution of the human P2Y1 receptor. Biochem Biophys Res Commun 221:588–593

Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, Schachter JB, Harden TK, Jacobson KA (1997) A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol Pharmacol 52:499–507

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A (2007) Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10:331–339

Kawamura M, Gachet C, Inoue K, Kato F (2004) Direct excitation of inhibitory interneurons by extracellular ATP mediated by P2Y1 receptors in the hippocampal slice. J Neurosci 24:10835–10845

Kłopocka W, Korczyński J, Pomorski P (2013) Cytoskeleton and nucleotide signaling in glioma C6 cells. Adv Exp Med Biol 986:103–119

Koch H, Franke H, Kruegel U (2010) Impact of purinergic receptors in prefrontal mediated cognitive and executive functions. Eur Neuropsychopharmacol 20:S43–S44

Koch H, Bespalov A, Drescher K, Franke H, Krügel U (2015) Impaired cognition after stimulation of P2Y1 receptors in the rat medial prefrontal cortex. Neuropsychopharmacology 40:305–314

Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K (2003) Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. Proc Natl Acad Sci USA 100:11023–11028

Kranenburg O, Moolenaar WH (2001) Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists. Oncogene 20:1540–1546

Krzemiński P, Supłat D, Czajkowski R, Pomorski P, Barańska J (2007) Expression and functional characterization of P2Y1 and P2Y 12 nucleotide receptors in long-term serum-deprived glioma C6 cells. FEBS J 274:1970–1982

Kubiatowski T, Jang T, Lachyankar MB, Salmonsen R, Nabi RR, Quesenberry PJ, Litofsky NS, Ross AH, Recht LD (2001) Association of increased phosphatidylinositol 3-kinase signaling with increased invasiveness and gelatinase activity in malignant gliomas. J Neurosurg 95:480–488

Kwon S-G, Roh D-H, Yoon S-Y, Moon J-Y, Choi S-R, Choi H-S, Kang S-Y, Han H-J, Beitz AJ, Oh SB, Lee J-H (2014) Acid evoked thermal hyperalgesia involves peripheral P2Y1 receptor mediated TRPV1 phosphorylation in a rodent model of thrombus induced ischemic pain. Mol Pain 10:1744-8069-10–2

Lalo U, Rasooli-Nejad S, Pankratov Y (2014) Exocytosis of gliotransmitters from cortical astrocytes: implications for synaptic plasticity and aging. Biochem Soc Trans 42:1275–1281

Léon C, Vial C, Cazenave JP, Gachet C (1996) Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. Gene 171:295–297

Léon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C (1997) The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. FEBS Lett 403:26–30

Li G, Zhang Z, Wang R, Ma W, Yang Y, Wei J, Wei Y (2013) Suppression of STIM1 inhibits human glioblastoma cell proliferation and induces G0/G1 phase arrest. J Exp Clin Cancer Res 32:20

Luthardt J, Borvendeg SJ, Sperlagh B, Poelchen W, Wirkner K, Illes P (2003) P2Y(1) receptor activation inhibits NMDA receptor-channels in layer V pyramidal neurons of the rat prefrontal and parietal cortex. Neurochem Int 42:161–172

Mamedova LK, Gao Z-G, Jacobson KA (2006) Regulation of death and survival in astrocytes by ADP activating P2Y1 and P2Y12 receptors. Biochem Pharmacol 72:1031–1041

Mendoza-Fernández V, Andrew RD, Barajas-López C (2000) ATP inhibits glutamate synaptic release by acting at P2Y receptors in pyramidal neurons of hippocampal slices. J Pharmacol Exp Ther 293:172–179

Messens J, Slegers H (1992) Synthesis of glial fibrillary acidic protein in rat C6 glioma in chemically defined medium: cyclic AMP-dependent transcriptional and translational regulation. J Neurochem 58:2071–2080

Miller EK, Cohen JD (2001) An integrative theory of prefrontal cortex function. Annu Rev Neurosci 24:167–202

Mitchell C, Syed N -i.-H, Tengah A, Gurney AM, Kennedy C (2012) Identification of contractile P2Y1, P2Y6, and P2Y12 receptors in rat intrapulmonary artery using selective ligands. J Pharmacol Exp Ther 343:755–762

Montero TD, Orellana JA (2015) Hemichannels: new pathways for gliotransmitter release. Neuroscience 286:45–59

Moolenaar WH (1999) Bioactive lysophospholipids and their G protein-coupled receptors. Exp Cell Res 253:230–238

Moore D, Chambers J, Waldvogel H, Faull R, Emson P (2000) Regional and cellular distribution of the P2Y (1) purinergic receptor in the human brain: striking neuronal localisation. J Comp Neurol 421:374–384

Moore D, Chambers J, Wahlin J, Tan K, Moore G, Jenkins O, Emson P, Murdock P (2001) Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. Biochim Biophys Acta 1521:107–119

Moraga-Amaro R, Jerez-Baraona JM, Simon F, Stehberg J (2014) Role of astrocytes in memory and psychiatric disorders. J Physiol 108:240–251

Morán-Jiménez MJ, Matute C (2000) Immunohistochemical localization of the P2Y(1) purinergic receptor in neurons and glial cells of the central nervous system. Brain Res Mol Brain Res 78:50–58

Nakata H, Yoshioka K, Saitoh O (2003) Hetero-oligomerization between adenosine A1 and P2Y1 receptors in living cells: formation of ATP-sensitive adenosine receptors. Drug Dev Res 58:340–349

Naviaux RK, Zolkipli Z, Wang L, Nakayama T, Naviaux JC, Le TP, Schuchbauer MA, Rogac M, Tang Q, Dugan LL, Powell SB (2013) Antipurinergic therapy corrects the autism-like features in the Poly(IC) mouse model. PLoS One 8:e57380

Naviaux JC, Schuchbauer MA, Li K, Wang L, Risbrough VB, Powell SB, Naviaux RK (2014) Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. Transl Psychiatry 4:e400

O'Grady SM, Elmquist E, Filtz TM, Nicholas RA, Harden TK (1996) A guanine nucleotide-independent inwardly rectifying cation permeability is associated with P2Y1 receptor expression in Xenopus oocytes. J Biol Chem 271:29080–29087

Pankratov Y, Lalo U, Castro E, Miras-Portugal MT, Krishtal O (1999) ATP receptor-mediated component of the excitatory synaptic transmission in the hippocampus. Prog Brain Res 120:237–249

Pankratov Y, Lalo U, Verkhratsky A, North RA (2006) Vesicular release of ATP at central synapses. Pflügers Arch – Eur J Physiol 452:589–597

Pankratov Y, Lalo U, Verkhratsky A, North RA (2007) Quantal release of ATP in mouse cortex. J Gen Physiol 129:257–265

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul J-Y, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113–116

Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A (2012) Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc Natl Acad Sci USA 109:E197–E205

Putney JW (1990) Capacitative calcium entry revisited. Cell Calcium 11:611–624

Putney JW, Bird GSJ (1993) The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev 14:610–631

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

Rapaport E (1983) Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. J Cell Physiol 114:279–283

Rapaport E (1990) Mechanisms of anticancer activities of adenine nucleotides in tumor-bearing hosts. Ann N Y Acad Sci 603:142-9-50

Rapaport E, Fontaine J (1989) Generation of extracellular ATP in blood and its mediated inhibition of host weight loss in tumor-bearing mice. Biochem Pharmacol 38:4261–4266

Ribeiro-Filho AC, Buri MV, Barros CC, Dreyfuss JL, Nader HB, Justo GZ, Craveiro RB, Pesquero JB, Miranda A, Ferreira AT, Paredes-Gamero EJ (2016) Functional and molecular evidence for heteromeric association of P2Y1 receptor with P2Y2 and P2Y4 receptors in mouse granulocytes. BMC Pharmacol Toxicol 17:29

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA (2005) Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. J Neurosci 25:6286–6295

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Veliçelebi G, Stauderman KA (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445

Sabala P, Czajkowski R, Przybyłek K, Kalita K, Kaczmarek L, Barańska J (2001) Two subtypes of G protein-coupled nucleotide receptors, P2Y(1) and P2Y (2) are involved in calcium signalling in glioma C6 cells. Br J Pharmacol 132:393–402

Saitow F, Murakoshi T, Suzuki H, Konishi S (2005) Metabotropic P2Y purinoceptor-mediated presynaptic and postsynaptic enhancement of cerebellar GABAergic transmission. J Neurosci 25:2108–2116

Sak K, Illes P (2005) Neuronal and glial cell lines as model systems for studying P2Y receptor pharmacology. Neurochem Int 47:401–412

Salińska E, Danysz W, Łazarewicz JW (2005) The role of excitotoxicity in neurodegeneration. Folia Neuropathol 43:322–339

Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK (1996) Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor. Br J Pharmacol 118:167–173

Sellers LA, Simon J, Lundahl TS, Cousens DJ, Humphrey PP, Barnard EA (2001) Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. J Biol Chem 276:16379–16390

Soulet C, Hechler B, Gratacap M-P, Plantavid M, Offermanns S, Gachet C, Payrastre B (2005) A differential role of the platelet ADP receptors P2Y1 and P2Y12 in Rac activation. J Thromb Haemost 3:2296–2306

Stork PJS, Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12:258–266

Suplat D, Krzemiński P, Pomorski P, Barańska J (2007) P2Y1 and P2Y12 receptor cross-talk in calcium signalling: evidence from nonstarved and long-term serum-deprived glioma C6 cells. Purinergic Signal 3:221–230

Tokuyama Y, Hara M, Jones EMC, Fan Z, Bell GI (1995) Cloning of Rat and Mouse P2Y Purinoceptors. Biochem Biophys Res Commun 211:211–218

Tremblay M-È, Majewska AK (2011) A role for microglia in synaptic plasticity? Commun Integr Biol 4:220–222

Uehara K, Uehara A (2011) P2Y1, P2Y6, and P2Y12 receptors in rat splenic sinus endothelial cells: an immunohistochemical and ultrastructural study. Histochem Cell Biol 136:557–567

Van Kolen K, Slegers H (2006) Integration of P2Y receptor-activated signal transduction pathways in G protein-dependent signalling networks. Purinergic Signal 2:451–469

Van Kolen K, Gilany K, Moens L, Esmans EL, Slegers H (2006) P2Y12 receptor signalling towards PKB proceeds through IGF-I receptor cross-talk and requires activation of Src, Pyk2 and Rap1. Cell Signal 18:1169–1181

von Kügelgen I, Koch H, Starke K (1997) P2-receptor-mediated inhibition of serotonin release in the rat brain cortex. Neuropharmacology 36:1221–1227

Waldo GL, Harden TK (2004) Agonist binding and Gq-stimulating activities of the purified human P2Y1 receptor. Mol Pharmacol 65:426–436

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, Barnard EA (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett 324:219–225

Webb TE, Feolde E, Vigne P, Neary JT, Runberg A, Frelin C, Barnard EA (1996) The P2Y purinoceptor in rat brain microvascular endothelial cells couple to inhibition of adenylate cyclase. Br J Pharmacol 119:1385–1392

Wei Q, Costanzi S, Liu Q-Z, Gao Z-G, Jacobson KA (2011) Activation of the P2Y1 receptor induces apoptosis and inhibits proliferation of prostate cancer cells. Biochem Pharmacol 82:418–425

White N, Burnstock G (2006) P2 receptors and cancer. Trends Pharmacol Sci 27:211–217

White N, Ryten M, Clayton E, Butler P, Burnstock G (2005) P2Y purinergic receptors regulate the growth of human melanomas. Cancer Lett 224:81–91

Wypych D, Barańska J (2013) Cross-talk in nucleotide signaling in glioma C6 cells. Adv Exp Med Biol 986:31–59

Wypych D, Pomorski P (2012) P2Y1 nucleotide receptor silencing and its effect on glioma C6 calcium signaling. Acta Biochim Pol 59:711–717

Wypych D, Pomorski P (2013) Calcium signaling in glioma cells – the role of nucleotide receptors. Adv Exp Med Biol 986:61–79

Yang S, Zhang JJ, Huang X-Y (2009) Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. Cancer Cell 15:124–134

Yang N, Tang Y, Wang F, Zhang H, Xu D, Shen Y, Sun S, Yang G (2013) Blockade of store-operated Ca(2+) entry inhibits hepatocarcinoma cell migration and invasion by regulating focal adhesion turnover. Cancer Lett 330:163–169

Yang J, Yang H, Liu Y, Li X, Qin L, Lou H, Duan S, Wang H (2016) Astrocytes contribute to synapse elimination via type 2 inositol 1,4,5-trisphosphate receptor-dependent release of ATP. elife 5:e15043

Yoshida J, Iwabuchi K, Matsui T, Ishibashi T, Masuoka T, Nishio M (2012) Knockdown of stromal interaction molecule 1 (STIM1) suppresses store-operated calcium entry, cell proliferation and tumorigenicity in human epidermoid carcinoma A431 cells. Biochem Pharmacol 84:1592–1603

Yoshioka K, Saitoh O, Nakata H (2001) Heteromeric association creates a P2Y-like adenosine receptor. Proc Natl Acad Sci USA 98:7617–7622

Yoshioka K, Hosoda R, Kuroda Y, Nakata H (2002) Hetero-oligomerization of adenosine A1 receptors with P2Y1 receptors in rat brains. FEBS Lett 531:299–303

Zhang J, Wang H, Ye C, Ge W, Chen Y, Jiang Z, Wu C, Poo M, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971–982

Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD (2005) STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. Nature 437:902–905

Zhang D, Gao Z-G, Zhang K, Kiselev E, Crane S, Wang J, Paoletta S, Yi C, Ma L, Zhang W, Han GW, Liu H, Cherezov V, Katritch V, Jiang H, Stevens RC, Jacobson KA, Zhao Q, Wu B (2015) Two disparate ligand-binding sites in the human P2Y1 receptor. Nature 520:317–321

Zhang X, Qin J, Zou J, Lv Z, Tan B, Shi J, Zhao Y, Ren H, Liu M, Qian M, Du B (2016) Extracellular ADP facilitates monocyte recruitment in bacterial infection via ERK signaling. Cell Mol Immunol. doi:10.1038/cmi.2016.56

Zimmermann H (2006) Ectonucleotidases in the nervous system. Novartis Found Symp 276:113-28-30, 233–237, 275–281

Adv Exp Med Biol - Protein Reviews (2017) 19:91–106
DOI 10.1007/5584_2017_90

Published online: 17 August 2017

$P2Y_2$ Receptor Functions in Cancer: A Perspective in the Context of Colorectal Cancer

Fernand-Pierre Gendron, Morgane Placet, and Guillaume Arguin

Abstract

Purinergic signaling has recently emerged as a network of signaling molecules, enzymes and receptors that coordinates the action and behavior of cancerous cells. Extracellular adenosine 5′ triphosphate activates a plethora of P2 nucleotide receptors that can putatively modulate cancer cell proliferation, survival and dissemination. In this context, the G protein-coupled $P2Y_2$ receptor was identified as one of the entities coordinating the cellular and molecular events that characterize cancerous cells. In this chapter, we will look at the contribution of the $P2Y_2$ receptor in cancer outcomes and use this information to demonstrate that the $P2Y_2$ receptor represents a drug target of interest in the setting of colorectal cancer, for which the role and function of this receptor is poorly defined. More particularly, we will review how the $P2Y_2$ receptor modulates cancer cell proliferation and survival, while promoting cell dissemination and formation of metastases. Finally, we will investigate how the $P2Y_2$ receptor can contribute to the detrimental development of drug resistance that is often observed in cancerous cells.

Keywords

$P2Y_2$ receptor • Cancer • Colorectal cancer • Extracellular nucleotides

Abbreviations

AOM	Azoxymethane
APC	Adenomatous polyposis coli
ATP	Adenosine 5′-triphosphate
BMDC	Bone marrow-derived dendritic cell
C/EBPβ	CCAAT-enhancer-binding protein beta
CRC	Colorectal cancer

F.-P. Gendron (✉), M. Placet, and G. Arguin
Department of Anatomy and Cell Biology, Faculté de Médecine et des Sciences de la Santé, Pavillon de Recherche Appliquée sur le Cancer, Université de Sherbrooke, Sherbrooke, QC, Canada
e-mail: fernand-p.gendron@ushebrooke.ca

DSS	Dextran sulfate sodium
EGF	Epithelial growth factor
EMT	Epithelial-mesenchymal transition
GPCR	G protein-coupled receptor
IEC	Intestinal epithelial cell
IP_3	Inositol 1,4,5-triphosphate
ITGA5	Integrin alpha 5
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
MRP2	Multidrug resistance-associated protein 2
MT	Microtubule
NFκB	Nuclear factor-kappa B
NSCLC	Non-small cell lung carcinoma
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
SNP	Single nucleotide polymorphism
TGF-β	Tumor growth factor beta
TME	Tumor microenvironment
UTP	Uridine 5′-triphosphate
VEGF	Vascular epithelial growth factor

1 Colorectal Cancer: A Rapid Overview

Colorectal cancer (CRC) is the third most prevalent form of cancer worldwide and despite colossal research efforts, approximately 55% of CRC patients will die from this type of cancer (Andres et al. 2015; Morris et al. 2010). Western countries harbor most of the CRC cases, representing the fourth form of cancer-related deaths (Kuipers et al. 2015). However, the latter is becoming a worldwide concern due to the "westernization" of certain developing countries over the years (Kuipers et al. 2015). The etiology of CRC is multifactorial and involves both intrinsic and extrinsic factors that lead to mutations in oncogenes, tumor suppressor genes and genes related to DNA repair (Fearon 2011). Sporadic cancer, which represents 70% of all CRC, originates from the accumulation of genetic mutations and epigenetic deregulation of tumor suppressor genes and oncogenes, most prominently *APC*, *KRAS* and *TP53* genes in intestinal epithelial cells (IECs) (Fearon 2011). As a result, transformed IECs acquire an evolutionary growth advantage leading to the formation of tumors (Fearon 2011). Somatic mutations that inactivate *APC* are present in more than 80% of sporadic colorectal adenomas and carcinomas (see Fearon and references therein (Fearon 2011)). Truncated APC is thus unable to control β-catenin signaling, resulting in aberrant cell cycle stimulation and cell proliferation (Aoki and Taketo 2007; Polakis 2007). These mutations are involved in the initial growth of polyps but are insufficient for tumor progression (Fearon 2011). The progression from hyperplastic lesions, such as polyps, to invasive carcinoma requires the mutation of other genes, including *KRAS* and *TP53*. Amongst the extrinsic factors contributing to CRC, age represents the first risk factor (Levin et al. 2008). Chronic intestinal inflammation, as found in ulcerative colitis and Crohn's disease, dietary and physical activity habits (obesity) as well as tobacco smoking are all factors that also contribute to tumorigenic conditions (Marmol et al. 2017). Hence, the immune system is thereby molded by the tumor environment while directly contributing to the formation of the tumor niche and dissemination of cancerous cells (Lasry et al. 2016; Parcesepe et al. 2016). Consequently, solid cancers, such as CRC, are viewed not as a single disease but as an amalgam of numerous diseases as reflected by the observed cell heterogeneity found in tumors.

The contribution of growth factor receptors, such as epithelial growth factor (EGF) and vascular epithelial growth factor (VEGF) receptors, as well as Frizzled for WNT factors and the TGF-β type II receptor for TGFβ is well documented in CRC (Kuipers et al. 2015; De Rosa et al. 2016). Surprisingly, the role and function of G protein-coupled receptors (GPCRs) remain largely unknown in the setting of cancer, despite representing the largest family of signal-transducing receptors. Interestingly, GPCRs regulate cell functions associated with cancer cell behavior such as proliferation, resistance to

apoptosis, promotion of angiogenesis, immune tolerance and adaptation, as well as cell migration and dissemination (Bar-Shavit et al. 2016; Liu et al. 2016). Protease activated receptors, lysophosphatidic acid receptors, chemokine receptor CXCR4, endothelin receptors, prostaglandin receptors (PE2 and PE4), bradykinin receptor types 1 and 2 and sphingosine 1-phosphate receptor 1 are amongst the most studied GPCR in the context of cancer (Bar-Shavit et al. 2016). However, the understanding of GPCR signaling in colorectal cancer cell biology is practically abysmal or, at least, largely underappreciated. Despite recent reviews highlighting the potential influences of purinergic signaling in cancer biology, including CRC, the contribution of the G protein-coupled P2Y receptors in cancer, and in CRC carcinogenesis in particular, remains very limited.

In the following sections, we will examine various aspects associated with cancer and how the ATP and UTP-activated $P2Y_2$ receptor can contribute to these cancer hallmarks. We will review what it is known regarding the $P2Y_2$ receptor in the context of colorectal cancer and subsequently focus on the function and potential implication of the $P2Y_2$ receptor in cancerous intestinal epithelial cell proliferation, dissemination and metastasis. Finally, we will examine the potential implication of the $P2Y_2$ receptor in the complex mechanism of drug resistance of cancer cells.

2 $P2Y_2$ Receptor Expression in Cancer

The increased expression of the $P2Y_2$ receptor has been measured in various diseases, including, but not limited to neurological disorders (Weisman et al. 2012a, b), cardiovascular diseases (Djerada et al. 2016; Chen et al. 2017) and inflammatory bowel diseases (Grbic et al. 2008; Degagne et al. 2009, 2012). More recently, the participation of this receptor to the etiology of cancer has gained increasing interest given the ability of the receptor to modulate the proliferation of cancer cells and to increase their survival and dissemination. With the worldwide increase in cancer incidence, understanding and identifying new molecular entities allowing the development of disease-modifying agents is critical. Despite minute regional discrepancies and disparities between sexes, lung, breast, colorectal and prostate cancers remain the top four most incidental and prevalent forms of cancer (Ferlay et al. 2015). The following will thus focus on the modulation of $P2Y_2$ receptor expression in these four cancer forms.

Lung cancer is the most common cause of death from cancer worldwide with smoking remaining the principal cause of this disease (Ferlay et al. 2015). As in other diseases, the expression of the $P2Y_2$ receptor is upregulated in lung cancer cells when compared to normal epithelial cells. In fact, the receptor expression is increased by more than threefold in the adenocarcinoma-derived, non-small cell lung cancer H23 cell line and by more than 14-fold in the lung carcinoma A549 cell line when compared to the Beas-2b normal lung epithelial cell line (Song et al. 2016). However, these *in vitro* observations need to be validated both in animal models and in lung cancer patients.

Breast cancer is the most prevalent form of cancer in adult females in the world (Ferlay et al. 2015). The expression of *P2RY2* mRNA has been observed in MCF-7 and MDA-MB-231 breast cancer cell lines (Li et al. 2011; Zhang et al. 2017). However, the highly metastatic MDA-MB-231 breast cancer cell line displays an increased $P2Y_2$ receptor activity comparatively to low metastatic MCF-7 cells without significant modulations of receptor mRNA expression (Jin et al. 2014). Such results are not surprising since GPCR expression is a highly regulated process in which mRNA expression rarely correlates with protein expression level and receptor activity. Again, validation for $P2Y_2$ receptor expression must be confirmed *in vivo*.

Prostate cancer is the most prevalent form of cancer in males worldwide (Ferlay et al. 2015). The mRNA and protein expression of the $P2Y_2$ receptor has been studied in LNCap cells (androgen-sensitive human prostate

adenocarcinoma cells), DU-145 cells (hormone-insensitive human prostate adenocarcinoma cells) and in the PC-3M human prostate carcinoma cell line, as well as in the PC-3M highly metastatic subclone 1E8 and non-metastatic subclone 2B4 (Li et al. 2013). The expression of the $P2Y_2$ receptor was found in all of the studied cell types (Li et al. 2013). Unfortunately, it is not known whether receptor expression is increased in cancerous cells vs. non-cancerous prostate cells, as the authors of this latter study did not include non-cancerous prostate cells in their experiments. In PC-3 and DU-145 cells, the mRNA expression of the $P2Y_2$ receptor has been correlated with the activity of the receptor, as determined by the ability of ATP and UTP to stimulate inositol phosphate accumulation in an equipotent, equiactive and non-additive manner (Janssens and Boeynaems 2001). The protein expression of the $P2Y_2$ receptor has also been reported in human hormone refractory prostate cancer (HRPC) cells (Calvert et al. 2004).

Colorectal cancer is the third most common diagnosed cancer in the world and represents the fourth cause of cancer-related deaths in developed countries (Favoriti et al. 2016). In the intestine and in IECs, $P2Y_2$ receptor expression has been reported to be increased in response to inflammatory challenges (Grbic et al. 2008; Degagne et al. 2009, 2012). In this environment, the molecular mechanism regulating $P2Y_2$ receptor expression in IEC involves the cooperative action of NF-κB and C/EBPβ (Degagne et al. 2009, 2012). Although the precise molecular mechanisms regulating $P2Y_2$ expression in cancer cells are unknown, the expression of this receptor has been reported to be increased in human colorectal carcinoma tissues obtained from surgical resection for colon tumors (Nylund et al. 2007). Increased expression of $P2Y_2$ in CRC tissues has also been observed in tumors and corresponding margins harvested from mice with AOM/DSS-induced CRC (Fig. 1). The azoxymethane/dextran sodium sulfate (AOM/DSS) CRC mouse model is a highly suitable animal model for recapitulating the human colorectal adenoma-carcinoma sequence by displaying neoplastic lesions, aberrant crypt foci and adenocarcinomas (Bird and Good 2000; De Robertis et al. 2011). Of note, a different study reported that human *P2RY2* mRNA expression was down-regulated in colorectal cancer tissue samples as compared to normal tissues (Kunzli et al. 2011). This apparent discrepancy may stem from differences in experimental design between the Nylund (Nylund et al. 2007) and Kunzil (Kunzli et al. 2011) studies. Indeed, the Nylund study used matched tumor-free samples (Nylund et al. 2007), whereas the Kunzil study used non-CRC tissues obtained from various patients (Kunzli et al. 2011). Classically, comparison studies typically use matched tumor-free samples obtained from the same patient as in the Nylund publication.

Finally, amongst the other forms of cancer, the expression of the $P2Y_2$ receptor has also been found in biopsies of gastric cancer patients (Aquea et al. 2014), as well as in biopsies obtained from patients suffering from melanoma (White et al. 2005), cutaneous squamous cells (Greig et al. 2003) and human hepatocellular carcinoma (Xie et al. 2014; Tak et al. 2016).

3 $P2Y_2$ Receptor Signaling and Cell Proliferation and Survival

Colorectal cancer, as is the case for most solid tumors, is derived from the proliferation of transformed epithelial cells in which the tumor microenvironment (TME) is key in shaping tumor cell behaviors (Pancione et al. 2014; Peddareddigari et al. 2010; Wang and Karin 2015; Crotti et al. 2017). TME also molds the function of mesenchymal and immune cells so as to favor immune cell evasion, tumor cell growth and metastasis (Lasry et al. 2016; Parcesepe et al. 2016). These modifications in cell behaviors and molding are modulated by numerous growth factors, cytokines and extracellular nucleotides as recently highlighted (Ferrari et al. 2016; Di Virgilio and Adinolfi 2017). In fact, it is well documented that the TME is enriched in ATP (Pellegatti et al. 2008) and one can easily speculate that other nucleotides, such as UTP, are also

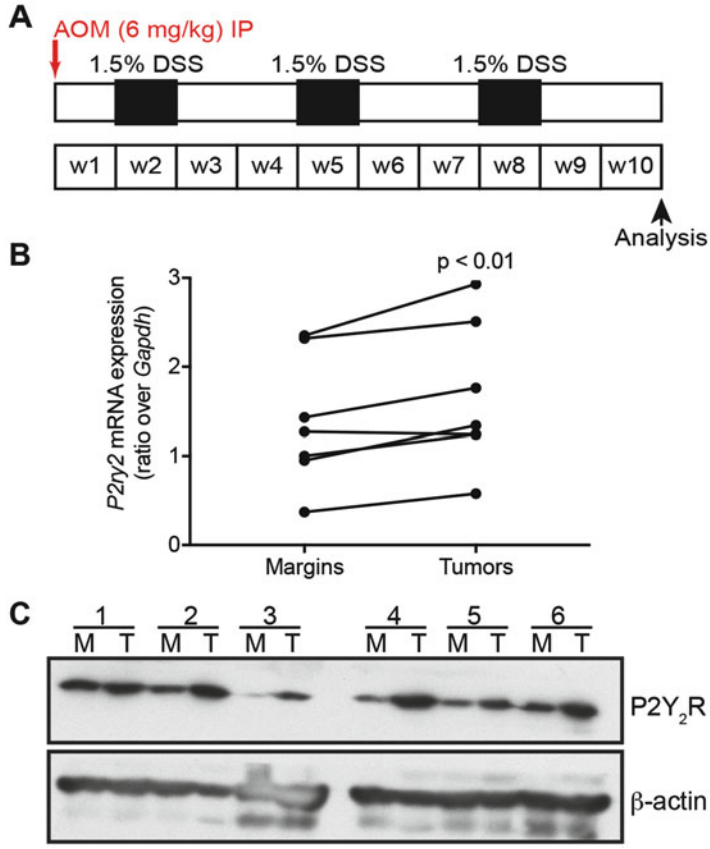

Fig. 1 P2Y$_2$ receptor mRNA and protein expression in mouse CRC tumors. Colorectal cancer was induced in three 3-month-old *C57BL/6J* mice (Jackson Laboratory) according to the protocol presented in *panel* (**A**). (**B**) *P2ry2* expression was determined by qPCR from mRNA isolated from tumor tissues (Tumors) and matched non-cancerous margins (Margins) obtained from mouse tumors (AOM/DSS) and matching margins. Two tumors and matching margins were isolated from every mouse. The qPCR assays were performed as previously described (Degagne et al. 2012). The statistical significance was determined by unpaired, two-tailed *t* test. (**C**) P2Y$_2$ receptor protein expression was determined by Western blot of protein lysates obtained from isolated mouse tumors (T) and matching margins (M). For Western blots, tissues were processed as previously described (Grbic et al. 2008). Immunoblots were performed using a 1:1000 dilution of rabbit anti-P2Y$_2$ receptor (Alomone Labs #APR-010) as previously described (Degagne et al. 2012). Specific protein bands were detected using a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG and visualized on autoradiographic film using the Millipore chemiluminescence system. Signal normalization was performed as described previously using an antibody against β-actin (Grbic et al. 2008)

present in the TME as reported in the literature (Di Virgilio and Adinolfi 2017).

Extracellular ATP and UTP are the equipotent agonists of the human P2Y$_2$ receptor which, upon stimulation, elicit a Gα_q-dependent activation of phospholipase C (PLC), leading to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol as well as the subsequent release of intracellular Ca^{2+} and activation of protein kinase C (PKC), the modulation of ion channel activities, and MAP kinase (MAPK) activation (Erb and Weisman 2012). Studies performed in Weisman's laboratory, at the University of Missouri-Columbia, and in Gendron's laboratory, at the Université de Sherbrooke in Canada, showed that P2Y$_2$ receptor signaling

also occurs via pathways that are dependent on $G\alpha_o$ and $G\alpha_{12}$ recruitment (Erb and Weisman 2012; Bagchi et al. 2005; Erb et al. 2001; Liao et al. 2007; Degagne et al. 2013). Contrary to the direct coupling of $P2Y_2$ receptor with Gαq, the interaction with $G\alpha_o$ and $G\alpha_{12}$ proteins requires the interaction between the $P2Y_2$ receptor-RGD domain with integrin α_v (Bagchi et al. 2005; Erb et al. 2001; Degagne et al. 2013). This interaction was first observed in astrocytoma 1321N1 cells overexpressing the recombinant human $P2Y_2$ receptor (Erb et al. 2001), and more recently with the endogenous $P2Y_2$ receptor expressed by intestinal epithelial cells (Degagne et al. 2013). The coupling of the $P2Y_2$ receptor with integrin signaling and $G\alpha_o$ and $G\alpha_{12}$ mobilization is important for the modulation of actin cytoskeleton dynamics, as elegantly reviewed by Erb and Weisman (Erb and Weisman 2012). In IECs, the cooperative interaction between the $P2Y_2$ receptor and integrin α_v in the recruitment of $G\alpha_o$ favors microtubule (MT) elongation and stabilization (Degagne et al. 2013). Accordingly, modulation of cytoskeleton dynamics is important for the maintenance of cell architecture and a key event for cell division and migration as previously reported (Parker et al. 2014). Cytoskeleton dynamics is thus crucial for cancer cell proliferation and dissemination (Davidson and Wood 2016; Fanale et al. 2015; Gehren et al. 2015), with the modulation of MT dynamics being a target of chemotherapeutic agents as recently reviewed (Fanale et al. 2015). Indeed, these drugs modulate MT dynamics so as to reduce cell migration and block cell proliferation by interfering with both cell mitosis and tumor vascularization (Fanale et al. 2015). In this setting, modulating $P2Y_2$ signaling could potentiate the effect of these MT-modifying drugs. For example, it has been shown that $P2Y_2$ activation stimulates the acetylation of α-tubulin on lysine-40 and the stabilization of MT elongation by a mechanism involving the recruitment of $G\alpha_o$ protein with the cooperative interaction of integrin α_v and the activation of PI3K/AKT and inhibition of GSK3β (Fig. 2) (Degagne et al. 2013). The end result is the stimulation of IEC migration, which is a prerequisite for wound healing. However, in the context of cancer, including CRC, this effect is seemingly detrimental to patients (Degagne et al. 2013). In general, $P2Y_2$ receptor activation appears to stimulate proliferation and survival of human A549 lung epithelial tumor cells (Schafer et al. 2003), cutaneous squamous cells (Greig et al. 2003), breast cancer cells (Chadet et al. 2014), human hepatocellular carcinoma (Xie et al. 2014), human PANC-1 cancerous pancreatic duct epithelial cells (Choi et al. 2013), and ARO cells derived from a human anaplastic thyroid carcinoma (Pines et al. 2006). Surprisingly, the $P2Y_2$-dependent signaling pathway, which mediates A549 lung epithelial tumor cell proliferation, is independent of PKC, ERK1/2, PI3K, or Src kinases, but is rather stimulated by PLC, Ca^{2+}/calmodulin-dependent protein kinase II and NFκB (Schafer et al. 2003). It would thus appear that $P2Y_2$ receptor signaling events leading to the stimulation of cancer cell proliferation may be as diverse as the number of affected organs and cell types. If one takes into account, that the $P2Y_2$ receptor can transactivate EGF and VEGF receptors (Liu et al. 2004; Li et al. 2015; Rumjahn et al. 2009; Seye et al. 2004), the number of potential signaling combinations is significantly enhanced. Furthermore, both G protein-coupled receptor kinase 2 (GRK2) and arrestin-2 have been shown to putatively modulate $P2Y_2$ receptor signaling (Morris et al. 2011, 2012), similar to signaling for other GPCR. The number of signaling possibilities triggered by the $P2Y_2$ receptor thus appears to be substantial. As such, investigators should keep in mind this myriad of potential signaling effectors associated with this particular receptor in cancer studies.

On the other hand, other studies have reported that the $P2Y_2$ receptor may suppress the proliferation of ovarian and endometrial cancer cells (Schultze-Mosgau et al. 2000; Katzur et al. 1999), as well as inhibit cell proliferation and induce apoptosis in colorectal and esophageal cancer cells (Maaser et al. 2002; Hopfner et al. 2001). However, these studies must be considered with caution since the concentration of nucleotides, namely ATP, was within the range

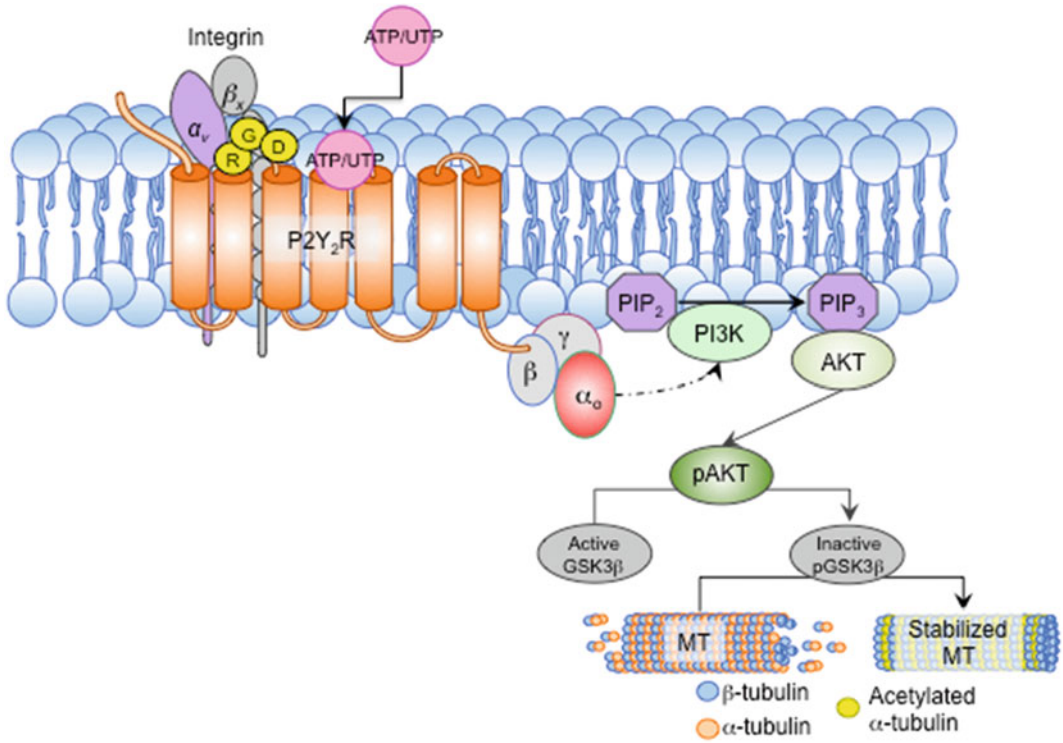

Fig. 2 Proposed signaling mechanisms involved in the stabilization of microtubules (MT) in response to the stimulation of the P2Y$_2$ receptor in intestinal epithelial cells as reported by Degagné et al. (2013). The stabilization of the MT was initiated by the interaction of the P2Y$_2$ receptor with integrin αv via the receptor RGD motif located in its first extracellular loop. This cooperative interaction recruits the Gαo protein and subsequent phosphorylation of AKT by phosphoinositide-3-kinase (PI3K). MT stabilization is achieved through the acetylation of α-tubulin in response to the AKT-dependent inhibitory phosphorylation of GSK3β as previously described (Degagne et al. 2013). PIP2: phosphatidylinositol (3,4)-bis- phosphate, PIP3: phosphatidylinositol (3,4,5)-tris- phosphate

necessary to activate the ionotropic P2X7 receptor, which is well known for its apoptotic effect.

4 The P2Y$_2$ Receptor Coordinates the Dissemination and Metastasis of Cancerous Cells

In the previous sections, we have shown that P2Y$_2$ receptor expression is often upregulated in different forms of cancer, including CRC. We also highlighted that the diversity of signaling pathways activated by the P2Y$_2$ receptor putatively contribute to cancer cell proliferation and survival, as well as in suppressing proliferation and inducing apoptosis under defined conditions and for particular cancer cell types. One of the hallmarks of cancer cells is their ability to infiltrate tissues and disseminate to other organs to form metastases (Hanahan and Weinberg 2011). Indeed, in all cancers, the presence of metastatic foci is correlated with poor prognosis and patient survival (Morris et al. 2010; Riihimaki et al. 2016). In order to disseminate and colonize distant organs, cancerous cells undergo a transformation process known as epithelial-mesenchymal transition (EMT) (Yilmaz and Christofori 2009; Heerboth et al. 2015). The mechanism leading to EMT is complex and still poorly understood. In brief, polarized IECs, in the case of CRC, lose their cell-cell junctions and transform into non-polarized, motile and invasive cells having

a mesenchymal phenotype (Yilmaz and Christofori 2009). EMT can be triggered by a number of factors among which include TGFβ (Zavadil and Bottinger 2005) and EGF (Lo et al. 2007). At the molecular level, EMT involves the engagement of small GTPases such as RhoA and Rac1 (Bakin et al. 2002; Bhowmick et al. 2001), Ras (Janda et al. 2002), PI3K (Bakin et al. 2000) and MAPK (Bakin et al. 2002) as well as other effectors as reviewed by Heerboth and coll (Heerboth et al. 2015). Of note, all of these signaling pathways are also activated by the $P2Y_2$ receptor as presented in this chapter.

As previously stated, the tumor microenvironment is enriched in extracellular nucleotides. However, the presence and potential functions of these signaling molecules have long been ignored until recently. In a recent review by Ferrari et al., purinergic signaling molecules, including ATP and UTP, were identified as important modulators of tumor biology as well as pro-metastatic factors (Ferrari et al. 2016). For instance, stimulation of the $P2Y_2$ receptor in the highly metastatic breast cancer cell line MDA-MB-231 contributes to the formation of a pre-metastatic niche by mediating the release of lysyl oxidase (LOX), which catalyzes the cross-linking of extracellular matrix proteins, and by promoting the recruitment of THP-1 monocytes *in vitro* (Joo et al. 2014). *In vivo*, subcutaneous injections of MDA-MB-231 cells in athymic nude mice lead to high levels of LOX secretion, cross-linking collagen and $CD11b^+$ BMDC recruitment to the lung (Joo et al. 2014). These effects are conversely abolished when MDA-MB-231-sh*P2RY2* cells are injected, thus elegantly demonstrating the contribution of the $P2Y_2$ receptor in the formation of a metastatic niche. Furthermore, the metastatic effect of the $P2Y_2$ receptor in breast cancer appears to rely on the crosstalk between cancer cells and endothelial cells in order to facilitate the extravasation of EMT-transformed breast cancer cells (Jin et al. 2014). Accordingly, $P2Y_2$ receptor stimulation in MDA-MB-231 breast cancer cells show an increased expression of EMT markers such as Snail, vimentin and N-cadherin (Eun et al. 2015). The increased expression of these EMT markers in MDA-MB-231 cells has furthermore been associated with $P2Y_2$ receptor-dependent activation of ERK and PKC pathways (Eun et al. 2015), which are signaling pathways classically linked to $P2Y_2$ receptor recruitment of Gαq protein as described earlier. Similar results have been reported using MCF-7 breast cancer cells (Chadet et al. 2014). In this latter study, $P2Y_2$ receptor activation was shown to increase MCF-7 migration via a MEK-ERK1/2-dependent signaling pathway (Chadet et al. 2014). More recently, UTP stimulation of the $P2Y_2$ receptor in human ovarian SKOV-3 adenocarcinoma-derived cell line was found to lead to EMT and stimulate cell migration through a crosstalk with the EGF receptor (Martinez-Ramirez et al. 2016). Similar observations have also been made using the PC-3M human prostate carcinoma cell line (Li et al. 2015). Although the signaling pathway was not clearly established, it would appear that the $P2Y_2$ receptor can trigger EMT and stimulate the formation of a metastasis niche.

In addition to reported migration of intestinal epithelial cells in response to $P2Y_2$ receptor activation *in vitro* and *in vivo* (Degagne et al. 2013), there is surprisingly little or no information regarding the potential participation of the $P2Y_2$ receptor in colorectal cancer cell dissemination and metastasis. Notwithstanding the latter, stimulation of the colorectal adenocarcinoma-derived Caco-2 cell line with ATP and UTP increases the expression of ICAM-1 at the surface of these cells by a mechanism involving the $P2Y_2$-dependent activation of NFκB (Langlois and Gendron 2009). This increase in ICAM-1 expression is similar to observations reported in the study by Jin et al. (Jin et al. 2014) in which a cross-talk between cancerous breast cells was shown to interact with endothelial cells via ICAM-1 in order to facilitate cancer cell dissemination. The expression of other adhesion proteins and matrix metalloproteinases (MMP) are also modulated in response to UTP stimulation of Caco-2 cells (Table 1). Among these proteins, the expression of integrin subunit α5 (ITGA5) is upregulated by more than 15-fold over non-stimulated cells. ITGA5 is furthermore associated with poor patient outcome in non-small-cell lung cancer

(Dingemans et al. 2010; Zheng et al. 2016) in addition to increasing metastases in lung cancer (Cimino et al. 2013; Valastyan et al. 2010). ITGA5 expression also promotes angiogenesis and carcinoma metastasis in ovarian and lung cancer glioblastoma and melanoma (Schaffner et al. 2013). Conversely, MMP8 expression is down-regulated by more than fivefold in UTP-stimulated cells (Table 1). MMP8 has been identified as a tumor suppressive protease and the loss of MMP8 expression is acknowledged to accelerate tumor onset (Thirkettle et al. 2013; Decock et al. 2015).

5 Cancer Drug Resistance: Is There a Role for the P2Y$_2$ Receptor?

An important aspect of cancer cell biology is resistance, or the acquisition of resistance, of cancerous cells to drug treatments. In effect, resistance to chemotherapy is a major crisis facing treating clinicians, which directly impact the well-being and chances of survival of cancer patients (Holohan et al. 2013). Although ATP infusions in patients with advanced non-small cell lung cancer (NSCLC) have been reported to be beneficial to the overall quality of life (Coolen et al. 2011; Agteresch et al. 2003), recent findings suggest, however, that P2Y$_2$ receptor stimulation may drive drug resistance. Indeed, P2Y receptors, including the P2Y$_2$ receptor, have been identified as drug resistance drivers in anaplastic lymphoma kinase-driven NSCLC (Wilson et al. 2015). This subset of NSCLC is particularly challenging as patients develop resistance to the chemotherapeutic agent crizotinib within the first 12 mo (Sullivan and Planchard 2016). Briefly, the expression of the *P2RY2* mRNA is upregulated in anaplastic lymphoma kinase inhibitor-resistant tumors. *In vitro*, the resistance of the H3122 NSCLC cell line to crizotinib as well as to certinib, two chemotherapeutic agents used for the treatment of anaplastic lymphoma kinase-driven NSCLC, has been linked to P2Y$_2$ receptor-dependent stimulation of PKC-δ (Wilson et al. 2015). Furthermore, P2Y$_2$ receptor stimulation of Src/p38/COX-2-pathways in the CRC HT-29 cell line and the prostate cancer DU145 cell line is associated with ursolic acid-induced apoptosis (Limami et al. 2012). Ursolic acid is a natural pentacyclic triterpenoid carboxylic acid shown to have potent anti-cancer properties in lung cancer as well as in other forms (Kim et al. 2015; Shanmugam et al. 2013). In another study, P2Y purinergic receptor signaling was identified as a potential biomarker for sensitivity of gallbladder cancer cells to gemcitabine (Yang et al. 2014). Unfortunately, the DNA microarray performed in this study did not determine the P2Y receptor subtype(s) nor whether P2Y receptor signaling was blocking or enhancing the effect of gemcitabine treatment. In the context of CRC, ATP, via the activation of the P2Y$_2$ receptor, has been reported to enhance colorectal cancer cell resistance to etoposide, cisplatin and doxorubicin (Vinette et al. 2015). The chemoresistance of cancerous epithelial cells is induced by the increased expression of the multidrug resistance-associated protein 2 (MRP2) (Vinette et al. 2015). MRP2 belongs to the ATP-binding cassette superfamily of proteins that interfere with the efficacy of cancer drugs by mediating their export out of the cells (Hlavata et al. 2012). This increase in MRP2 is stimulated by the P2Y$_2$ receptor activation of the MEK/ERK signaling pathway (Vinette et al. 2015).

In the present section, we have provided evidence that the P2Y$_2$ receptor may contribute to cancer drug resistance by modulating the signaling pathway activated by chemotherapeutic agents (Wilson et al. 2015; Limami et al. 2012) or by stimulating drug export out of the target cells (Vinette et al. 2015). Previously in this chapter, we have reported that the P2Y$_2$ receptor contributes to breast cancer cell EMT and provided clues that the P2Y$_2$ receptor may well drive CRC cell EMT and dissemination. If we accept that EMT participates in drug resistance by providing a resistance-promoting adaptive response, as recently reviewed (Holohan et al. 2013), we now have two mechanisms of action by which the P2Y$_2$ receptor could contribute to cancer drug resistance. Despite these recent

Table 1 Activation of the $P2Y_2$ receptor by UTP modulates the expression of adhesion proteins and matrix metalloproteinase (MMP) in human cancer Caco-2 intestinal epithelial cells as compared to nonstimulated cells

Targets	Fold variation (mean ± SEM)	Targets	Fold variation (mean ± SEM)
MMP8	−5.36 ± 1.87	LAMA1	2.00 ± 0.49
COL15A1	−5.04 ± 1.85	TGFBI	2.01 ± 0.34
ITGA4	−4.80 ± 2.6	SPG7	2.01 ± 0.46
SELE	−3.98 ± 1.26	CTNND1	2.07 ± 0.09
VCAM1	−3.71 ± 0.74	MMP11	2.07 ± 0.22
ADAMTS1	−3.59 ± 1.18	LAMC1	2.18 ± 0.24
CNTN1	−2.87 ± 0.63	ICAM1	2.20 ± 0.24
MMP12	−2.76 ± 0.57	KAL1	2.29 ± 0.87
CTNND2	−2.30 ± 0.19	CD44	2.31 ± 0.27
MMP16	−2.15 ± 0.39	TIMP1	2.41 ± 0.32
		LAMA3	2.41 ± 0.44
		ITGAV	2.44 ± 0.43
		CDH1	2.46 ± 0.31
		SGCE	2.49 ± 0.45
		ITGB5	2.51 ± 0.43
		PECAM1	2.53 ± 0.61
		VTN	2.54 ± 0.74
		TIMP2	2.58 ± 0.33
		ITGAL	2.61 ± 0.57
		CTNNA1	2.65 ± 0.42
		ITGB1	2.67 ± 0.45
		MMP14	2.76 ± 0.63
		COL16A1	2.78 ± 0.49
		LAMB3	2.78 ± 0.49
		THBS3	2.80 ± 0.76
		COL6A2	2.80 ± 0.44
		COL1A1	2.82 ± 0.21
		CTNNB1	2.82 ± 0.41
		MMP2	2.84 ± 0.80
		ECM1	2.86 ± 0.53
		COL6A1	2.92 ± 0.54
		MMP9	2.94 ± 0.03
		VCAN	3.15 ± 0.54
		MMP1	3.55 ± 0.04
		ITGA2	3.62 ± 0.01
		MMP15	3.99 ± 1.01
		COL12A1	4.10 ± 1.31
		ITGA6	4.46 ± 0.42
		HAS1	6.31 ± 1.46
		THBS1	8.50 ± 2.01
		CTGF	8.73 ± 1,84
		ITGA5	15.10 ± 1.78

Target mRNA expression was determined by quantitative real-time PCR using the Qiagen extracellular matrix and adhesion molecules RT^2 profiler PCR array. Only the targets presenting a twofold variation are displayed. Data are presented as the mean ± SEM of the fold variation of UTP-stimulated over nonstimulated Caco-2 cells

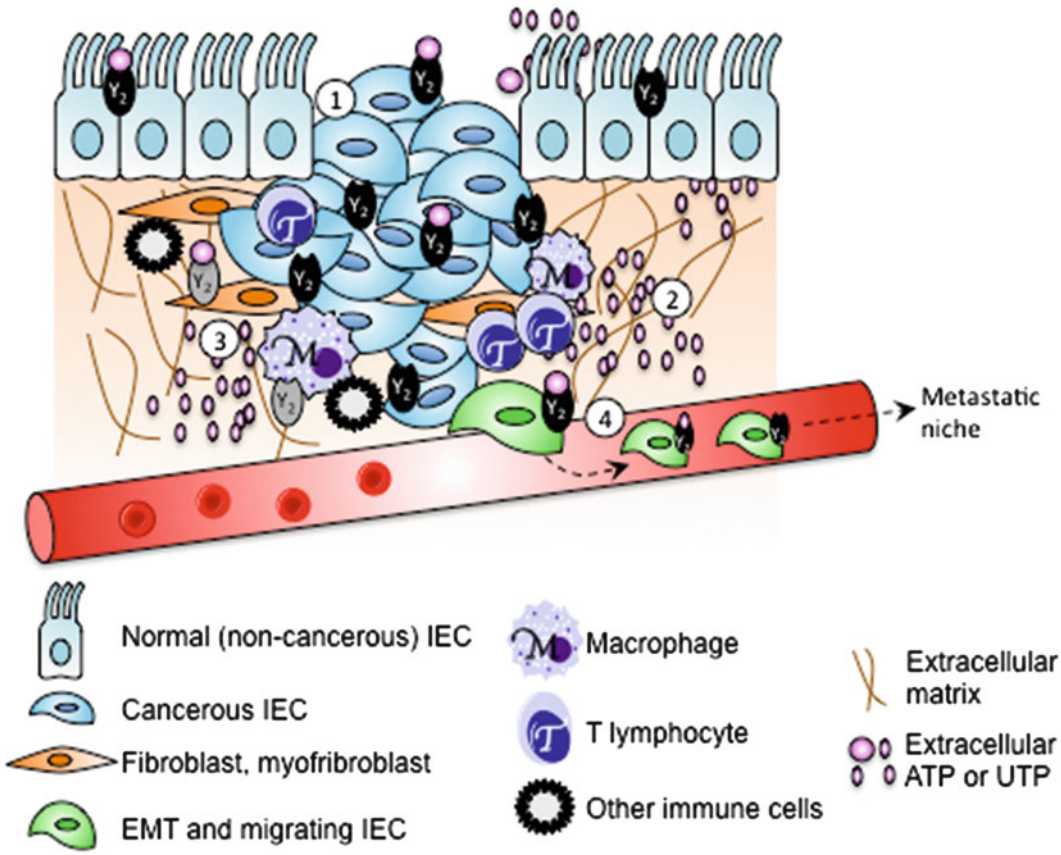

Fig. 3 Hypothetical model by which the P2Y$_2$ receptor could contribute to the formation of the colorectal tumor microenvironment promoting cancer cell proliferation and dissemination. ① Following a tumorigenic stress, the expression of the P2Y$_2$ receptor increases in cancerous IEC. ② The nucleotide (ATP and UTP)-enriched tumor microenvironment activates the P2Y$_2$ receptor to promote cancerous IEC proliferation and survival. ③ The extracellular ATP and UTP shape the tumorigenic immune environment by acting on macrophages, T lymphocytes and other immune cells such as dendritic cells, as well as on fibroblasts and myofibroblasts, which enhances cancerous IEC proliferation while stimulating tissue invasion. ④ The activity of the P2Y$_2$ receptor drives EMT that contributes to tissue invasion and migration to metastatic niches. Y$_2$: P2Y$_2$ receptor

findings, the contribution of the P2Y$_2$ receptor, as well as the other P2 receptors, in cancer drug therapy remains largely unknown. With the development of novel molecules aimed at modulating P2Y$_2$ receptor activity and function, it will be crucial to understand how this receptor ultimately interacts with existing chemotherapeutic agents and anti-cancer biologics. The cellular heterogeneity of solid tumors, such as that found in colorectal adenocarcinomas, call for a better identification of P2Y$_2$ receptor functions in the various cellular entities comprising tumors. Furthermore, the presence of single nucleotide polymorphism (SNP) for the P2Y$_2$ receptor has been linked to particular human diseases such as cardiovascular diseases (Wang et al. 2009a, b, 2010) and the risk of osteoporosis (Wesselius et al. 2013). Consequently, it would be of interest to determine whether there is a correlation between P2Y$_2$ receptor SNP and cancer.

6 Conclusion

In this chapter, we have highlighted the contribution of the P2Y$_2$ receptor to cancer cell

proliferation, dissemination and resistance to chemotherapeutic agents in various cancers. In the context of colorectal cancer, given that the $P2Y_2$ receptor can (i) drive immune and intestinal epithelial cell migration, (ii) modulate the expression of adhesion proteins and MMPs and that $P2Y_2$ receptor endogenous agonists have recently been identified as a guide for metastatic cells (Ferrari et al. 2016), it is plausible that the $P2Y_2$ receptor could contribute to the formation of the colorectal cancer microenvironment while facilitating cancer cell dissemination as illustrated in the hypothetical model presented in Fig. 3. However, it would be surprising that the $P2Y_2$ receptor is able to mediate these actions on its own. Indeed, the literature as well as our personal observations lead us to believe that the $P2Y_2$ receptor coordinates and modulates the action of other signaling molecules. As such, further studies are required to identify potential $P2Y_2$ receptor partners and to decipher the signaling events modulating receptor actions in the cancer microenvironment.

Acknowledgements F.P.G. is supported by a Canadian Institutes of Health Research operating grant (MOP-286567) and Natural Sciences and Engineering Research Council of Canada discovery grant (327128-2013). F.P.G. is a member of the FRQS-funded "Centre de Recherche du Centre hospitalier universitaire de Sherbrooke".

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical Approval All procedures were performed according to the protocol # 328-13B that was approved by the Université de Sherbrooke Animal Care Committee and the Canadian Guidelines for Care and Use of Experimental Animals.

References

Agteresch HJ, Burgers SA, van der Gaast A, Wilson JH, Dagnelie PC (2003) Randomized clinical trial of adenosine 5′-triphosphate on tumor growth and survival in advanced lung cancer patients. Anti-Cancer Drugs 14 (8):639–644

Andres A, Mentha G, Adam R, Gerstel E, Skipenko OG, Barroso E, Lopez-Ben S, Hubert C, Majno PE, Toso C (2015) Surgical management of patients with colorectal cancer and simultaneous liver and lung metastases. Br J Surg 102(6):691–699

Aoki K, Taketo MM (2007) Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. J Cell Sci 120(Pt 19):3327–3335

Aquea G, Bresky G, Lancellotti D, Madariaga JA, Zaffiri V, Urzua U, Haberle S, Bernal G (2014) Increased expression of P2RY2, CD248 and EphB1 in gastric cancers from Chilean patients. Asian Pac J Cancer Prev 15(5):1931–1936

Bagchi S, Liao Z, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, Erb L (2005) The P2Y2 nucleotide receptor interacts with alphav integrins to activate Go and induce cell migration. J Biol Chem 280 (47):39050–39057

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem 275 (47):36803–36810

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL (2002) p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci 115 (Pt 15):3193–3206

Bar-Shavit R, Maoz M, Kancharla A, Nag JK, Agranovich D, Grisaru-Granovsky S, Uziely B (2016) G protein-coupled receptors in cancer. Int J Mol Sci 17(8):1320

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL (2001) Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 12(1):27–36

Bird RP, Good CK (2000) The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. Toxicol Lett 112–113:395–402

Calvert RC, Shabbir M, Thompson CS, Mikhailidis DP, Morgan RJ, Burnstock G (2004) Immunocytochemical and pharmacological characterisation of P2-purinoceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. Anticancer Res 24(5A):2853–2859

Chadet S, Jelassi B, Wannous R, Angoulvant D, Chevalier S, Besson P, Roger S (2014) The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. Carcinogenesis 35(6):1238–1247

Chen X, Qian S, Hoggatt A, Tang H, Hacker TA, Obukhov AG, Herring PB, Seye CI (2017) Endothelial cell-specific deletion of P2Y2 receptor promotes plaque stability in atherosclerosis-susceptible ApoE-null mice. Arterioscler Thromb Vasc Biol 37(1):75–83

Choi JH, Ji YG, Lee DH (2013) Uridine triphosphate increases proliferation of human cancerous pancreatic duct epithelial cells by activating P2Y2 receptor. Pancreas 42(4):680–686

Cimino D, De Pitta C, Orso F, Zampini M, Casara S, Penna E, Quaglino E, Forni M, Damasco C, Pinatel E, Ponzone R, Romualdi C, Brisken C, De Bortoli M, Biglia N, Provero P, Lanfranchi G, Taverna D (2013) miR148b is a major coordinator of breast cancer progression in a relapse-associated microRNA signature by targeting ITGA5, ROCK1, PIK3CA, NRAS, and CSF1. FASEB J 27(3):1223–1235

Coolen EJ, Arts IC, Bekers O, Vervaet C, Bast A, Dagnelie PC (2011) Oral bioavailability of ATP after prolonged administration. Br J Nutr 105(3):357–366

Crotti S, Piccoli M, Rizzolio F, Giordano A, Nitti D, Agostini M (2017) Extracellular matrix and colorectal cancer: how surrounding microenvironment affects cancer cell behavior? J Cell Physiol 232(5):967–975

Davidson AJ, Wood W (2016) Unravelling the actin cytoskeleton: a new competitive edge? Trends Cell Biol 26 (8):569–576

Decock J, Hendrickx W, Thirkettle S, Gutierrez-Fernandez A, Robinson SD, Edwards DR (2015) Pleiotropic functions of the tumor- and metastasis-suppressing matrix metalloproteinase-8 in mammary cancer in MMTV-PyMT transgenic mice. Breast Cancer Res 17:38

Degagne E, Grbic DM, Dupuis AA, Lavoie EG, Langlois C, Jain N, Weisman GA, Sevigny J, Gendron FP (2009) P2Y2 receptor transcription is increased by NF-kappa B and stimulates cyclooxygenase-2- expression and PGE2 released by intestinal epithelial cells. J Immunol 183(7):4521–4529

Degagne E, Turgeon N, Moore-Gagne J, Asselin C, Gendron FP (2012) P2Y(2) receptor expression is regulated by C/EBPbeta during inflammation in intestinal epithelial cells. FEBS J 279(16):2957–2965

Degagne E, Degrandmaison J, Grbic DM, Vinette V, Arguin G, Gendron FP (2013) P2Y2 receptor promotes intestinal microtubule stabilization and mucosal re-epithelization in experimental colitis. J Cell Physiol 228(1):99–109

De Robertis M, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, Signori E, Fazio VM (2011) The AOM/DSS murine model for the study of colon carcinogenesis: from pathways to diagnosis and therapy studies. J Carcinog 10:9

De Rosa M, Rega D, Costabile V, Duraturo F, Niglio A, Izzo P, Pace U, Delrio P (2016) The biological complexity of colorectal cancer: insights into biomarkers for early detection and personalized care. Therap Adv Gastroenterol 9(6):861–886

Dingemans AM, van den Boogaart V, Vosse BA, van Suylen RJ, Griffioen AW, Thijssen VL (2010) Integrin expression profiling identifies integrin alpha5 and beta1 as prognostic factors in early stage non-small cell lung cancer. Mol Cancer 9:152

Di Virgilio F, Adinolfi E (2017) Extracellular purines, purinergic receptors and tumor growth. Oncogene 36 (3):293–303

Djerada Z, Feliu C, Richard V, Millart H (2016) Current knowledge on the role of P2Y receptors in cardioprotection against ischemia-reperfusion. Pharmacol Res 118:5–18

Erb L, Weisman GA (2012) Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdiscip Rev Membr Transp Signal 1(6):789–803

Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Perez LI, Gonzalez FA, Gresham HD, Turner JT, Weisman GA (2001) An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G(o)-mediated signal transduction. J Cell Biol 153(3):491–501

Eun SY, Ko YS, Park SW, Chang KC, Kim HJ (2015) P2Y2 nucleotide receptor-mediated extracellular signal-regulated kinases and protein kinase C activation induces the invasion of highly metastatic breast cancer cells. Oncol Rep 34(1):195–202

Fanale D, Bronte G, Passiglia F, Calo V, Castiglia M, Di Piazza F, Barraco N, Cangemi A, Catarella MT, Insalaco L, Listi A, Maragliano R, Massihnia D, Perez A, Toia F, Cicero G, Bazan V (2015) Stabilizing versus destabilizing the microtubules: a double-edge sword for an effective cancer treatment option? Anal Cell Pathol (Amst) 2015:690916

Favoriti P, Carbone G, Greco M, Pirozzi F, Pirozzi RE, Corcione F (2016) Worldwide burden of colorectal cancer: a review. Updat Surg 68(1):7–11

Fearon ER (2011) Molecular genetics of colorectal cancer. Annu Rev Pathol 6:479–507

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136(5):E359–E386

Ferrari D, Malavasi F, Antonioli L (2016) A purinergic trail for metastases. Trends Pharmacol Sci 38:277–290

Gehren AS, Rocha MR, de Souza WF, Morgado-Diaz JA (2015) Alterations of the apical junctional complex and actin cytoskeleton and their role in colorectal cancer progression. Tissue Barriers 3(3):e1017688

Grbic DM, Degagne E, Langlois C, Dupuis AA, Gendron FP (2008) Intestinal inflammation increases the expression of the P2Y6 receptor on epithelial cells and the release of CXC chemokine ligand 8 by UDP. J Immunol 180(4):2659–2668

Greig AV, Linge C, Healy V, Lim P, Clayton E, Rustin MH, McGrouther DA, Burnstock G (2003) Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells. J Invest Dermatol 121(2):315–327

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674

Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A, Sarkar S (2015) EMT and tumor metastasis. Clin Transl Med 4:6

Hlavata I, Mohelnikova-Duchonova B, Vaclavikova R, Liska V, Pitule P, Novak P, Bruha J, Vycital O, Holubec L, Treska V, Vodicka P, Soucek P (2012) The role of ABC transporters in progression and

clinical outcome of colorectal cancer. Mutagenesis 27 (2):187–196

Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG (2013) Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 13(10):714–726

Hopfner M, Maaser K, Barthel B, von Lampe B, Hanski C, Riecken EO, Zeitz M, Scherubl H (2001) Growth inhibition and apoptosis induced by P2Y2 receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. Int J Color Dis 16(3):154–166

Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grunert S (2002) Ras and TGF [beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 156(2):299–313

Janssens R, Boeynaems JM (2001) Effects of extracellular nucleotides and nucleosides on prostate carcinoma cells. Br J Pharmacol 132(2):536–546

Jin H, Eun SY, Lee JS, Park SW, Lee JH, Chang KC, Kim HJ (2014) P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. Breast Cancer Res 16(5):R77

Joo YN, Jin H, Eun SY, Park SW, Chang KC, Kim HJ (2014) P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell MDA-MB-231 contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. Oncotarget 5(19):9322–9334

Katzur AC, Koshimizu T, Tomic M, Schultze-Mosgau A, Ortmann O, Stojilkovic SS (1999) Expression and responsiveness of P2Y2 receptors in human endometrial cancer cell lines. J Clin Endocrinol Metab 84 (11):4085–4091

Kim SH, Ryu HG, Lee J, Shin J, Harikishore A, Jung HY, Kim YS, Lyu HN, Oh E, Baek NI, Choi KY, Yoon HS, Kim KT (2015) Ursolic acid exerts anticancer activity by suppressing vaccinia-related kinase 1-mediated damage repair in lung cancer cells. Sci Rep 5:14570

Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, van de Velde CJ, Watanabe T (2015) Colorectal cancer. Nat Rev Dis Primers 1:15065

Kunzli BM, Bernlochner MI, Rath S, Kaser S, Csizmadia E, Enjyoji K, Cowan P, d'Apice A, Dwyer K, Rosenberg R, Perren A, Friess H, Maurer CA, Robson SC (2011) Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer. Purinergic Signal 7(2):231–241

Langlois C, Gendron FP (2009) Promoting MPhi transepithelial migration by stimulating the epithelial cell P2Y(2) receptor. Eur J Immunol 39 (10):2895–2905

Lasry A, Zinger A, Ben-Neriah Y (2016) Inflammatory networks underlying colorectal cancer. Nat Immunol 17(3):230–240

Levin B, Lieberman DA, McFarland B, Smith RA, Brooks D, Andrews KS, Dash C, Giardiello FM, Glick S, Levin TR, Pickhardt P, Rex DK, Thorson A, Winawer SJ, American Cancer Society Colorectal Cancer Advisory G, Force USM-ST, American College of Radiology Colon Cancer C (2008) Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA Cancer J Clin 58(3):130–160

Li HJ, Wang LY, Qu HN, Yu LH, Burnstock G, Ni X, Xu M, Ma B (2011) P2Y2 receptor-mediated modulation of estrogen-induced proliferation of breast cancer cells. Mol Cell Endocrinol 338(1–2):28–37

Li WH, Qiu Y, Zhang HQ, Liu Y, You JF, Tian XX, Fang WG (2013) P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. Br J Cancer 109 (6):1666–1675

Li WH, Qiu Y, Zhang HQ, Tian XX, Fang WG (2015) P2Y2 receptor and EGFR cooperate to promote prostate cancer cell invasion via ERK1/2 pathway. PLoS One 10(7):e0133165

Liao Z, Seye CI, Weisman GA, Erb L (2007) The P2Y2 nucleotide receptor requires interaction with alpha v integrins to access and activate G12. J Cell Sci 120 (Pt 9):1654–1662

Limami Y, Pinon A, Leger DY, Pinault E, Delage C, Beneytout JL, Simon A, Liagre B (2012) The P2Y2/Src/p38/COX-2 pathway is involved in the resistance to ursolic acid-induced apoptosis in colorectal and prostate cancer cells. Biochimie 94(8):1754–1763

Liu J, Liao Z, Camden J, Griffin KD, Garrad RC, Santiago-Perez LI, Gonzalez FA, Seye CI, Weisman GA, Erb L (2004) Src homology 3 binding sites in the P2Y2 nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. J Biol Chem 279 (9):8212–8218

Liu Y, An S, Ward R, Yang Y, Guo XX, Li W, Xu TR (2016) G protein-coupled receptors as promising cancer targets. Cancer Lett 376(2):226–239

Lo HW, Hsu SC, Xia W, Cao X, Shih JY, Wei Y, Abbruzzese JL, Hortobagyi GN, Hung MC (2007) Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. Cancer Res 67(19):9066–9076

Maaser K, Hopfner M, Kap H, Sutter AP, Barthel B, von Lampe B, Zeitz M, Scherubl H (2002) Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. Br J Cancer 86 (4):636–644

Marmol I, Sanchez-de-Diego C, Pradilla Dieste A, Cerrada E, Rodriguez Yoldi MJ (2017) Colorectal carcinoma: a general overview and future perspectives in colorectal cancer. Int J Mol Sci 18(1):197

Martinez-Ramirez AS, Garay E, Garcia-Carranca A, Vazquez-Cuevas FG (2016) The P2RY2 receptor induces carcinoma cell migration and EMT through cross-talk with epidermal growth factor receptor. J Cell Biochem 117(4):1016–1026

Morris EJ, Forman D, Thomas JD, Quirke P, Taylor EF, Fairley L, Cottier B, Poston G (2010) Surgical management and outcomes of colorectal cancer liver metastases. Br J Surg 97(7):1110–1118

Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RA, Willets JM (2011) G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. Cardiovasc Res 89(1):193–203

Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RA, Willets JM (2012) Arrestins 2 and 3 differentially regulate ETA and P2Y2 receptor-mediated cell signaling and migration in arterial smooth muscle. Am J Physiol Cell Physiol 302(5):C723–C734

Nylund G, Hultman L, Nordgren S, Delbro DS (2007) P2Y2- and P2Y4 purinergic receptors are overexpressed in human colon cancer. Auton Autacoid Pharmacol 27(2):79–84

Pancione M, Giordano G, Remo A, Febbraro A, Sabatino L, Manfrin E, Ceccarelli M, Colantuoni V (2014) Immune escape mechanisms in colorectal cancer pathogenesis and liver metastasis. J Immunol Res 2014:686879

Parcesepe P, Giordano G, Laudanna C, Febbraro A, Pancione M (2016) Cancer-associated immune resistance and evasion of immune surveillance in colorectal cancer. Gastroenterol Res Pract 2016:6261721

Parker AL, Kavallaris M, McCarroll JA (2014) Microtubules and their role in cellular stress in cancer. Front Oncol 4:153

Peddareddigari VG, Wang D, Dubois RN (2010) The tumor microenvironment in colorectal carcinogenesis. Cancer Microenviron 3(1):149–166

Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F (2008) Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One 3(7): e2599

Pines A, Bivi N, Vascotto C, Romanello M, D'Ambrosio C, Scaloni A, Damante G, Morisi R, Filetti S, Ferretti E, Quadrifoglio F, Tell G (2006) Nucleotide receptors stimulation by extracellular ATP controls Hsp90 expression through APE1/Ref-1 in thyroid cancer cells: a novel tumorigenic pathway. J Cell Physiol 209(1):44–55

Polakis P (2007) The many ways of Wnt in cancer. Curr Opin Genet Dev 17(1):45–51

Riihimaki M, Hemminki A, Sundquist J, Hemminki K (2016) Patterns of metastasis in colon and rectal cancer. Sci Rep 6:29765

Rumjahn SM, Yokdang N, Baldwin KA, Thai J, Buxton IL (2009) Purinergic regulation of vascular endothelial growth factor signaling in angiogenesis. Br J Cancer 100(9):1465–1470

Schafer R, Sedehizade F, Welte T, Reiser G (2003) ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. Am J Physiol Lung Cell Mol Physiol 285(2): L376–L385

Schaffner F, Ray AM, Dontenwill M (2013) Integrin alpha5beta1, the fibronectin receptor, as a pertinent therapeutic target in solid tumors. Cancers (Basel) 5 (1):27–47

Schultze-Mosgau A, Katzur AC, Arora KK, Stojilkovic SS, Diedrich K, Ortmann O (2000) Characterization of calcium-mobilizing, purinergic P2Y(2) receptors in human ovarian cancer cells. Mol Hum Reprod 6 (5):435–442

Seye CI, Yu N, Gonzalez FA, Erb L, Weisman GA (2004) The P2Y2 nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). J Biol Chem 279(34):35679–35686

Shanmugam MK, Dai X, Kumar AP, Tan BK, Sethi G, Bishayee A (2013) Ursolic acid in cancer prevention and treatment: molecular targets, pharmacokinetics and clinical studies. Biochem Pharmacol 85 (11):1579–1587

Song S, Jacobson KN, McDermott KM, Reddy SP, Cress AE, Tang H, Dudek SM, Black SM, Garcia JG, Makino A, Yuan JX (2016) ATP promotes cell survival via regulation of cytosolic [Ca2+] and Bcl-2/Bax ratio in lung cancer cells. Am J Physiol Cell Physiol 310(2):C99–114

Sullivan I, Planchard D (2016) ALK inhibitors in non-small cell lung cancer: the latest evidence and developments. Ther Adv Med Oncol 8(1):32–47

Tak E, Jun DY, Kim SH, Park GC, Lee J, Hwang S, Song GW, Lee SG (2016) Upregulation of P2Y2 nucleotide receptor in human hepatocellular carcinoma cells. J Int Med Res 44(6):1234–1247

Thirkettle S, Decock J, Arnold H, Pennington CJ, Jaworski DM, Edwards DR (2013) Matrix metalloproteinase 8 (collagenase 2) induces the expression of interleukins 6 and 8 in breast cancer cells. J Biol Chem 288(23):16282–16294

Valastyan S, Chang A, Benaich N, Reinhardt F, Weinberg RA (2010) Concurrent suppression of integrin alpha5, radixin, and RhoA phenocopies the effects of miR-31 on metastasis. Cancer Res 70(12):5147–5154

Vinette V, Placet M, Arguin G, Gendron FP (2015) Multidrug resistance-associated protein 2 expression is upregulated by adenosine 5′-triphosphate in colorectal cancer cells and enhances their survival to chemotherapeutic drugs. PLoS One 10(8):e0136080

Wang K, Karin M (2015) Tumor-elicited inflammation and colorectal cancer. Adv Cancer Res 128:173–196

Wang Z, Nakayama T, Sato N, Yamaguchi M, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Ozawa Y, Ma Y, Doba N, Hinohara S (2009a) Purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene is associated with cerebral infarction in Japanese subjects. Hypertens Res 32(11):989–996

Wang ZX, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Matsumoto K, Ozawa Y, Ma YT, Doba N, Hinohara S (2009b) Association of the purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene with myocardial infarction in Japanese men. Circ J 73(12):2322–2329

Wang Z, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Ozawa Y, Ma Y (2010) The purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene associated with essential hypertension in Japanese men. J Hum Hypertens 24 (5):327–335

Weisman GA, Woods LT, Erb L, Seye CI (2012a) P2Y receptors in the mammalian nervous system: pharmacology, ligands and therapeutic potential. CNS Neurol Disord Drug Targets 11(6):722–738

Weisman GA, Ajit D, Garrad R, Peterson TS, Woods LT, Thebeau C, Camden JM, Erb L (2012b) Neuroprotective roles of the P2Y(2) receptor. Purinergic Signal 8(3):559–578

Wesselius A, Bours MJ, Henriksen Z, Syberg S, Petersen S, Schwarz P, Jorgensen NR, van Helden S, Dagnelie PC (2013) Association of P2Y(2) receptor SNPs with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients. Purinergic Signal 9(1):41–49

White N, Ryten M, Clayton E, Butler P, Burnstock G (2005) P2Y purinergic receptors regulate the growth of human melanomas. Cancer Lett 224(1):81–91

Wilson FH, Johannessen CM, Piccioni F, Tamayo P, Kim JW, Van Allen EM, Corsello SM, Capelletti M, Calles A, Butaney M, Sharifnia T, Gabriel SB, Mesirov JP, Hahn WC, Engelman JA, Meyerson M, Root DE, Janne PA, Garraway LA (2015) A functional landscape of resistance to ALK inhibition in lung cancer. Cancer Cell 27(3):397–408

Xie R, Xu J, Wen G, Jin H, Liu X, Yang Y, Ji B, Jiang Y, Song P, Dong H, Tuo B (2014) The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. J Biol Chem 289(27):19137–19149

Yang C, Xu M, Shen HJ, Zhu HY, Li F, He M, Chen T, Wang J, Shi WJ, Ji F (2014) Potential biomarkers for sensitivity of gallbladder cancer cells to gemcitabine. Int J Clin Exp Pathol 7(2):521–528

Yilmaz M, Christofori G (2009) EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 28 (1–2):15–33

Zavadil J, Bottinger EP (2005) TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 24 (37):5764–5774

Zhang JL, Liu Y, Yang H, Zhang HQ, Tian XX, Fang WG (2017) ATP-P2Y2-beta-catenin axis promotes cell invasion in breast cancer cells. Cancer Sci 108:1318–1327

Zheng W, Jiang C, Li R (2016) Integrin and gene network analysis reveals that ITGA5 and ITGB1 are prognostic in non-small-cell lung cancer. Onco Targets Ther 9:2317–2327

Adv Exp Med Biol - Protein Reviews (2017) 19:107–122
DOI 10.1007/5584_2017_89

Published online: 27 September 2017

$P2Y_{11}$ Receptors: Properties, Distribution and Functions

Charles Kennedy

Abstract

The $P2Y_{11}$ receptor is a G protein-coupled receptor that is stimulated by endogenous purine nucleotides, particularly ATP. Amongst P2Y receptors it has several unique properties; (1) it is the only human P2Y receptor gene that contains an intron in the coding sequence; (2) the gene does not appear to be present in the rodent genome; (3) it couples to stimulation of both phospholipase C and adenylyl cyclase. Its absence in mice and rats, along with a limited range of selective pharmacological tools, has hampered the development of our knowledge and understanding of its properties and functions. Nonetheless, through a combination of careful use of the available tools, suppression of receptor expression using siRNA and genetic screening for SNPs, possible functions of native $P2Y_{11}$ receptors have been identified in a variety of human cells and tissues. Many are in blood cells involved in inflammatory responses, consistent with extracellular ATP being a damage-associated signalling molecule in the immune system. Thus proposed potential therapeutic applications relate, in the main, to modulation of acute and chronic inflammatory responses.

Keywords

$P2Y_{11}$ receptor • ATP • NF157 • NF340 • NF546

Abbreviations

ADP	adenosine 5′-diphosphate
AMI	acute myocardial infarction
ATP	adenosine 5′-triphosphate
$cP2Y_{11}$ receptor	canine $P2Y_{11}$ receptor

C. Kennedy (✉)
Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK
e-mail: c.kennedy@strath.ac.uk

DAMPs	damage-associated molecular patterns
GPCR	G protein-coupled receptors
GTP	guanosine 5′-triphosphate
$hP2Y_{11}$ receptor	human $P2Y_{11}$ receptor
IP_3	inositol 1,4,5-trisphosphate
IPs	inositol phosphates
ITP	inosine 5′-triphosphate
LXA_4	lipoxin A_4
MDC	Malmo Diet and Cancer
$NAADP^+$	nicotinic acid adenine dinucleotide phosphate
NAD^+	nicotinamide adenine dinucleotide
PKC	protein kinase C
PLC	phospholipase C
SNPs	single nucleotide polymorphisms
UDP	uridine 5′diphosphate
UTP	uridine 5′-triphosphate

1 Introduction

P2Y receptors are G protein-coupled receptors (GPCR) that are stimulated by the endogenous nucleotides, adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5-′-triphosphate (UTP), uridine 5′diphosphate (UDP) and UDP-glucose. Eight human subtypes have been cloned ($P2Y_{1,2,4,6,11,12,13,14}$) (Abbracchio et al. 2005, 2006) and are expressed throughout the body (Burnstock and Kennedy 1985; Burnstock and Knight 2004; Kennedy et al. 2013). The missing numbers represent non-mammalian orthologues or proteins proposed to be P2Y receptors, but at which the nucleotides were subsequently found to be inactive. The eight genuine P2Y subtypes show differential sensitivities to the endogenous agonists and the $P2Y_{11}$ receptor is activated by ATP and ADP, but not the uridine nucleotides. In addition, the $P2Y_{11}$ receptor has several unique properties; (1) it is the only human P2Y receptor gene that contains an intron in the coding sequence; (2) the gene does not appear to be present in the rodent genome; (3) it couples to stimulation of both phospholipase C (PLC) and adenylyl cyclase. Here, we will discuss these properties and review the pharmacological profile and possible physiological functions of the $P2Y_{11}$ receptor.

2 Cloning and Sequence of $P2Y_{11}$ Receptors

Humans The human $P2Y_{11}$ ($hP2Y_{11}$) receptor was first cloned from placenta using probes based on the nucleotide sequence of the $P2Y_4$ receptor (Communi et al. 1997). The *P2RY11* gene (AJ298334) is on chromosome 19 (19p13.2) in humans, is 1125 base pairs long and codes a protein of 274 amino acids (CAC29362.1) (Communi et al. 2001). Unlike all other P2Y subtypes, the gene has a 1.9 kb intron that separates two exons, which encode the first six amino acids and the rest of the receptor respectively. The $P2Y_{11}$ protein has relatively low sequence identity with the other P2Y subtypes, ranging from 23% ($P2Y_{14}$) to 34% ($P2Y_6$) (Table 1). Multiple single nucleotide polymorphisms (SNPs) have been identified in the human genome and two of these, rs3745601 and rs2305795, will be discussed below when considering possible functions of $P2Y_{11}$ receptors.

Initially, the *P2RY11* receptor sequence was reported to comprise 1113 base pairs (AF030335), coding 371 amino acids (AAB88674.1), but subsequently, the same group reported that this cDNA sequence arose from intergenic splicing of the *P2RY11* gene and that of *PPAN*, the human orthologue of Ssf1, a nuclear protein that is involved in mating in *Saccharomyces cerevisiae*, which lies adjacent and upstream (Communi et al. 2001; Suarez-Huerta et al. 2000). The first *P2RY11* exon was thus revealed to code for the protein sequence MAANVS, rather than MDR, as initially reported. The *P2RY11*/*PPAN* fusion transcript (AJ300588) lacks the first exon of *P2RY11* and the last two thirds of the final exon in *PPAN* and is of unknown function. It appears to be

Table 1 Human P2Y receptor subtype amino acid sequence identity

% Identity								
Subtype	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
$P2Y_1$	–	38	44	46	32	24	24	27
$P2Y_2$		–	41	41	29	25	26	26
$P2Y_4$			–	43	32	25	26	28
$P2Y_6$				–	34	24	24	23
$P2Y_{11}$					–	22	21	23
$P2Y_{12}$						–	48	48
$P2Y_{13}$							–	47
$P2Y_{14}$								–

expressed widely throughout the body and, as will be discussed below, this has been a problem when studying the distribution of *P2RY11* mRNA.

Species Orthologues The canine *P2RY11* receptor (NM_001204441) was subsequently cloned (Zambon et al. 2001). The gene is found in the same synteny as the human gene and the resultant protein has 70% amino acid sequence identity with the $hP2Y_{11}$ receptor. An amphibian p2y receptor (AM040941) that has 35% amino acid sequence identity with the $hP2Y_{11}$ receptor is proposed to be the species homologue of *Xenopus (*Devader et al. 2007*)*. Putative orthologues are identified in a number of other species in the NCBI HomoloGene database, but intriguingly, the gene does not appear where expected in the rat and mouse genomes and convincing *P2RY11* mRNA transcripts have not been identified in rodent tissues (Abbracchio et al. 2006; Vassilatis et al. 2003; Dreisig and Kornum 2016). Furthermore, it is unlikely that the gene has translocated to a different chromosomal region and can still be transcribed to produce a functional protein, as using the human sequence to search the UniProt protein database for similar mouse proteins, Dreisig and Kornum (2016) found the closest match to be the $P2Y_1$ receptor, at 32% similarity. This is the same degree of similarity as seen between the human $P2Y_1$ and $P2Y_{11}$ receptors (Table 1). Thus there is no evidence for expression of *P2RY11* mRNA and $P2Y_{11}$ protein in rodents that resemble those present in humans, which must be taken into account when considering pharmacological studies in rodent tissues, as will be discussed below.

3 Coupling of $P2Y_{11}$ Receptors to 2nd Messenger Systems

Direct Activation of Signalling Pathways As P2Y receptors are GPCR, they couple to heterotrimeric G proteins and in this respect they fall into two groups: the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ subtypes couple mainly to $G\alpha_{q/11}$, as indicated by a rise in cytoplasmic levels of inositol 1,4,5-trisphosphate (IP_3) and related inositol phosphates (IPs), whilst the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors couple to $G\alpha_{i/o}$ (Abbracchio et al. 2006; Von Kügelgen and Harden 2011). The $P2Y_{11}$ receptor is unique in that it couples to both $G\alpha_{q/11}$ and $G\alpha_s$ (Communi et al. 1999). When activated, $G\alpha_{q/11}$ stimulates PLCβ, which cleaves phosphatidylinositol 4,5-bisphosphate in the plasma membrane into (1) IP_3, which binds to IP_3 receptors in the endoplasmic reticulum to release Ca^{2+} stores; and (2) diacylglycerol, which activates protein kinase C (PKC). $G\alpha_q$ can also bind to and activate the guanine nucleotide exchange factor, p63RhoGEF, which in turn stimulates Rho GTPases, such as RhoA (Lutz et al. 2005). $G\alpha_s$, on the other hand, activates adenylyl cyclase, leading to increased synthesis of cAMP and subsequently stimulation of protein kinase A.

Initially, ATP was reported to activate adenylyl cyclase with similar (Communi et al.

1997) or greater potency (Communi et al. 1999) compared to PLC activation. The experiments were, however, carried out in different cell lines; 1321 N1 human astrocytoma cells for IP accumulation and CHO-K1 cells for cAMP synthesis. Since agonist potency is greatly influenced by the level of receptor expression (Kenakin 1997; Palmer et al. 1998), the coupling of the $hP2Y_{11}$ receptor to these signalling pathways was studied further in the same cell line (Qi et al. 2001a). In 1321 N1 cells, ATP promoted IP accumulation with low μM potency (EC_{50} = 8.5 ± 0.1 μM) and was 15-fold less potent in raising cAMP (EC_{50} = 130 ± 10 μM). In CHO-K1 cells, ATP evoked IP accumulation with slightly higher potency (EC_{50} = 3.6 ± 1.3 μM) than in 1321 N1 cells, but it was still 15-fold less potent in promoting cAMP synthesis (EC_{50} = 62.4 ± 15.6 μM). Comparable differences in potencies for promoting the 2 s messenger responses were observed with other adenosine nucleotide analogues. Thus the $P2Y_{11}$ receptor appears to couple more effectively with the $G\alpha_{q/11}$ signalling pathway than that of $G\alpha_s$.

Indirect Activation of Adenylyl Cyclase It is important to note that some isoforms of adenylyl cyclase can be activated by PKC, independently of $G\alpha_s$ (Sunahara et al. 1996). For example, $P2Y_1$ receptors can activate PKCζ, which increases the activity of isoform 5 of adenylyl cyclase (del Puerto et al. 2012). Such a mechanism does not appear to underlie the rise in cAMP mediated by $P2Y_{11}$ receptors, as inhibition of PKC did not supress cAMP production elicited by stimulation of the canine $P2Y_{11}$ ($cP2Y_{11}$) receptor stably expressed in CF2Th cells (Zambon et al. 2001). Concomitant activation of PKC may, however, potentiate cAMP synthesis, as down-regulating PKC by chronic treatment with a phorbol ester decreased ATP-promoted cAMP accumulation by 60–80%, with no change in ATP's potency, in both 1321 N1 and CHO-K1 cells expressing the $hP2Y_{11}$ receptor (Qi et al. 2001a). Likewise, chelation of intracellular Ca^{2+} decreased ATP-promoted cAMP accumulation by ~45% in 1321 N1 cells, whereas chelation had no effect on either the efficacy or potency of ATP in CHO-K1 cells. Thus the capacity of the $P2Y_{11}$ receptor to elicit cAMP accumulation appears to be via $G\alpha_s$, and this can be potentiated by the concomitant activation of PKC and mobilisation of intracellular Ca^{2+} stores in a cell-type- and/or species subtype-dependent manner.

4 Pharmacological Properties of $P2Y_{11}$ Receptors

Agonists The $hP2Y_{11}$ receptor is adenine-nucleotide-preferring, with ATP more potent than ADP, whilst UTP, UDP and the nucleotide triphosphates, guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate, thymidine 5′-triphosphate and inosine 5′-triphosphate (ITP), are inactive (Communi et al. 1997; Qi et al. 2001a; Morrow et al. 2014). The rank order of agonist potency for increasing either cAMP or IP_s is ARC67085MX ≥ ATPγS ≈ BzATP > dATP > ATP ≈ 2-methythioATP ≈ ATPαS > ADPβS >2-methylthioADP > ADP (Communi et al. 1997, 1999; Qi et al. 2001a, b; van der Weyden et al. 2000; Meis et al. 2010). In addition, the diphosphates, ADP, ADPβS and 2-methylthioADP are partial agonists, with apparent efficacies of 60–80% of maximal response to ATP (Qi et al. 2001b). Note that AR-C67085XX, the most potent $P2Y_{11}$ agonist, also antagonises $P2Y_{12}$ receptors (Von Kügelgen and Harden 2011), which limits its usefulness in characterising the functions of native $P2Y_{11}$ receptors. These studies all used the original $hP2Y_{11}$ clone, in which the N terminal amino acid sequence begins MDR. When the receptor with the correct sequence, MAANVS, was expressed, there was, however, no significant difference in the potency of ATP and related molecules between the two isoforms (Meis et al. 2010).

Screening of a library of sulphonic and phosphonic acid derivatives identified NF546, a full agonist at $hP2Y_{11}$ receptors (Meis et al. 2010). It is 2.5-fold less potent than ATP, but

has a degree of selectivity over other P2Y subtypes as its EC_{50} value at $hP2Y_{11}$ receptors was 28-fold, 102-fold and 604-fold lower than that at human $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors respectively and it had little or no effect at human $P2Y_1$ and $P2Y_4$ receptors. Its activity at other P2X and P2Y subtypes has not been reported. Apparent agonist potency in bioassays depends, however, on the level of receptor expression, so whilst NF546 is a very useful tool for the pharmacological characterisation of native P2Y receptor subtypes, an action over a particular concentration range is not necessarily evidence for the presence of $P2Y_{11}$ receptors and an appropriate antagonist action profile and/or knockdown of the receptor using siRNA must also be obtained.

Nicotinamide adenine dinucleotide (NAD^+) and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), which both play important roles in intracellular signalling, are also $hP2Y_{11}$ agonists (Moreschi et al. 2006, 2008). Furthermore, it has been suggested that NAD^+ can be released from cells via connexin 43 hemichannels and so may act endogenously (Fruscione et al. 2011). Note, however, that NAD^+ also stimulates other P2 subtypes, including P2X1, P2X4, P2X7 and $P2Y_1$ receptors (Grahnert et al. 2009; Klein et al. 2009). In addition, NAD^+ can be metabolised to cyclic ADP-ribose, a potent agonist for release of intracellular Ca^{2+} stores (Grahnert et al. 2011), so a pharmacological effect of NAD^+ cannot, on its own, be automatically taken as evidence for the expression of $P2Y_{11}$ receptors and again and an appropriate antagonist action profile and/or knockdown of the receptor using siRNA must also be obtained.

Antagonists Suramin and PPADS are general, non-selective P2X and P2Y antagonists (Kennedy et al. 2013; Burnstock and Kennedy 2011) and whilst suramin, at low μM concentrations, is a surmountable $hP2Y_{11}$ antagonist, PPADS is inactive (Communi et al. 1999; van der Weyden et al. 2000). The suramin analogue, NF157, is a competitive antagonist with $pA_2 = 7.77$, which makes it more potent than suramin, and it is at least 650-fold selective over $P2Y_1$ and $P2Y_2$ receptors (Ullmann et al. 2005). In addition, NF157 is non-selective over P2X1 receptors and has low (P2X2, P2X3) to moderate (P2X4, P2X7) selectivity over other P2X subtypes (Ullmann et al. 2005). Its activity at other P2X and P2Y subtypes has not been reported. These factors can limit its usefulness when studying native $P2Y_{11}$ receptors and care must be taken when using NF157 to obtain supporting data, such as lack of effect of a range of other P2X and P2Y antagonists and/or depression of receptor expression using tools such as siRNA.

Another suramin analogue, NF340, is also a competitive antagonist, with a pA_2 of 8.02 (Meis et al. 2010). 10 μM NF340 inhibited responses at recombinant human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors by less than 10% and so NF340 was calculated to have at least 520-fold selectivity over other P2Y receptors. Similarly, stimulation of recombinant human P2X1, P2X2 and P2X2/3 receptors was unaffected by 3 μM NF340. This compound may, therefore be more useful in determining the physiological functions of native $P2Y_{11}$ receptors, but again, its activity at other P2X and P2Y subtypes has not been reported and supporting data should be obtained.

UTP Is Not a Biased Agonist at $P2Y_{11}$ Receptors Biased agonism describes a multistate model of GPCR activation in which each ligand induces a unique structural conformation of the receptor, such that the receptor couples differentially to G proteins and other intracellular proteins. For example, activation of the $P2Y_2$ receptor by UTP causes similar translocation of β-arrestin-1 and 2 from the cytoplasm to the plasma membrane, whereas ATP induced a greater translocation of β-arrestin-1 (Hoffmann et al. 2008). These differential effects of ATP and UTP had a downstream effect on cell signalling, as both agonists increased the levels of phosphorylated extracellular-signal regulated kinase, but the effect of UTP was transient, whereas that of ATP was prolonged.

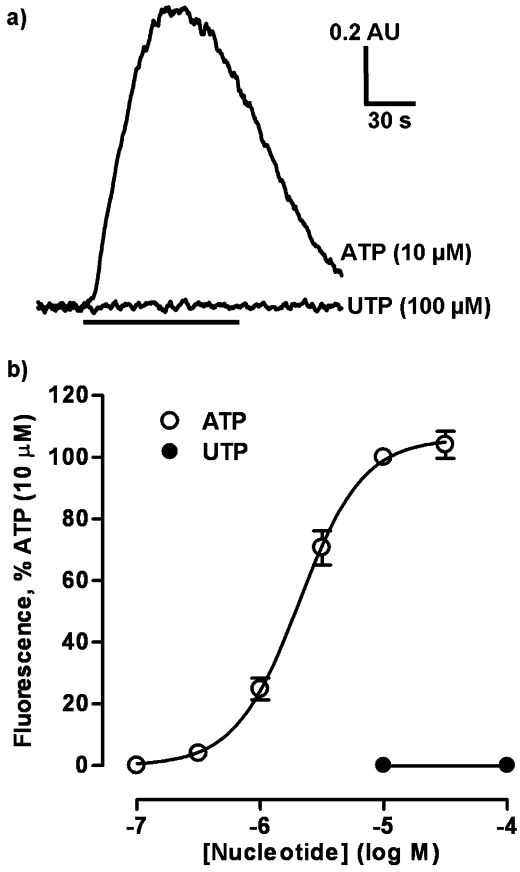

Fig. 1 ATP, but not UTP increases intracellular Ca^{2+} in 1321 N1-$hP2Y_{11}$ cells (**a**) The superimposed traces show fluo-4 fluorescence during superfusion with ATP (10 μM) (*upper trace*) and UTP (100 μM) (*lower trace*) for 90 s, as indicated by the *horizontal bar*. Both records are from the same population of cells (**b**) The mean peak amplitude of responses evoked by ATP (n = 5) and UTP (10 μM, n = 6 and 100 μM, n = 12) are shown. Responses are expressed as % of the response to ATP (10 μM). *Vertical lines* show S.E.M. (From Morrow et al. 2014)

As discussed above, all of the initial studies found UTP to be ineffective at raising IPs or cAMP in cells expressing $hP2Y_{11}$ receptors. A subsequent report proposed, however, that UTP was a biased $P2Y_{11}$ agonist, as it increased cytosolic $[Ca^{2+}]$, but did not induce accumulation of IPs, whereas ATP did both (White et al. 2003). This was an intriguing proposal, so we studied the action of UTP at $P2Y_{11}$ receptors in greater detail (Morrow et al. 2014).

We found that ATP evoked a rapid, concentration-dependent rise in intracellular Ca^{2+} with an EC_{50} that was essentially the same as that reported by (White et al. 2003). This is not surprising, as the 1312 N1-$hP2Y_{11}$ cell line used in both studies was generated by the present author whilst on sabbatical at the University of North Carolina. Despite this, we did not observe a rise in intracellular Ca^{2+} when UTP was applied, even at 100 μM, a concentration that was almost maximally effective in the earlier study (Fig. 1). Consistent with this, another group also failed to see a UTP-induced rise in intracellular Ca^{2+} levels in 1321 N1 cells stably expressing the $hP2Y_{11}$ receptor (Meis et al. 2010). Furthermore, we found that coapplying a high concentration of UTP with ATP did not inhibit either the rise in Ca^{2+} or IPs evoked by ATP (Fig. 2), indicating that UTP does not bind to this receptor.

In contrast, UTP was slightly, but significantly more potent than ATP in evoking a rise in intracellular Ca^{2+} in 1321 N1 cells stably expressing the human $P2Y_2$ receptor, with no difference in the maximum response. Thus the lack of response to UTP at $hP2Y_{11}$ receptors was not due to a problem with the UTP solution. So, contrary to the previous report, we found no evidence for an agonist action of UTP at the $hP2Y_{11}$ receptor, nor does UTP act as an antagonist. In our view the most feasible explanation for the discrepancy in the activity of UTP is contamination of the UTP solution by a non-nucleotide agent acting at a receptor that is not a P2Y receptor in the earlier study.

$cP2Y_{11}$ Pharmacology Interestingly, the $cP2Y_{11}$ receptor is adenine diphosphate-preferring, with reported rank order of agonist potencies of ADPβS = 2-methylthioADP ≈ 2-methylthioATP >> ADP > ATP (Zambon et al. 2001) and 2-methylthioADP > ADPβS >2-methylthioATP > ADP > ATPγS ≥ ATP (Qi et al. 2001b) for both IP and cAMP production. Furthermore, ADP, ADPβS and 2-methylthioADP are full agonists (Qi et al. 2001b). Like the $hP2Y_{11}$ receptor, UTP, UDP,

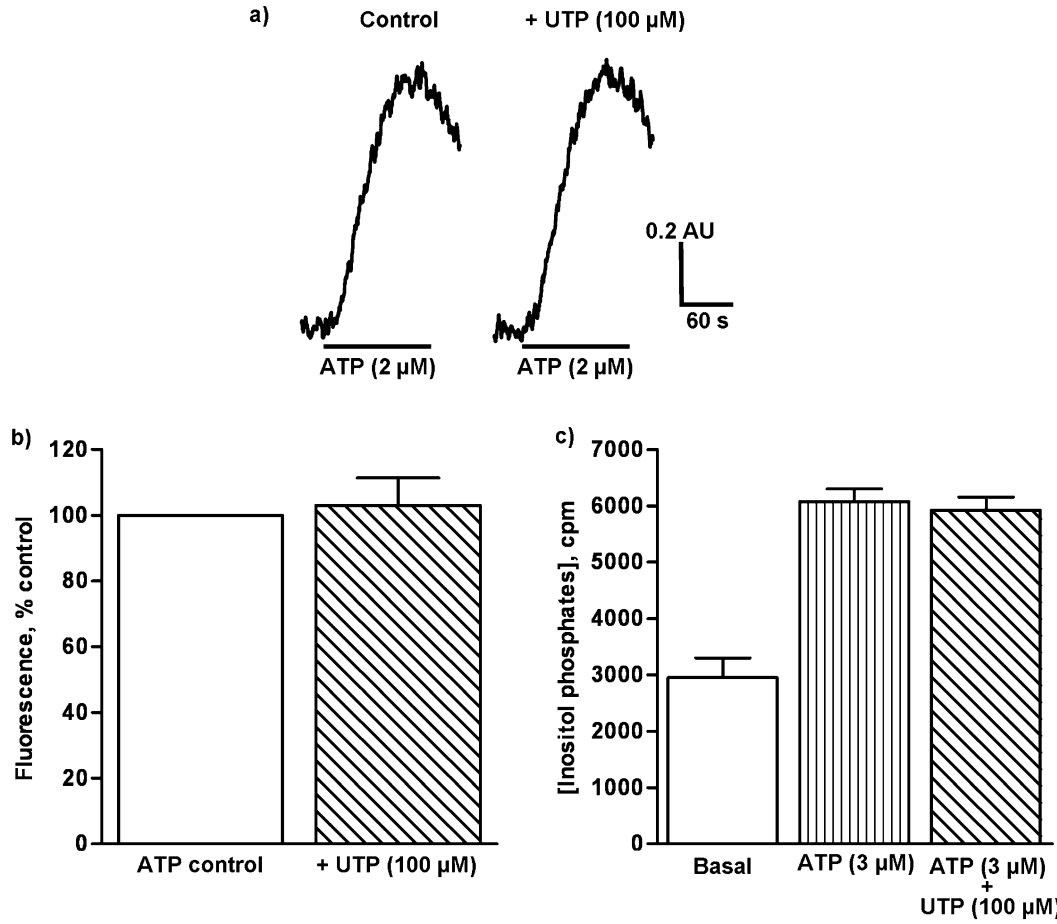

Fig. 2 UTP does not inhibit ATP-evoked responses in 1321 N1-hP2Y$_{11}$ cells (**a**) The traces show the rise in intracellular Ca^{2+} evoked by ATP (2 μM) in the same population of cells before (*left-hand side*) and after superfusion for 10 min with UTP (100 μM) (*right-hand side*), as indicated by the *horizontal bars* (**b**) The mean peak amplitude of responses evoked by ATP (2 μM) in the absence and presence of UTP (100 μM, n = 5) are shown. Responses are expressed as % of the control response to ATP (2 μM) (**c**) The mean basal level of IPs (*left-hand column*) and the mean amplitude of responses evoked by ATP (3 μM) in the absence (*middle column*) and presence of UTP (100 μM) (*right-hand column*) are shown. n = 3. *Vertical lines* indicate S.E.M. (From Morrow et al. 2014)

GTP and ITP were inactive, and suramin, but not PPADS inhibited these responses.

The human and canine receptors have only 70% amino acid identity and a mutagenesis study targeted the arginine residue (Arg268) that is present at the junction between TM6 and the third extracellular loop of the hP2Y$_{11}$ receptor, by replacing it with glutamine, which is present in the analogous position in the cP2Y$_{11}$ receptor (Qi et al. 2001b). This change increased both the potency and efficacy of ADP relative to ATP, such that they were now nearly equipotent and equi-efficacious. Likewise, mutating the glutamine in the canine receptor to arginine increased the efficacy and potency of ATP relative to ADP, and they now had essentially identical potency and efficacy. Thus this single amino acid within the P2Y$_{11}$ receptor is at least partially responsible for the species differences in its pharmacological properties.

Ligand Binding Site Computational modelling and mutational analysis have provided insight to some of the amino acid residues that likely

play a role in ligand binding. As noted above, Arg268 in the $hP2Y_{11}$ receptor is at least partially responsible for the differences in the relative potency of ATP and ADP compared with at the canine orthologue (Qi et al. 2001b). This amino acid also plays a major role in agonist stereo-selectivity. The $hP2Y_{11}$ receptor is preferentially activated by the Rp stereoisomer of ATPαS, but mutating Arg268 to an uncharged residue caused the stereo-selectivity to be lost (Ecke et al. 2008a). This mutational approach has also implicated arginine 106 and 307, glutamate 168 and alanine 313 in the ATP binding pocket (Zylberg et al. 2007). Determination the three dimensional structure of the $P2Y_{11}$ receptor through crystallisation or similar high resolution techniques is required, however, to confirm this.

5 $P2Y_1$/$P2Y_{11}$ Heteromultimers

GPCR were long considered to be monomeric entities that couple to G proteins on a 1:1 stoichiometric basis. It is now clear, however, that GPCR, including P2Y receptors, can interact to form dimeric or higher ordered oligomeric complexes, which may couple to one or more G proteins, with implications for the regulation of receptor subcellular localisation and trafficking, ligand binding and functional activity (Ferré et al. 2009, 2014; Kamal et al. 2011; Milligan 2013). Constitutive formation of $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ homodimers has been shown using biochemical techniques (d'Ambrosi et al. 2006; Savi et al. 2006; Choi et al. 2008) and publication of the crystal structure of the $P2Y_{12}$ receptor confirmed the latter (Zhang et al. 2014). Furthermore, different P2Y subtypes can interact physically to form function heterodimers, with altered pharmacological and signalling properties (see, for example, (d'Ambrosi et al. 2007; Shakya Shrestha et al. 2010; Ribeiro-Filho et al. 2016).

This is true also for the $P2Y_{11}$ receptor, which forms a complex with $P2Y_1$ receptor that has a greatly altered sensitivity to the antagonists, MRS2179 and NF157 (Ecke et al. 2008b). In addition, the $P2Y_{11}$ receptor did not undergo agonist-induced endocytosis when expressed on its own, consistent with the early report that $P2Y_{11}$ receptors do not desensitise (Communi et al. 1999). However, after cotransfection of the $P2Y_1$ subtype, ATP induced endocytosis of the $P2Y_{11}$ protein, which was not inhibited by the $P2Y_{11}$ antagonist NF157 (Ecke et al. 2008b). The authors, therefore, concluded that agonist-induced endocytosis of the $P2Y_{11}$ receptor requires coexpression of the $P2Y_1$ receptor and in a subsequent study identified alanine 87 of the $P2Y_{11}$ receptor as playing a crucial role in the interaction with $P2Y_1$ receptors (Haas et al. 2014). In addition, G protein receptor kinase 2 may also be required for $P2Y_{11}$ internalisation (Hoffmann et al. 2008).

6 Expression of $P2Y_{11}$ Receptors

mRNA Expression Many studies using RT-PCR or northern blotting have reported detection of *P2RY11* mRNA in a range of tissues and species (Abbracchio et al. 2006), but this is complicated by the discovery that the *P2RY11/PPAN* fusion mRNA transcript is expressed widely (see (Dreisig and Kornum 2016) for an extensive overview). The latter transcript comprises the second exon of *P2RY11* and most of the sequence of *PPAN*, so RT-PCR and northern blotting will only distinguish between the two sequences if one of the primers is designed to recognise at least part of the first exon of *P2RY11* and/or part of the untranslated upstream sequence.

After a detailed and extensive review of the published literature, Dreisig and Kornum (Dreisig and Kornum 2016) identified only 5 papers out of 89 in which detection of the fusion transcript could confidently be discounted, all in human cells (Ding et al. 2011; Kornum et al. 2011; Gao et al. 2013; Higgins et al. 2014; Azimi et al. 2016). Thus it is clear that care must be taken when designing primers and that many reports that claimed to show *P2RY11* mRNA expression need to be revisited and reviewed. The same group subsequently reported

detection of *P2RY11* mRNA in distinct regions of macaque monkey brain (Dreisig et al. 2016).

Protein Expression The presence of mRNA may not necessarily mean that the corresponding protein is expressed, so it is essential to confirm protein expression using a selective antibody. This approach has been used widely to study expression of all P2Y receptor subtypes, but in most cases the specificity of the antibody has not been unequivocally confirmed, for example, by showing that staining is absent in the appropriate receptor knock-out animal. Indeed, a commonly-used anti-$P2Y_1$ antibody has been reported to be nonspecific (Vial et al. 2006) and we found that several commercially-available P2Y subtype antibodies show staining in 1321 N1 cells, which do not express endogenous P2Y receptors (Kennedy, unpublished observations). Thus care must be taken when interpreting antibody staining data.

This holds true for data obtained using anti-$P2Y_{11}$ antibodies, as a review of the published literature identified several issues with the commercially-available anti-$P2Y_{11}$ antibodies (Dreisig and Kornum 2016). Most of these target a region of the C-terminus of the receptor that has significant sequence similarity with the C-terminus of other P2Y subtypes, so there is a reasonable chance that this antibody may not be specific for the $P2Y_{11}$ receptor. Although the predicted size of the $P2Y_{11}$ protein is 40 kDa, western blot data from different groups, in which the most commonly used antibody (#APR-015) was utilised, reported the molecular mass to be 33–60 kDa. All claimed that their band represented the monomeric $P2Y_{11}$ receptor (see (Dreisig and Kornum 2016) for full details).

Anti-$P2Y_{11}$ antibodies also bind proteins in rodent tissues (Abbracchio et al. 2006; Dreisig and Kornum 2016), even though the *$P2RY_{11}$* gene is not present in the rodent genome, further casting doubt upon their specificity. An attempt to verify the specificity of three commercially-available anti-$P2Y_{11}$ antibodies was made by first determining if they recognised the recombinant $hP2Y_{11}$ receptor when expressed in a cell line (Dreisig et al. 2016). One antibody that targeted the 3rd cytoplasmic loop of the $hP2Y_{11}$ receptor did not show any signal of the expected band size in western blots, but instead produced two bands of 142 and 195 kDa, including in untransfected cells. The other two antibodies, targeted against the 3rd extracellular loop or the C-terminus, both produced multiple bands, including of the expected size. These two antibodies stained neuronal-like cells in macaque monkey brain, but an identical staining pattern was also seen in rat brain. Thus the specificity of these antibodies could not be validated.

Finally, the targeted C-terminus region is also present in the $P2Y_{11}$/PPAN fusion protein, which has a predicted size of 90 kDa, so that too will be detected by anti-$P2Y_{11}$ antibodies. For example, an antibody generated against a sequence at the end of the $P2Y_{11}$ C-terminus produced a band of around 90 kDa in western blots using CHO-K1 cells transfected with the *P2RY11*/*PPAN* fusion plasmid, whereas transfection with the *P2RY11* plasmid resulted in bands of around 45 kDa (Communi et al. 2001). Transfection with the empty vector produced no bands.

7 Functions of $P2Y_{11}$ Receptors

Criteria for Identifying Receptor Function Ideally, a comprehensive understanding of the function of any receptor arises from characterisation of the effects of highly selective agonists and antagonists on cellular and tissue activity and correlation of these with the expression of its mRNA and protein and perhaps also with the effects of deleting the gene or supressing translation of the mRNA. Unfortunately, since the *P2RY11* gene appears to be absent in mice and rats, the species used in genetic deletion studies, it is not possible to produce $P2Y_{11}$-knock-out animals. In addition, as discussed above, doubt has been cast on the specificity of commercially-available anti-$P2Y_{11}$ antibodies. Nonetheless, *possible* functions of native $P2Y_{11}$ receptors have been identified in a range of

non-rodent cells by applying two or more of the following criteria: a) detection of mRNA using appropriately designed primers; b) demonstration of appropriate effects of appropriate concentrations of NF546, NF157 and NF340; c) lack of effect of agonists and antagonists selective at other P2Y and P2X subtypes; d) inhibition of the proposed $P2Y_{11}$ function by *P2RY11* RNA interference. Detection of a band close to the predicted size of the $P2Y_{11}$ receptor, ~40 kDa, in western blots, although not direct evidence in itself, might also be considered consistent with $P2Y_{11}$ expression in a tissue.

Mostly, the proposed functions are based on single reports, though they may be supported by other studies in the same cell type in which only one of the above criteria are fulfilled. The majority of studies used siRNA knock-down of $hP2Y_{11}$ receptors to support pharmacological data and many are in blood cells involved in immune responses, consistent with extracellular ATP being a member of the damage-associated molecular patterns (DAMPs) family (Rubartelli and Lotze 2007).

Granulocytes β-NAD^+ elicited rises in cytoplasmic Ca^{2+}, IP_3 and cAMP in freshly isolated human granulocytes, which were inhibited by 1 μM NF157, (a concentration that is 50-times higher than the antagonist's affinity for $hP2Y_{11}$ receptors) and downregulation of $P2Y_{11}$ expression by siRNA (Moreschi et al. 2006). Furthermore, NF157 inhibited β-NAD^+-induced granulocyte chemotaxisand it was suggested that β-NAD^+ is an endogenous $hP2Y_{11}$ agonist and acts as a proinflammatory cytokine. Subsequently, similar effects were seen with another endogenous $P2Y_{11}$ agonist, $NAADP^+$ (Moreschi et al. 2008).

Neutrophils Human neutrophils undergo constitutive apoptosis, which was delayed by ATP, NAD^+ and BzATP, but not UTP and appeared to be dependent upon cAMP activation of PKA (Vaughan et al. 2007), consistent with earlier reports of a $P2Y_{11}$-mediated increase in cAMP in human HL-60 cells, which are comprised mainly of neutrophil promyelocyte precursor cells (Choi and Kim 1997; Suh et al. 2000). The delay in apoptosis was inhibited by 500 nM NF157, but unaffected by P2X7 antagonism. Western blotting identified a protein of approximately 45 kDa. Similar results were reported in (Pliyev et al. 2014), which further demonstrated that the delay in neutrophil apoptosis was due to inhibition of the mitochondrial, but not the extrinsic, pathway of apoptosis. Thus endogenous ATP, and possibly also NAD^+, may act via $P2Y_{11}$ receptors to increase neutrophil survival, which will prolong the inflammatory response and the ability of neutrophils to phagocytose and destroy foreign particles and invading microorganisms. Further supporting evidence is, however, required.

Macrophages THP-1 cells are a human acute monocytic leukemia cell line that differentiate into the M1-proinflammatory type of macrophage after priming with lipopolysaccharide (LPS) and interferon-γ. This was inhibited by apyrase, a soluble enzyme that dephosphorylates ATP, and by 50 μM NF157 (Sakaki et al. 2013). This is a high concentration of NF157 (2500-times greater than its K_B at $P2Y_{11}$ receptors) and is enough to produce substantial blockade of numerous P2X and P2Y subtypes. THP-1 differentiation, was, however, unaffected by a range of other antagonists, PPADS (non-selective P2X and P2Y antagonist), NF449 (P2X1 antagonist), A438079 (P2X7 antagonist), MRS2179 ($P2Y_1$ antagonist), MRS2578 ($P2Y_6$ antagonist), clopidogrel ($P2Y_{12}$ antagonist), MRS2211 ($P2Y_{13}$ antagonist), consistent with NF157 acting here via $P2Y_{11}$ receptors. Furthermore, THP-1 differentiation was also supressed by down-regulation of $P2Y_{11}$ receptors by specific siRNA.

LPS also induced the release of the pro-inflammatory cytokine, IL-6, and this too was inhibited by NF157 and $P2Y_{11}$-specific siRNA. Inhibition of adenylyl cyclase, similarly, depressed IL-6 release, consistent with IL-6 release being mediated by $P2Y_{11}$ receptors. Based on these and other results, the authors concluded that LPS induces vesicular exocytosis

of ATP from macrophage precursor cells, which then acts in an autocrine manner at $P2Y_{11}$ receptors to cause these cells to differentiate into M1-pro-inflammatory macrophages that release pro-inflammatory cytokines, such as IL-6. In addition, they proposed that $P2Y_{11}$ receptor antagonists might potentially be useful in the treatment of inflammatory diseases, such as sepsis.

Dendritic Cells The first clear evidence for functional expression of $P2Y_{11}$ receptors in human dendritic cells was that the $P2Y_{11}$ agonist, NF546, induced a rise in intracellular Ca^{2+} concentration that was abolished by NF340 (Meis et al. 2010), which is consistent with expression of *P2RY11* mRNA in these cells (Kornum et al. 2011). In contrast to the studies above, which indicate pro-inflammatory roles for $P2Y_{11}$ receptors, an anti-inflammatory role is indicated in human cultured, monocyte-derived dendritic cells (Chadet et al. 2015). Here, ATP and BzATP, but not ADP, UTP or UDP, induced dendritic cell maturation, which was inhibited by 10 μM NF340 and suramin, but unaffected by PPADS and P2X4 and P2X7 antagonists. In these cells, LPS caused release of the inflammatory cytokine, IL-12 and this was inhibited by ATP, an action that was reversed by both NF340 and down-regulation of $P2Y_{11}$ receptors by specific siRNA. Exposing dendritic cells to hypoxia for 5 h, followed by reoxygenation, also suppressed the inhibitory effects of ATP and in addition, down-regulated *P2RY11* mRNA. The authors proposed that this inhibition of $P2Y_{11}$ receptor activity may cause dendritic cells to become pro-inflammatory and so contribute to post ischaemia/reperfusion injury.

Bronchial Epithelial Cells The human bronchial epithelial cell lines, NuLi-1 and CuFi-1, are amongst the small number of cell types in which expression of *P2RY11* mRNA has been demonstrated with confidence (Higgins et al. 2014). This group further showed that in these cells, lipoxin A4 (LXA_4), a regulator of adaptive immunity that has been proposed to reduce inflammation, acts by inducing ATP release, which in turn stimulates $P2Y_{11}$ receptors. In these cells, LXA_4 raised cytoplasmic Ca^{2+} and cAMP, increased airway surface liquid height, induced cell proliferation and migration and improved wound repair, all of which were inhibited by NF340 (0.1–1 μM). In addition, knock-down of the $P2Y_{11}$ receptor using siRNA inhibited the increase in airway surface liquid height. Thus in this situation it is stimulation, rather than inhibition, of $P2Y_{11}$ receptors that is likely to be beneficial therapeutically.

Keratinocytes $P2Y_{11}$ receptor-mediated release of pro-inflammatory cytokines is also indicated in keratinocytes (Ishimaru et al. 2013). Interferon-γ induced IL-6 release from the human HaCaT keratinocyte cell line and this was inhibited by apyrase, suramin and NF157. The concentration of NF157 used, 100 μM, was very high, but IL-6 release was unaffected by a range of other antagonists; PPADS (non-selective P2X and P2Y antagonist), AZ10606120 (P2X7 antagonist), MRS2179 ($P2Y_1$ antagonist), MRS2578 ($P2Y_6$ antagonist), clopidogrel ($P2Y_{12}$ antagonist), MRS2211 ($P2Y_{13}$ antagonist). Furthermore, IL-6 release was also inhibited by down-regulation of $P2Y_{11}$ receptors using specific siRNA. Thus $P2Y_{11}$ receptors may play a role in inflammatory conditions of the skin, such as psoriasis and blocking them could facilitate skin repair.

Mesenchymal Cells NAD^+ evoked rises in cytoplasmic Ca^{2+}, cAMP and cyclic ADP-ribose in human bone marrow-derived mesenchymal stem cells, which were all abolished by 1 μM NF157 (Fruscione et al. 2011). Although alternative antagonists were not employed to rule out the involvement of other P2X and P2Y receptor subtypes, the effects of NAD^+ were also inhibited by $P2Y_{11}$ receptor down-regulation by siRNA, consistent with NAD^+ acting here via $P2Y_{11}$ receptors. In the same study, NAD^+ also induced NF157-sensitive chemotaxis of the mesenchymal cells, a small increase in cell proliferation, release of a variety of cytokines and

increased nuclear translocation of a number of cAMP/Ca^{2+}-dependent transcription factors. The cells also released NAD^+ and ATP via connexin43 hemi-channels, suggesting that these nucleotides may act as endogenous modulators of mesenchymal cell function.

8 *P2RY11* Polymorphisms as an Indicator of Function

Numerous SNPs of the *P2RY11* gene are identified in the NCBI Single Nucleotide Polymorphism database and whilst their functional consequences are mostly unknown, genetic screening has implicated two SNPs in disease states.

Cardiovascular Disease Genotyping of participants in the Malmo Diet and Cancer (MDC) study identified a small increased risk of acute myocardial infarction (AMI) in the A87T polymorphism (rs3745601) and the risk was greater in patients with a family history of AMI and/or early onset AMI (Amisten et al. 2007). In addition, patients in the MDC cardiovascular risk group had elevated plasma levels of C-reactive protein, a marker of inflammation and a strong prognostic factor for the development of AMI. Thus this $P2Y_{11}$ polymorph appears to be associated with increased systemic inflammation and risk of cardiovascular disease.

Position 87 is near the extracellular end of the second transmembrane spanning region of the $P2Y_{11}$ receptor and the $P2Y_{11}$A87T variant replaces a hydrophobic alanine residue with a polar threonine. How this could lead to the changes seen in the MDC cohort is unclear, but the A87T mutation had no effect on the potency and efficacy of BzATP compared with wild-type $P2Y_{11}$ receptors expressed on their own, but BzATP became inactive when h$P2Y_{11}$A87T was coexpressed with $P2Y_1$ receptors (Haas et al. 2014). This was not mimicked by replacing A87 with serine or tyrosine. Additionally, the A87T mutation rendered the receptor insensitive to agonist-induced internalisation and the authors concluded that it disrupts the $P2Y_1$/$P2Y_{11}$ heteromeric receptor interaction.

Narcolepsy Increasing evidence indicates that narcolepsy is an autoimmune disease that leads to a loss of hypocretin (orexin)-releasing neurones in the hypothalamus. Genome-wide association studies have identified a SNP (rs2305795) that is associated with narcolepsy in European and Asian (Kornum et al. 2011), Chinese (Han et al. 2013) and Japanese (Yamasaki et al. 2016) patients. The SNP is in a region of the 3′ untranslated sequence of *P2RY11* that appears to regulate transcription, as it correlated with substantially reduced expression of *P2RY11* mRNA in $CD8^+$ T lymphocytes and natural killer cells of the carriers, which in turn was associated with a reduced ability of $P2Y_{11}$ receptors to inhibit ATP-induced monocyte cell death (Kornum et al. 2011). These data are consistent with roles for $P2Y_{11}$ receptors in modulation of immune cell function, as discussed above.

9 $P2Y_{11}$-Like Receptors in Rodents

As discussed in Sect. 2, the *P2RY11* gene has not been identified in rodent genomes and careful analysis has failed to reveal good evidence for expression of *P2RY11* mRNA and $P2Y_{11}$ protein in rodents that resemble those present in humans. Nonetheless, there is a body of reports that propose functional $P2Y_{11}$ receptor expression in rodent tissues. Some are based on the ability of ATP to raise cytosolic cAMP, but, as discussed in Sect. 3, some isoforms of adenylyl cyclase can be activated independently of $G\alpha_s$ and so an increase in cAMP cannot, on its own, be taken as evidence for $P2Y_{11}$ expression. In the main, the reports are based on pharmacological data using agonists, such as NAD^+, and/or NF157, as a supposedly selective $P2Y_{11}$ antagonist. As discussed in Sect. 4, however, both compounds have multiple sites of action and should not be

described as "selective". Furthermore, no report has convincingly excluded these other sites from the effects of NAD^+ or NF157 by using a variety of subtype-selective antagonists. In addition, data obtained using NF340 or NF546, which are more selective that NAD^+ and NF157, cannot be supported by knock-down of the receptor by siRNA due to the lack of the *P2RY11* gene. Consequently, until a solution to the apparent lack of *P2RY11* gene in rodent genomes has been found, these receptors should, at best, only be termed $P2Y_{11}$-like.

10 Concluding Remarks

In the 20 years since the $P2Y_{11}$ receptor was first cloned, our knowledge and understanding of its properties and functions have improved relatively slowly. This has been, in part, due to its apparent absence in mice and rats, the most commonly used species for studying receptors and drug action and which prevent genetic deletion studies from being performed. In common with many other purinergic receptor subtypes, progress has also been hampered by the limited range of selective pharmacological tools. Nonetheless, by careful use of the available tools, along with suppression of receptor expression using siRNA and also genetic screening for SNPs, possible functions of native $P2Y_{11}$ receptors have been identified in a range of human cells and tissues. Many are in blood cells involved in inflammatory responses, consistent with extracellular ATP being a DAMP signalling molecule in the immune system. Thus proposed potential therapeutic applications relate, in the main, to modulation of acute and chronic inflammatory responses. Further study of these in animals will require the development of non-rodent models, whilst selective ligands with suitable pharmacokinetic properties are needed before potential trials in humans can go ahead. Regardless of these difficulties it is clear that the $P2Y_{11}$ receptor is a viable, novel therapeutic target.

Conflicts of Interest The author declares that he has no conflict of interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA (2005) The recently deorphanized GPR80 (GPR99) proposed to be the $P2Y_{15}$ receptor is not a genuine P2Y receptor. Trends Pharmacol Sci 26:8–9

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology: update of the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341

Amisten S, Melander O, Wihlborg AK, Berglund G, Erlinge D (2007) Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in carriers of the Thr-87 variant of the ATP receptor $P2Y_{11}$. Eur Heart J 28:13–18

Azimi I, Beilby H, Davis FM, Marcial DL, Kenny PA, Thompson EW, Roberts-Thomson SJ, Monteith GR (2016) Altered purinergic receptor-Ca^{2+} signaling associated with hypoxia-induced epithelial-mesenchymal transition in breast cancer cells. Mol Oncol 10:166–178

Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 16:433–440

Burnstock G, Kennedy C (2011) P2X receptors in health and disease. Adv Pharmacol 61:333–372

Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 240:31–304

Chadet S, Ivanes F, Benoist L, Salmon-Gandonnière C, Guibon R, Velge-Roussel F, Babuty D, Baron C, Roger S, Angoulvant D (2015) Hypoxia/reoxygenation inhibits $P2Y_{11}$ receptor expression and its immunosuppressive activity in human dendritic cells. J Immunol 195:651–660

Choi SY, Kim KT (1997) Extracellular ATP-stimulated increase of cytosolic cAMP in HL-60 cells. Biochem Pharmacol 53:429–432

Choi RCY, Simon J, Tsim KWK, Barnard EA (2008) Constitutive and agonist-induced dimerizations of the $P2Y_1$ receptor: relationship to internalization and scaffolding. J Biol Chem 283:11050–11063

Communi D, Govaerts C, Parmentier M, Boeynaems JM (1997) Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J Biol Chem 272:31969–31973

Communi D, Robaye B, Boeynaems J-M (1999) Pharmacological characterization of the human $P2Y_{11}$ receptor. Br J Pharmacol 128:1199–1206

Communi D, Suarez-Huerta N, Dussossoy D, Savi P, Boeynaems JM (2001) Cotranscription and intergenic splicing of human P2Y11 and SSF1 genes. J Biol Chem 276:16561–16566

d'Ambrosi N, Iafrate M, Vacca F, Amadio S, Tozzi A, Mercuri N, Volonté C (2006) The $P2Y_4$ receptor forms homo-oligomeric complexes in several CNS and PNS neuronal cells. Purinergic Signal 2:575–582

d'Ambrosi N, Iafrate M, Saba E, Rosa P, Volonté C (2007) Comparative analysis of $P2Y_4$ and $P2Y_6$ receptor architecture in native and transfected neuronal systems. Biochim Biophys Acta 1768:1592–1599

del Puerto A, Diaz-Hernandez JI, Tapia M, Gomez-Villafuertes R, Benitez MJ, Zhang J, Miras-Portugal MT, Wandosell F, Diaz-Hernandez M, Garrido JJ (2012) Adenylate cyclase 5 coordinates the action of ADP, $P2Y_1$, $P2Y_{13}$ and ATP-gated P2X7 receptors on axonal elongation. J Cell Sci 125:176–188

Devader C, Drew CM, Geach TJ, Tabler J, Townsend-Nicholson A, Dale L (2007) A novel nucleotide receptor in *Xenopus* activates the cAMP second messenger pathway. FEBS Lett 581:5332–5336

Ding L, Ma W, Littmann T, Camp R, Shen J (2011) The $P2Y_2$ nucleotide receptor mediates tissue factor expression in human coronary artery endothelial cells. J Biol Chem 286:27027–27038

Dreisig K, Kornum B (2016) A critical look at the function of the $P2Y_{11}$ receptor. Purinergic Signal 12:427–437

Dreisig K, Degn M, Sund L, Hadaczek P, Samaranch L, San Sebastian W, Bankiewicz K, Rahbek Kornum B (2016) Validation of antibodies for neuroanatomical localization of the $P2Y_{11}$ receptor in macaque brain. J Chem Neuroanat 78:25–33

Ecke D, Fischer B, Reiser G (2008a) Diastereoselectivity of the $P2Y_{11}$ nucleotide receptor: mutational analysis. Br J Pharmacol 155:1250–1255

Ecke D, Hanck T, Tulapurkar ME, Schäfer R, Kassack M, Stricker R, Reiser G (2008b) Hetero-oligomerization of the $P2Y_{11}$ receptor with the $P2Y_1$ receptor controls the internalization and ligand selectivity of the $P2Y_{11}$ receptor. Biochem J 409:107–116

Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, Durroux T, Fuxe K, George SR, Javitch JA, Lohse MJ, Mackie K, Milligan G, Pfleger KDG, Pin JP, Volkow ND, Waldhoer M, Woods AS, Franco R (2009) Building a new conceptual framework for receptor heteromers. Nat Chem Biol 5:131–134

Ferré S, Casadó V, Devi LA, Filizola M, Jockers R, Lohse MJ, Milligan G, Pin JP, Guitart X (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol Rev 66:413–434

Fruscione F, Scarfì S, Ferraris C, Bruzzone S, Benvenuto F, Guida L, Uccelli A, Salis A, Usai C, Jacchetti E, Ilengo C, Scaglione S, Quarto R, Zocchi E, De Flora A (2011) Stem Cells Dev 20:1183–1198

Gao Z-G, Wei Q, Jayasekara MPS, Jacobson KA (2013) The role of $P2Y_{14}$ and other P2Y receptors in degranulation of human LAD2 mast cells. Purinergic Signal 9:31–40

Grahnert A, Klein C, Hauschildt S (2009) Involvement of P2X receptors in the NAD^+-induced rise in $[Ca^{2+}]$i in human monocytes. PUSI 5:309–319

Grahnert A, Grahnert A, Klein C, Schilling E, Wehrhahn J, Hauschildt S (2011) NAD^+: a modulator of immune functions. Innate Immun 17:212–233

Haas M, Shaaban A, Reiser G (2014) Alanine-(87)-threonine polymorphism impairs signaling and internalization of the human $P2Y_{11}$ receptor, when co-expressed with the $P2Y_1$ receptor. J Neurochem 129:602–613

Han F, Faraco J, Dong XS et al (2013) Genome wide analysis of narcolepsy in China implicates novel immune loci and reveals changes in association prior to versus after the 2009 H1N1 influenza pandemic. PLoS Genet 9:e1003880

Higgins G, Buchanan P, Perriere M, Al-Alawi M, Costello RW, Verriere V, McNally P, Harvey BJ, Urbach V (2014) Activation of *P2RY11* and ATP release by lipoxin A4 restores the airway surface liquid layer and epithelial repair in cystic fibrosis. Am J Respir Cell Mol Biol 51:178–190

Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ (2008) Agonist-selective, receptor-specific interaction of human P2Y receptors with β-arrestin-1 and -2. J Biol Chem 283:30933–30941

Ishimaru M, Tsukimoto M, Harada H, Kojima S (2013) Involvement of $P2Y_{11}$ receptor in IFN-γ -induced IL-6 production in human keratinocytes. Eur J Pharmacol 703:67–73

Kamal M, Maurice P, Jockers R (2011) Expanding the concept of G protein-coupled receptor (GPCR) dimer asymmetry towards GPCR-interacting proteins. Pharmaceuticals 4:273–284

Kenakin T (1997) Pharmacologic analysis of drug-receptor interaction, 3rd edn. Lippencott-Raven Publishers, Philadelphia

Kennedy C, Chootip K, Mitchell C, Syed NH, Tengah A (2013) P2X and P2Y nucleotide receptors as targets in cardiovascular disease. Future Med Chem 5:431–439

Klein C, Grahnert A, Abdelrahman A, Müller CE, Hauschildt S (2009) Extracellular NAD^+ induces a rise in $[Ca^{2+}]_i$ in activated human monocytes via engagement of $P2Y_1$ and $P2Y_{11}$ receptors. Cell Calcium 46:263–272

Kornum BR, Kawashima M, Faraco J et al (2011) Common variants in *P2RY11* are associated with narcolepsy. Nat Genet 43:66–71

Lutz S, Freichel-Blomquist A, Yang Y, Rümenapp U, Jakobs KH, Schmidt M, Wieland T (2005) The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. J Biol Chem 280:11134–11139

Meis S, Hamacher A, Hongwiset D, Marzian C, Wiese M, Eckstein N, Royer HD, Communi D, Boeynaems JM, Hausmann R, Schmalzing G, Kassack MU (2010) NF546 [4,4′-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-alpha, alpha'--diphosphonic acid) tetrasodium salt] is a non-nucleotide $P2Y_{11}$ agonist and stimulates release of interleukin-8 from human monocytes. J Pharmacol Exp Ther 332:238–247

Milligan G (2013) The prevalence, maintenance, and relevance of G protein-coupled receptor oligomerization. Mol Pharmacol 84:158–169

Moreschi I, Bruzzone S, Nicholas RA, Fruscione F, Sturla L, Benvenuto F, Usai C, Meis S, Kassack MU, Zocchi E, De Flora A (2006) Extracellular NAD^+ is an agonist of the human $P2Y_{11}$ purinergic receptor in human granulocytes. J Biol Chem 281:31419–31429

Moreschi I, Bruzzone S, Bodrato N, Usai C, Guida L, Nicholas RA, Kassack MU, Zocchi E, De Flora A (2008) $NAADP^+$ is an agonist of the human $P2Y_{11}$ purinergic receptor. Cell Calcium 43:344–355

Morrow GB, Nicholas RA, Kennedy C (2014) UTP is not a biased agonist at human $P2Y_{11}$ receptors. Purinergic Signal 10:581–585

Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK (1998) Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. Mol Pharmacol 54:1118–1123

Pliyev BK, Ivanova AV, Savchenko VG (2014) Extracellular NAD^+ inhibits human neutrophil apoptosis. Apoptosis 19:581–593

Qi AD, Kennedy C, Harden TK, Nicholas RA (2001a) Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase. Br J Pharmacol 132:318–326

Qi AD, Zambon AC, Insel PA, Nicholas RA (2001b) An arginine/glutamine difference at the juxtaposition of transmembrane domain 6 and the third extracellular loop contributes to the markedly different nucleotide selectivities of human and canine $P2Y_{11}$ receptors. Mol Pharmacol 60:1375–1382

Ribeiro-Filho AC, Buri MV, Barros CC, Dreyfuss JL, Nader HB, Justo GZ, Craveiro RB, Pesquero JB, Miranda A, Ferreira AT, Paredes-Gamero EJ (2016) Functional and molecular evidence for heteromeric association of $P2Y_1$ receptor with $P2Y_2$ and $P2Y_4$ receptors in mouse granulocytes. BMC Pharmacol Toxicol 17:29

Rubartelli A, Lotze MT (2007) Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol 28:429–436

Sakaki H, Tsukimoto M, Harada H, Moriyama Y, Kojima S (2013) Autocrine regulation of macrophage activation via exocytosis of ATP and activation of $P2Y_{11}$ receptor. PLoS One 8:e59778

Savi P, Zachayus JL, Delesque-Touchard N, Labouret C, Hervé C, Uzabiaga MF, Pereillo JM, Culouscou JM, Bono F, Ferrara P, Herbert JM (2006) The active metabolite of Clopidogrel disrupts $P2Y_{12}$ receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci U S A 103:11069–11074

Shakya Shrestha S, Parmar M, Kennedy C, Bushell T (2010) Two-pore potassium ion channels are inhibited by both $G_{q/11}$- and G_i-coupled P2Y receptors. Mol Cell Neurosci 43:363–369

Suarez-Huerta N, Boeynaems JM, Communi D (2000) Cloning, genomic organization, and tissue distribution of human Ssf-1. Biochem Biophys Res Commun 275:37–42

Suh BC, Kim TD, Lee IS, Kim KT (2000) Differential regulation of $P2Y_{11}$ receptor-mediated signalling to phospholipase C and adenylyl cyclase by protein kinase C in HL-60 promyelocytes. Br J Pharmacol 131:489–497

Sunahara RK, Dessauer CW, Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. Ann Rev Pharmacol Toxicol 36:461–480

Ullmann H, Meis S, Hongwiset D, Marzian C, Wiese M, Nickel P, Communi D, Boeynaems JM, Wolf C, Hausmann R, Schmalzing G, Kassack MU (2005) Synthesis and structure-activity relationships of suramin-derived $P2Y_{11}$ receptor antagonists with nanomolar potency. J Med Chem 48:7040–7048

van der Weyden L, Adams DJ, Luttrell BM, Conigrave AD, Morris MB (2000) Pharmacological characterisation of the $P2Y_{11}$ receptor in stably transfected haematological cell lines. Mol Cell Biochem 213:75–81

Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA (2003) The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A 100:4903–4908

Vaughan KR, Stokes L, Prince LR, Marriott HM, Meis S, Kassack MU, Bingle CD, Sabroe I, Surprenant A, Whyte MK (2007) Inhibition of neutrophil apoptosis by ATP is mediated by the $P2Y_{11}$ receptor. J Immunol 179:8544–8553

Vial C, Fung CY, Goodall AH, Mahaut-Smith MP, Evans RJ (2006) Differential sensitivity of human platelet P2X1 and $P2Y_1$ receptors to disruption of lipid rafts. Biochem Biophys Res Comm 343:415–419

Von Kügelgen I, Harden TK (2011) Molecular pharmacology, physiology, and structure of the P2Y receptors. Adv Pharmacol 61:373–415

White PJ, Webb TE, Boarder MR (2003) Characterization of a Ca^{2+} response to both UTP and ATP at human $P2Y_{11}$ receptors: evidence for agonist-specific signalling. Mol Pharmacol 63:1356–1363

Yamasaki M, Miyagawa T, Toyoda H, Khor SS, Liu X, Kuwabara H, Kano Y, Shimada T, Sugiyama T, Nishida H, Sugaya N, Tochigi M, Otowa T, Okazaki Y, Kaiya H, Kawamura Y, Miyashita A, Kuwano R, Kasai K, Tanii H, Sasaki T, Honda Y, Honda M, Tokunaga K (2016) Evaluation of polygenic risks for narcolepsy and essential hypersomnia. J Hum Gene 61:873–878

Zambon AC, Brunton LL, Barrett KE, Hughes RJ, Torres B, Insel PA (2001) Cloning, expression, signaling mechanisms, and membrane targeting of $P2Y_{11}$ receptors in Madin Darby canine kidney cells. Mol Pharmacol 60:26–35

Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, Moss SM, Paoletta S, Kiselev E, Lu W, Fenalti G, Zhang W, Müller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014) Structure of the human $P2Y_{12}$ receptor in complex with an antithrombotic drug. Nature 509:115–118

Zylberg J, Ecke D, Fischer B, Reiser G (2007) Structure and ligand-binding site characteristics of the human $P2Y_{11}$ nucleotide receptor deduced from computational modelling and mutational analysis. Biochem J 405:277–286

Adv Exp Med Biol - Protein Reviews (2017) 19:123–138
DOI 10.1007/5584_2017_98

Published online: 12 September 2017

Structure, Pharmacology and Roles in Physiology of the $P2Y_{12}$ Receptor

Ivar von Kügelgen

Abstract

P2Y receptors are G-protein-coupled receptors (GPCRs) for extracellular nucleotides. The platelet ADP-receptor which has been denominated $P2Y_{12}$ receptor is an important target in pharmacotherapy. The receptor couples to $G_{\alpha i2}$ mediating an inhibition of cyclic AMP accumulation and additional downstream events including the activation of phosphatidylinositol-3-kinase and Rap1b proteins. The nucleoside analogue ticagrelor and active metabolites of the thienopyridine compounds ticlopidine, clopidogrel and prasugrel block $P2Y_{12}$ receptors and, thereby, inhibit ADP-induced platelet aggregation. These drugs are used for the prevention and therapy of cardiovascular events such as acute coronary syndromes or stroke. The recently published three-dimensional crystal structures of the human $P2Y_{12}$ receptor in complex with agonists and antagonists will facilitate the development of novel therapeutic agents with reduced adverse effects. $P2Y_{12}$ receptors are also expressed on vascular smooth muscle cells and may be involved in the pathophysiology of atherogenesis. $P2Y_{12}$ receptors on microglial cells operate as sensors for adenine nucleotides released during brain injury. A recent study indicated the involvement of microglial $P2Y_{12}$ receptors in the activity-dependent neuronal plasticity. Interestingly, there is evidence for changes in $P2Y_{12}$ receptor expression in CNS pathologies including Alzheimer's diseases and multiple sclerosis. $P2Y_{12}$ receptors may also be involved in systemic immune modulating responses and the susceptibility to develop bronchial asthma.

Keywords

Adenine nucleotides • Atherogenesis • Crystal structure • Inflammation • Microglial cells • $P2Y_{12}$ receptor • Platelet aggregation • Receptor ligands

I. von Kügelgen (✉)
Department of Pharmacology and Toxicology, Pharma Center, University of Bonn, D-53127 Bonn, Germany
e-mail: kugelgen@uni-bonn.de

Abbreviations

ADPßS	adenosine 5-O-2-thiodiphosphate
Akt	proteinkinase B
AR-C66096	2-(propylthio)adenosine-5′-O-(β,γ-difluoromethylene) triphosphate
AR-C67085	2-propylthio-β,-γ-dichloromethylene-D-ATP
AR-C69931MX	N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,-γ-dichloromethylene-ATP)
AZD1283	ethyl 6-(4-[(benzylsulfonyl) carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate)
AZD6140	ticagrelor
EL	extracellular loops
FITC	fluorescein isothiocyanate
GPCRs	G-protein-coupled receptors
LPS	lipopolysaccharide
PDZ	postsynaptic density 95/disc large/zonula occludens-1–binding domain
PSB-0739	1-Amino-9,10-dihydro-9,10-dioxo-4-[[4-(phenylamino)-3-sulfophenyl]amino]-2-anthracenesulfonic acid
RASA3	Ras GTPase-activating protein 3
TM	transmembrane regions

1 Introduction

The physiological roles and molecular properties of G-protein-coupled receptors for extracellular nucleotides – P2Y receptors – have previously been summarized by several comprehensive articles (Ralevic and Burnstock 1998, 2003; Müller 2002; Abbracchio et al. 2006; von Kügelgen 2006; Burnstock 2007; von Kügelgen and Harden 2011; Burnstock and Boeynaems 2014; von Kügelgen and Hoffmann 2016). Based on structural properties and G protein signaling mammalian P2Y receptors belong to either the $P2Y_1$-subfamily (including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors coupling to activation of G_q) or the $P2Y_{12}$-subfamiliy ($P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors coupling to G_i) (Abbracchio et al. 2006; von Kügelgen 2006). This chapter is focused on novel findings in structure, molecular mechanisms and pharmacology of the $P2Y_{12}$ receptor. The $P2Y_{12}$ receptor plays a prominent role in ADP-induced platelet aggregation (together with platelet $P2Y_1$ receptors) (Hollopeter et al. 2001; Cattaneo et al. 2003; Dorsam and Kunapuli 2004; Gachet 2006; Liverani et al. 2014a; Scavone et al. 2017). This is the basis for the use of $P2Y_{12}$ receptor antagonists for the prevention and therapy of cardiovascular events such as acute coronary syndromes (Dorsam and Kunapuli 2004; Gachet 2006). Active metabolites of the thienopyridine compounds clopidogrel and prasugrel as well as direct acting compounds including the nucleoside analogue ticagrelor inhibit platelet aggregation by a blockade of $P2Y_{12}$ receptors (Savi et al. 2006; Algaier et al. 2008; Hoffmann et al. 2014; Hechler and Gachet 2015). $P2Y_{12}$ receptors are also expressed on the membranes of a number of cells including vascular smooth muscle cells (Wihlborg et al. 2004) and microglial cells (Sasaki et al. 2003) as discussed below in detail.

2 Gene, Receptor Structure and Receptor Polymorphisms

The gene sequence encoding for the platelet ADP receptor protein was identified in 2001 (Hollopeter et al. 2001; Takasaki et al. 2001; Zhang et al. 2001). The gene P2RY12 contains no introns and is located on human chromosome 3 (3q25.1) very close to the genes P2RY13 and P2RY14. The predicted human $P2Y_{12}$ receptor protein consists of 342 amino acids. It shares 43.5 and 44.9% identical amino acids with the $P2Y_{13}$ and $P2Y_{14}$ receptors, respectively, but only 18.8% identical amino acids with the $P2Y_1$ receptor in agreement with the existence of two subfamilies of P2Y receptors (Abbracchio et al. 2006; von Kügelgen 2006).

Recently, crystal structural data of the human $P2Y_{12}$ receptor bound to both agonists and antagonists have been obtained by high-

resolution x-ray analysis (Zhang et al. 2014a, b). The crystals had been grown in complex with the non-nucleotide antagonist AZD1283 (ethyl 6-(4-[(benzylsulfonyl) carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate) (Zhang et al. 2014a) or the agonists 2-methylthio-ADP or 2-methylthio-ATP (Zhang et al. 2014b). The observed structures confirm general features of GPCRs including seven hydrophobic transmembrane regions (TM) connected by three extracellular loops (EL) and three intracellular loops. Moreover, the structures bound to agonists show a disulfide bridge in the extracellular compartment between TM3 and EL2 which is highly conserved among family A GPCRs (Zhang et al. 2014b) in agreement with previous data from mutational analysis (Savi et al. 2006; Algaier et al. 2008; Ding et al. 2009). In contrast to many structures of GPCRS, the x-ray analysis of the human $P2Y_{12}$ showed an unusual straight α–helical conformation of TM5 (Zhang et al. 2014a).

The binding pocket of the inactive, antagonist bound $P2Y_{12}$ receptor is very wide and consists of two subpockets (Zhang et al. 2014a). Interestingly, no disulfide bridge between TM3 and EL2 was observed in the inactive, antagonist bound structure (Zhang et al. 2014a). The cysteine residue $Cys97^{3.25}$ forming this bridge with Cys175 has been shown to interact with the active metabolites of the thienopyridine compounds clopidogrel and prasugrel which are irreversible acting $P2Y_{12}$ receptor inhibitors (Savi et al. 2006; Algaier et al. 2008). The antagonist AZD1283 is bound in subpocket 1 formed by regions of TMs 3–7 (Zhang et al. 2014a). Key residues involved in AZD1283 binding include the positively charged residues $Arg256^{6.55}$, and $Lys280^{7.35}$ (Zhang et al. 2014a). A classical ionic lock mechanism between charged residues in the intracellular portions of TM3 and TM6 stabilizes the inactive state of many rhodopsin-like GPCRs (Park et al. 2008; Jacobson et al. 2015). This mechanism, however, is absent in the human $P2Y_{12}$ receptor (Zhang et al. 2014a; Jacobson et al. 2015).

The agonist bound $P2Y_{12}$ receptor showed large-scale rearrangements (Zhang et al. 2014b). The agonists are also bound within subpocket 1, but the total binding pocket of the agonist bound receptor is markedly contracted. The negatively charged phosphate groups of the nucleotide agonists interact with positively charged residues including $Arg93^{3.21}$, $Arg256^{6.55}$, and $Lys280^{7.35}$ and hydrogen-bonding groups (for a predicted two dimensional structure see Fig. 1). Adenine of the agonist 2-methylthio-ADP forms a p-p interaction with $Tyr105^{3.33}$ (Zhang et al. 2014b). These structural data markedly extend published data of mutational studies (Hoffmann et al. 2008; Cöster et al. 2012; Schmidt et al. 2013).

The crystal structural data also show that the extracellular regions of TM6 and TM7 are bent inward toward the bound agonist closing the entrance to the binding pocket (Zhang et al. 2014b; Jacobson et al. 2015). As mentioned above, the highly conserved disulfide bridge between TM3 and EL2 is present in the agonist bound receptor (Zhang et al. 2014b). The agonist bound structures show only small minor rearrangements in the intracellular portions of TM6 and TM7 indicating that the analyzed structures represent an agonist-bound inactive state (Zhang et al. 2014b). The published structural data are now used to optimize receptor models for a guided drug design (Jacobson et al. 2015; Paoletta et al. 2015; Ahn et al. 2016; Zhang et al. 2016). These models explain interactions of $P2Y_{12}$ ligands such as ticagrelor with residues of the receptor protein (Hoffmann et al. 2014; Paoletta et al. 2015).

Interestingly, some patients with a loss or a reduction of platelet function carry changes in the gene P2RY12 (Hollopeter et al. 2001; Cattaneo et al. 2003; Scavone et al. 2017; Nicholas 2015; Cattaneo 2011). These changes include Arg122Cys, Lys174Glu, His187Gln, Arg256Gln, P258T, R265Trp and P341A (Cattaneo et al. 2003; Scavone et al. 2017; Cattaneo 2011; Remijn et al. 2007; Daly et al. 2009; Nisar et al. 2011; Patel et al. 2014; Lecchi

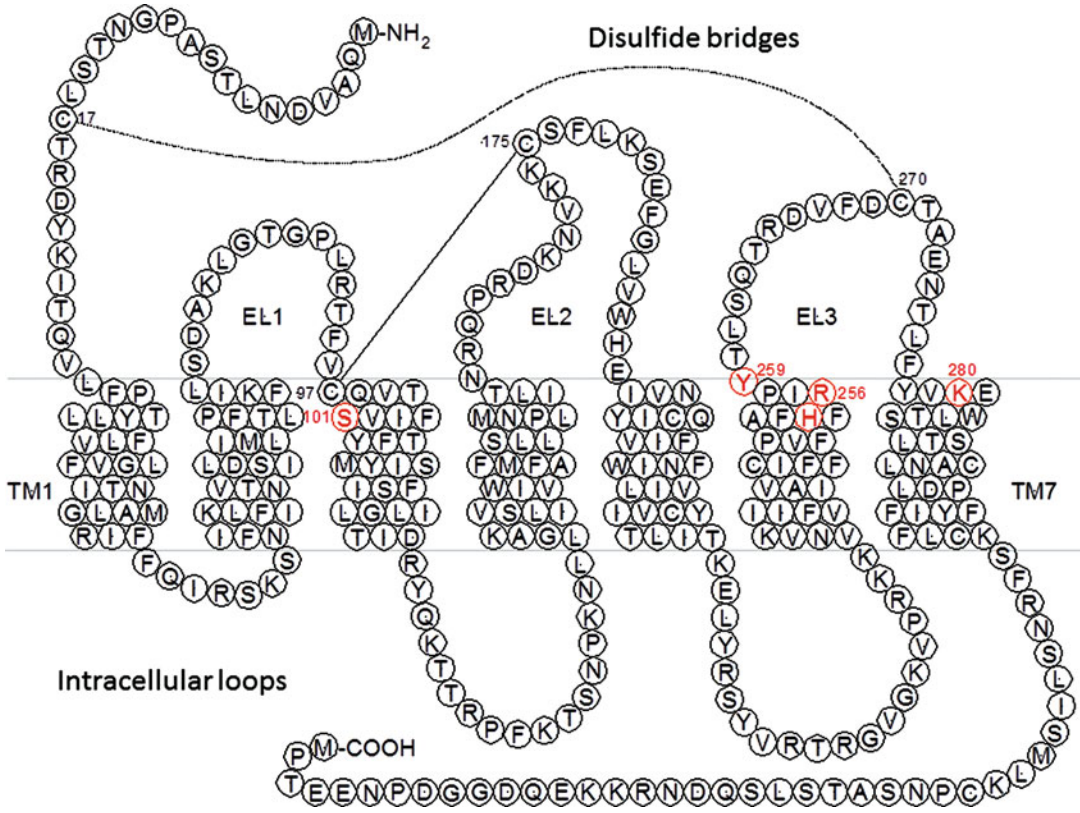

Fig. 1 Predicted two-dimensional structure of the human $P2Y_{12}$ receptor (*TM* transmembrane region, *EL* extracellular loop, *lines* indicate disulfide bridges; modified from (von Kügelgen 2006))

et al. 2015). The mutations alter ligand binding, the activation process or, possibly, the function of the postsynaptic density 95/disc large/zonula occludens-1 (PDZ)–binding domain at the C-terminus of the receptor protein (Nisar et al. 2011).

There is ongoing search for P2RY12 gene polymorphisms possibly associated with altered platelet functions or a changed responsiveness toward antiplatelet treatment (Fontana et al. 2003; Ou et al. 2016; Siasos et al. 2016; Yang et al. 2016; Yi et al. 2016, 2017). A study analyzing the $P2Y_{13}$ receptor M158 T polymorphism, which was shown to be in linkage disequilibrium with the $P2Y_{12}$ locus, revealed no association with the risk of acute myocardial infarction (Amisten et al. 2008).

3 Signaling Transduction Pathways

Cooper and Rodbell showed in 1979 that ADP promotes inhibition of adenylyl cyclase in platelet membranes (Cooper and Rodbell 1979). Two decades later, Hollopeter et al. identified the gene sequence encoding the ADP-activated $P2Y_{12}$ receptor and demonstrated the coupling of the recombinant receptor via G_i proteins (Hollopeter et al. 2001). A number of studies with recombinant $P2Y_{12}$ receptors confirmed the coupling of the $P2Y_{12}$ receptor to activation of $G_{\alpha i2}$ leading to inhibition of adenylyl cyclase activity (Takasaki et al. 2001; Zhang et al. 2001; Bodor et al. 2003; Chhatriwala et al. 2004). In platelet aggregation, $P2Y_{12}$ receptors also signal via phosphatidylinositol-3-kinase to inhibition of Ras GTPase-activating protein 3 (RASA3) to

promote GTPase Rap1b activity and integrin activation (Dorsam and Kunapuli 2004; Hechler and Gachet 2015; Kim and Kunapuli 2011; Kamae et al. 2006; Stefanini et al. 2015; Stefanini and Bergmeier 2016). Additional downstream signaling events include the activation of protein kinases C isoforms or K+ channels (von Kügelgen and Harden 2011; Hollopeter et al. 2001; Dorsam and Kunapuli 2004; Hechler and Gachet 2015; Mundell et al. 2006; Guidetti et al. 2008). The phosphorylation status of vasodilator-stimulated phosphoprotein is analyzed to monitor platelet $P2Y_{12}$ receptor activation (Hechler and Gachet 2015; Kim and Kunapuli 2011). $P2Y_{12}$ receptors in platelets have been demonstrated to be rapidly relocalized at the level of the cell membrane following agonist-induced desensitization and internalization in platelets; this could represent a mechanism to maintain haemostatic properties of platelets (Mundell et al. 2006; Baurand et al. 2005; Mundell et al. 2008). Interestingly, recent studies demonstrated the heterodimerization and co-internalization of the $P2Y_{12}$ receptor and the protease-activated receptor-4 and the subsequent signaling via Akt/proteinkinase B (Khan et al. 2014; Smith et al. 2017).

4 Pharmacology

ADP is the endogenous agonist of the $P2Y_{12}$ receptor (Hollopeter et al. 2001; Bodor et al. 2003). In studies performed on platelets, ATP has been shown to act as an antagonist (Gachet 2006; Kauffenstein et al. 2004). However, ATP may also act as an agonist at the $P2Y_{12}$ receptor natively expressed in tissues or on cells (Simon et al. 2001). Very potent agonists with half-maximal concentrations in the nanomolar range are 2-methylthio-ADP and 2-methylthio-ATP (von Kügelgen 2006; Zhang et al. 2014b). In contrast, 2-methylthio-AMP is a low affinity antagonist (Hollopeter et al. 2001). Analogues of ATP, including cangrelor (AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP) and AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP) are potent and competitive $P2Y_{12}$ receptor antagonists (von Kügelgen 2006; Ingall et al. 1999). However, both cangrelor and AR-C67085 also show antagonistic activities at $P2Y_{13}$ receptors (Marteau et al. 2003). [^{3}H]PSB-0413 is a derivative of AR-C67085; it is used as a radioligand with a K_D value of 5 nM at the $P2Y_{12}$ receptor (El-Tayeb et al. 2005; Ohlmann et al. 2013). Recently, diadenosine tetraphosphate derivatives have been developed as antagonists acting in the nanomolar range at the $P2Y_{12}$ receptor (Yanachkov et al. 2016). Uridine triphosphate thio analogues also exert antagonistic activities at the $P2Y_{12}$ receptor (Gündüz et al. 2017a). Farnesyl pyrophosphate is an endogenous, low affinity antagonist blocking $P2Y_{12}$ receptor-mediated effects (Högberg et al. 2012). In addition to nucleotides and farnesyl pyrophosphate, derivatives of nucleosides block the $P2Y_{12}$ receptor. Ticagrelor (AZD6140) is an orally active $P2Y_{12}$ receptor antagonist (Springthorpe et al. 2007; James et al. 2009; van Giezen et al. 2009; Olivier et al. 2014). When used in low concentrations in vitro, ticagrelor blocks the recombinant human $P2Y_{12}$ receptor in a competitive manner with a pA_2 value of 8.7 (Hoffmann et al. 2014). Depending on the expression level and the cellular system ticagrelor may also act as an inverse agonist at the $P2Y_{12}$ receptor (Aungraheeta et al. 2016). There is evidence for an antagonistic action of ticagrelor at the $P2Y_{13}$ receptor in addition to the $P2Y_{12}$ receptor (Björquist et al. 2016). Moreover, ticagrelor has been shown to block the equilibrative nucleoside transporter 1 and to increase, thereby, the extracellular concentration of adenosine (Aungraheeta et al. 2016). There are adverse effects: Ticagrelor has been reported to cause dyspnea in some patients (Cattaneo and Faioni 2012; Parker et al. 2017).

A number of compounds with very different structural properties have been identified or

developed as antagonists acting at the $P2Y_{12}$ receptor (Zetterberg and Svensson 2016). Several P2 receptor subtypes including the $P2Y_{12}$ receptor are blocked by suramin and reactive blue-2 (von Kügelgen 2006; von Kügelgen and Wetter 2000). An analogue of reactive blue-2, PSB-0739 (Baqi et al. 2009; Baqi 2016), was shown to act as a potent and competitive antagonist at the recombinant human $P2Y_{12}$ receptor (pA_2-value 9.8) (Hoffmann et al. 2009). A number of new scaffolds have been developed. These include 6-amino-2-mercapto-3H-pyrimidine-4-one derivatives (Crepaldi et al. 2009), piperazinyl glutamates (Parlow et al. 2010; Zech et al. 2012), morpholine analogues (Ahn et al. 2016), flavonolignans (Bijak et al. 2017) as well as ethyl 6-aminonicotinate acyl sulfonamides (Bach et al. 2013; Zhou et al. 2016). The ethyl 6-aminonicotinate acyl sulfonamide compound AZD1283 showed a K_D value of 11 nM at the $P2Y_{12}$ receptor (Bach et al. 2013). As mentioned above, AZD1283 was used to grow crystals of the human $P2Y_{12}$ receptor in an inactive state (Zhang et al. 2014a). Another clinical candidate is ACT-246475 with an IC_{50} of about 5 nM in the binding assay (Caroff et al. 2014; Caroff et al. 2015). Some of its analogues even displayed higher affinities at the receptor (Caroff et al. 2014).

For decades, thienopyridine compounds have been used in pharmacotherapy to inhibit platelet aggregation for the prevention of myocardial infarction or stroke (Dorsam and Kunapuli 2004; Gachet 2006). Ticlopidine (Maffrand et al. 1988), clopidogrel (Savi et al. 2006; Herbert et al. 1993), and prasugrel (Sugidachi et al. 2000; Sugidachi et al. 2016) are prodrugs. As already discussed above, their active metabolites have been demonstrated to interact in an irreversible manner with the cysteine residue Cys-97 of the human $P2Y_{12}$ receptor (Savi et al. 2006; Algaier et al. 2008; Ding et al. 2009). The active metabolite of prasugrel has been shown to modify the $P2Y_{12}$ receptor function without any interaction with the platelet $P2Y_1$ receptor (Niitsu et al. 2005; Hashimoto et al. 2007). Several studies indicate that treatment of patients with prasugrel or ticagrelor is more effective than treatment with clopidogrel (Olivier et al. 2014; Angiolillo et al. 2016; Bednar et al. 2016). Treatment using prasugrel or clopidogrel has been shown to inhibit neutrophil function and to attenuate lung injury and platelet sequestration in sepsis- or LPS-induced inflammation (Liverani et al. 2013, 2014b, 2016).

5 Platelet Aggregation

The role of the ADP receptor in platelet aggregation is well established (Gaarder et al. 1961; Born 1962; Burnstock 2015). In 1979, ADP was shown to strongly inhibit adenylyl cyclase activity in membranes of platelets (Cooper and Rodbell 1979). About two decades later, Hollopeter et al. identified the gene sequence encoding for the G_i coupled $P2Y_{12}$ receptor (Hollopeter et al. 2001). Interestingly, mutations within the human P2Y12 gene have been demonstrated to be associated with bleeding disorders in patients as described above (Hollopeter et al. 2001; Cattaneo et al. 2003; Scavone et al. 2017; Nicholas 2015; Cattaneo 2011; Remijn et al. 2007; Daly et al. 2009; Nisar et al. 2011; Patel et al. 2014; Lecchi et al. 2015). Studies with genetic knockout $P2Y_{12}(-/-)$ mice further confirmed the function of the $P2Y_{12}$ receptor in platelet aggregation (Dorsam and Kunapuli 2004; Foster et al. 2001; Andre et al. 2003). Platelets express $P2Y_1$ receptors and $P2X_1$ receptors in addition to $P2Y_{12}$ receptors (Dorsam and Kunapuli 2004; Wang et al. 2003). A study revealed the rank order of mRNA encoding the respective receptors in platelets to be $P2Y_{12}$ greater $P2X_1$ greater $P2Y_1$ (Wang et al. 2003). The numbers of $P2Y_{12}$ binding sites per single human platelet have been estimated to amount to 425 in average (Ohlmann et al. 2013). There is recently published evidence for a de novo synthesis of $P2Y_{12}$ receptors in platelets (Sirotkina et al. 2016). Despite possible differences in numbers of receptors per platelet, both $P2Y_{12}$ receptors and $P2Y_1$ receptors contribute to ADP-induced aggregation (Dorsam and Kunapuli 2004; Gachet 2006; Nylander et al. 2003; Nylander et al. 2004; Gremmel et al.

2016). As already described in the pharmacology section, the inhibition of platelet $P2Y_{12}$ receptors by directly acting agents such as ticagrelor or by active metabolites of thienopyridine compounds such as clopidogrel is commonly used for the prevention and therapy of cardiovascular diseases (Dorsam and Kunapuli 2004; Gachet 2006; Hechler and Gachet 2015; Angiolillo et al. 2016). Blockade of platelet $P2Y_{12}$ receptors reduces the inhibition of adenylyl cyclase and potentiates thereby the platelet inhibitory actions of prostacyclin and nitric oxide (Cattaneo and Lecchi 2007; Kirkby et al. 2013; Chan et al. 2015). The blockade of platelet activity by $P2Y_{12}$ receptor antagonists may also exert anti-inflammatory effects by a decreased release of pro-inflammatory granule contents and a decreased formation of platelet-leukocyte aggregates (Hechler and Gachet 2015; Cattaneo 2015; Thomas and Storey 2015). Moreover, a reduction in platelet activity by ticagrelor has been suggested to be involved in the inhibition of metastasis and in the improvement of survival in mouse models of cancer (Gebremeske et al. 2015).

6 Vascular and Renal Receptors

$P2Y_{12}$ receptors had been shown to be expressed in vascular smooth muscle cells and to contribute to vasoconstriction (Wihlborg et al. 2004; Shanker et al. 2006; Rauch et al. 2010; Mitchell et al. 2012; West et al. 2014). These smooth muscle $P2Y_{12}$ receptors also promote migration and atherogenesis (West et al. 2014; Niu et al. 2017). Smooth muscle cells within plaques showed increased $P2Y_{12}$ receptor like immunoreactivity (Niu et al. 2017). Interestingly, the treatment of apolipoprotein E Null mice with clopidogrel had recently been demonstrated to reduce both the number of $P2Y_{12}$ receptor positive smooth muscle cells in plaques and the plaque progression (Niu et al. 2017). There were also benefits in patients treated with clopidogrel (Niu et al. 2017). A reduction of the release of platelet contents may contribute to a reduced vessel wall remodeling (Södergren et al. 2016). Endothelial cells also express $P2Y_{12}$ receptors (Uehara and Uehara 2011; Gündüz et al. 2017b). The $P2Y_{12}$ receptor antagonist AR-C66096 but not ticagrelor was reported to increase endothelial cAMP levels and to counteract thrombin-induced hyper-permeability (Gündüz et al. 2017b). Pharmacological blockade of the $P2Y_{12}$ receptor increases the renal urine concentrating ability in agreement with a function of $P2Y_{12}$ receptors in the kidney (Zhang et al. 2015).

7 Control of Microglial Function and Inflammation

Within the adult CNS $P2Y_{12}$ receptors have been demonstrated to be specifically expressed on the cell membrane of microglial cells, but not on the membranes of neurons, oligodendrocytes or astroglial cells (Sasaki et al. 2003; Honda et al. 2001; Haynes et al. 2006; Butovsky et al. 2014; Moore et al. 2015; Tsuda and Inoue 2016; Mildner et al. 2017). Microglial cells are known to function as resident macrophages in the CNS (Tsuda and Inoue 2016). A recent study showed that microglial $P2Y_{12}$ receptors are expressed at a constant level in the human brain throughout lifetime (Mildner et al. 2017). Microglial $P2Y_{12}$ receptors operate as a sensor for adenine nucleotides released at the site of CNS injury (Honda et al. 2001; Haynes et al. 2006). Microglia of knockout $P2Y_{12}(-/-)$ mice have a reduced capacity to polarize, migrate, or generate process extensions toward the released nucleotides (Haynes et al. 2006; Tsuda and Inoue 2016; Koizumi et al. 2013). Intracellular responses to activation of microglial $P2Y_{12}$ receptors also include integrin-β1-activation (Ohsawa et al. 2010), calcium-dependent signaling (Eyo et al. 2015; Sunkaria et al. 2016), opening of potassium channels (Swiatkowski et al. 2016) and the activation of nuclear factor of activated T cell signaling leading to C-C

chemokine 3 expression (Tozaki-Saitoh et al. 2017). Under CNS inflammatory conditions such as infection with Schistosoma mekongi a high expression of $P2Y_{12}$ receptors on human microglial cells has been shown (Moore et al. 2015).

The microglial $P2Y_{12}$ receptor also plays roles in physiology. The involvement of $P2Y_{12}$ receptors on mature microglia in the activity-dependent synaptic plasticity within the mouse visual cortex has recently been demonstrated (Sipe et al. 2016). $P2Y_{12}(-/-)$ mice as well as wild type mice treated with clopidogrel showed a decreased ramification of microglial cells and a defect in the ocular dominance plasticity (Sipe et al. 2016). Moreover, $P2Y_{12}$ receptor-mediated chemotaxis of microglial processes has been shown to be required for the rapid closure of the blood brain barrier (Lou et al. 2016). Gene disruption as well as treatment of wild type mice with clopidogrel diminished the closing of laser-induced lesions (Lou et al. 2016).

Interestingly, in patients with CNS pathologies including Alzheimer's disease and multiple sclerosis a decrease in $P2Y_{12}$ receptor immune-reactivity within plaques or lesions has been observed (Moore et al. 2015; Mildner et al. 2017; Amadio et al. 2014). There are conflicting results in two recent studies analyzing autoimmune encephalomyelitis in $P2Y_{12}(-/-)$ mice. One study showed more severe symptoms in an experimental model of autoimmune encephalomyelitis in $P2Y_{12}(-/-)$ mice (Zhang et al. 2017), whereas in a second study $P2Y_{12}$ deficiency led to a reduced leukocyte infiltration and less extensive demyelination (Qin et al. 2017). The role of the $P2Y_{12}$ receptor in autoimmune encephalomyelitis has to be studied in more detail. There is evidence for a neuroprotective role of $P2Y_{12}$ receptors in the epileptic brain (Eyo et al. 2014). The $P2Y_{12}$ receptor-dependent microglial closure of the injured blood–brain barrier described above may contribute to neuroprotective mechanisms (Lou et al. 2016).

Activation of microglial $P2Y_{12}$ receptors may also acerbate symptoms in other pathologies. There is emerging evidence for a role of $P2Y_{12}$ receptor activation in the induction of neuropathic pain and cancer-induced pain (Tsuda and Inoue 2016; Tozaki-Saitoh et al. 2008; Horváth et al. 2014; Beko et al. 2017; Tatsumi et al. 2015; Gu et al. 2016; Tamagawa et al. 2016; Liu et al. 2017). $P2Y_{12}$ receptor antagonists including PSB-0739 reduced pain responses (Horváth et al. 2014; Beko et al. 2017) and genetic deficiency of the $P2Y_{12}$ receptor ameliorated pain hypersensitivities (Beko et al. 2017; Gu et al. 2016). $P2Y_{12}$ receptors also play a role in brain ischemia (Pedata et al. 2016). $P2Y_{12}(-/-)$ mice showed reduced damages after brain ischemia (Webster et al. 2013) and treatment of wild type rats with ticagrelor reduced ischemic lesions (Gelosa et al. 2014). In addition to the absence or the blockade of microglial $P2Y_{12}$ receptors, a reduction in platelet aggregation may have contributed to these effects in brain ischemia (Sugidachi et al. 2016; Pedata et al. 2016).

Systemic immune modulating roles of $P2Y_{12}$ receptors have been discussed (Burnstock and Boeynaems 2014; Hechler and Gachet 2015; Cattaneo 2015; Thomas and Storey 2015; Idzko et al. 2014). These immune modulating effects may partially be due to indirect mechanisms including the reduced release of pro-inflammatory granule contents from platelets after blockade of aggregation by $P2Y_{12}$ receptor inhibition (Burnstock and Boeynaems 2014; Hechler and Gachet 2015; Cattaneo 2015; Thomas and Storey 2015; Idzko et al. 2014). However, lymphocytes express $P2Y_{12}$ receptors in agreement with direct effects (Wang et al. 2004; Diehl et al. 2010). Studies performed on knock-out animals confirm the involvement of direct cellular mechanisms. Dentric cells of $P2Y_{12}(-/-)$ mice showed no ADPβS-mediated FITC-dextran and oval albumin uptake (Ben Addi et al. 2010). Activation of $P2Y_{12}$ receptors may play a role in bronchial asthma (Burnstock and Boeynaems 2014; Cattaneo 2015; Thomas and Storey 2015; Idzko et al. 2014). Allergen exposure induces the migration of platelets to the lung tissue and the subsequent activation (Knauer et al. 1981; Pitchford et al. 2008). Released components then contribute to the inflammatory responses (Cattaneo 2015;

Cameron 2012). P2RY12 gene variations have been associated with the risk of family-based asthma (Bunyavanich et al. 2012). Moreover, treatment with prasugrel caused a slight reduction of bronchial hyper-reactivity in patients (Lussana et al. 2015). And $P2Y_{12}$ receptor blockade attenuated eosinophilic inflammation and airway hyper-responsiveness in a mouse model of asthma (Suh et al. 2016). However, there are conflicting data arguing for a role of platelet $P2Y_1$ receptors, but not $P2Y_{12}$ receptors, in allergic inflammation (Amison et al. 2015). The underlying mechanisms have to be studied in more detail.

8 Neuronal Receptors

As discussed above, the expression of $P2Y_{12}$ receptors in the adult CNS appears to occur exclusively in microglial cells (e.g Mildner et al. 2017). In the autonomous nervous system, however, functional studies have suggested a role for $P2Y_{12}$ receptors in modulating neurotransmission (Kulick and von Kügelgen 2002; Kubista et al. 2003; Lechner et al. 2004; Quintas et al. 2009). A recent study now demonstrated the expression and function of $P2Y_{12}$ receptors in neurons of the rat trigeminal ganglion (Kawaguchi et al. 2015) in agreement with differences in the expression of $P2Y_{12}$ receptors in the CNS and the peripheral nervous system, respectively.

9 Bone Homeostasis

Osteoclast activity had been shown to be decreased in $P2Y_{12}(-/-)$ mice and these $P2Y_{12}(-/-)$ mice were partially protected from pathological bone loss (Su et al. 2012). However, studies with mice treated with clopidogrel revealed conflicting data with increased (Su et al. 2012) and decreased bone formation (Syberg et al. 2012). There is evidence for an association of exposure to higher doses of clopidogrel with an increased risk of fractures in patients and evidence for an association of exposure to lower doses of clopidogrel with a decreased risk of fractures (Jorgensen et al. 2012).

10 Conclusions and Outlook

Blockade of $P2Y_{12}$ receptors by directly acting antagonists such as ticagrelor or active metabolites of thienopyridine compounds such as clopidogrel is an important clinical strategy in the prevention and therapy of cardiovascular events. The published crystal structural data of the receptor will facilitate the development of more selective blockers with fewer side effects. In addition to platelets, $P2Y_{12}$ receptors are found on number of cells in the periphery and CNS including vascular smooth muscle cells and microglial cells. Blockade of these receptors may cause beneficial effects in some cases, but may also induce yet not understood adverse effects. The mechanisms involved should be studied in more detail.

Compliance with Ethical Standards

Conflicts of Interest The author declares that he has no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341

Ahn YH, Lee JY, Park HD, Kim TH, Park MC, Choi G, Kim S (2016) Identification of a new morpholine

scaffold as a P2Y12 receptor antagonist. Molecules 21:E1114

Algaier I, Jakubowski JA, Asai F, von Kügelgen I (2008) Interaction of the active metabolite of prasugrel, R-138727, with cysteine 97 and cysteine 175 of the human P2Y12 receptor. J Thromb Haemost 6:1908–1914

Amadio S, Parisi C, Montilli C, Carrubba AS, Apolloni S, Volonté C (2014) P2Y12 receptor on the verge of a neuroinflammatory breakdown. Mediat Inflamm 2014:975849

Amison RT, Momi S, Morris A, Manni G, Keir S, Gresele P, Page CP, Pitchford SC (2015) RhoA signaling through platelet P2Y1 receptor controls leukocyte recruitment in allergic mice. J Allergy Clin Immunol 135:528–538

Amisten S, Braun OO, Johansson L, Ridderstråle M, Melander O, Erlinge D (2008) The P2Y 13 Met-158-Thr polymorphism, which is in linkage disequilibrium with the P2Y 12 locus, is not associated with acute myocardial infarction. PLoS One 3:e1462

Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR, Conley PB (2003) P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest 112:398–406

Angiolillo DJ, Franchi F, Waksman R, Sweeny JM, Raveendran G, Teng R, Zhao Y, Carlson G, Khan N, Mehran R (2016) Effects of ticagrelor versus clopidogrel in troponin-negative patients with low-risk ACS undergoing ad hoc PCI. J Am Coll Cardiol 67:603–613

Aungraheeta R, Conibear A, Butler M, Kelly E, Nylander S, Mumford A, Mundell SJ (2016) Inverse agonism at the P2Y12 receptor and ENT1 transporter blockade contribute to platelet inhibition by ticagrelor. Blood 128:2717–2728

Bach P, Boström J, Brickmann K, van Giezen JJ, Groneberg RD, Harvey DM, Harvey DM, O'Sullivan M, Zetterberg F (2013) Synthesis, structure-property relationships and pharmacokinetic evaluation of ethyl 6-aminonicotinate sulfonylureas as antagonists of the P2Y12 receptor. Eur J Med Chem 65:360–375

Baqi Y (2016) Anthraquinones as a privileged scaffold in drug discovery targeting nucleotide-binding proteins. Drug Discov Today 21:1571–1577

Baqi Y, Atzler K, Köse M, Glänzel M, Müller CE (2009) High-affinity, non-nucleotide-derived competitive antagonists of platelet P2Y12 receptors. J Med Chem 52:3784–3793

Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, Cazenave JP, Léon C, Gachet C (2005) Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? Mol Pharmacol 67:721–733

Bednar F, Kroupa J, Ondrakova M, Osmancik P, Kopa M, Motovska Z (2016) Antiplatelet efficacy of P2Y12 inhibitors (prasugrel, ticagrelor, clopidogrel) in patients treated with mild therapeutic hypothermia after cardiac arrest due to acute myocardial infarction. J Thromb Thrombolysis 41:549–555

Beko K, Koványi B, Gölöncsér F, Horváth G, Dénes Á, Környei Z, Botz B, Helyes Z, Müller CE, Sperlágh B (2017) Contribution of platelet P2Y12 receptors to chronic complete Freund's adjuvant-induced inflammatory pain. J Thromb Haemost 15:1223–1235

Ben Addi A, Cammarata D, Conley PB, Boeynaems JM, Robaye B (2010) Role of the P2Y12 receptor in the modulation of murine dendritic cell function by ADP. J Immunol 185:5900–5906

Bijak M, Szelenberger R, Saluk J, Nowak P (2017) Flavonolignans inhibit ADP induced blood platelets activation and aggregation in whole blood. Int J Biol Macromol 95:682–688

Björquist A, Di Buduo CA, Femia EA, Storey RF, Becker RC, Balduini A, Nylander S, Cattaneo M (2016) Studies of the interaction of ticagrelor with the P2Y13 receptor and with P2Y13-dependent pro-platelet formation by human megakaryocytes. Thromb Haemost 116:1079–1088

Bodor ET, Waldo GL, Hooks SB, Corbitt J, Boyer JL, Harden TK (2003) Purification and functional reconstitution of the human P2Y12 receptor. Mol Pharmacol 64:1210–1216

Born GV (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927–929

Bunyavanich S, Boyce JA, Raby BA, Weiss ST (2012) Gene-by-environment effect of house dust mite on purinergic receptor P2Y12 (P2RY12) and lung function in children with asthma. Clin Exp Allergy 42:229–437

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87:659–797

Burnstock G (2015) Blood cells: an historical account of the roles of purinergic signalling. Purinergic Signal 11:411–434

Burnstock G, Boeynaems JM (2014) Purinergic signalling and immune cells. Purinergic Signal 10:529–564

Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, PM W, Doykan CE, Fanek Z, Liu L, Chen Z, Rothstein JD, Ransohoff RM, Gygi SP, Antel JP, Weiner HL (2014) Identification of a unique TGF-β-dependent molecular and functional signature in microglia. Nat Neurosci 17:131–143

Cameron L (2012) The LTE(4)-P2Y12 pathway in asthma. Clin Exp Allergy 42:176–179

Caroff E, Meyer E, Treiber A, Hilpert K, Riederer MA (2014) Optimization of 2-phenyl-pyrimidine-4-carboxamides towards potent, orally bioavailable and selective P2Y(12) antagonists for inhibition of platelet aggregation. Bioorg Med Chem Lett 24:4323–4331

Caroff E, Hubler F, Meyer E, Renneberg D, Gnerre C, Treiber A, Rey M, Hess P, Steiner B, Hilpert K, Riederer MA (2015) 4-((R)-2-{[6-((S)-3-

Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-3-phosphonopropionyl)piperazine-1-carboxylic acid butyl ester (ACT-246475) and its prodrug (ACT-281959), a novel P2Y12 receptor antagonist with a wider therapeutic window in the rat than clopidogrel. J Med Chem 58:9133–9153

Cattaneo M (2011) The platelet P2Y12 receptor for adenosine diphosphate: congenital and drug-induced defects. Blood 117:2102–2112

Cattaneo M (2015) The platelet P2 receptors in inflammation. Hamostaseologie 2015:262–266

Cattaneo M, Faioni EM (2012) Why does ticagrelor induce dyspnea? Thromb Haemost 108:1031–1036

Cattaneo M, Lecchi A (2007) Inhibition of the platelet P2Y12 receptor for adenosine diphosphate potentiates the antiplatelet effect of prostacyclin. J Thromb Haemost 5:577–582

Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, Conley PB, Ware J, Ruggeri ZM (2003) Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. Proc Natl Acad Sci U S A 100:1978–1983

Chan MV, Knowles RBM, Lundberg MH, Tucker AT, Mohamed NA, Kirkby NS, Armstrong PCJ, Mitchell JA, Warner TD (2015) P2Y12 Receptor blockade synergizes strongly with nitric oxide and prostacyclin to inhibit platelet activation. Br J Clin Pharmacol 81:621–633

Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA, Harden TK (2004) Induction of novel agonist selectivity for the ADP-activated P2Y1 receptor versus the ADP-activated P2Y12 and P2Y13 receptors by conformational constraint of an ADP analog. J Pharmacol Exp Ther 311:1038–1043

Cooper DM, Rodbell M (1979) ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. Nature 282:517–518

Cöster M, Wittkopf D, Kreuchwig A, Kleinau G, Thor D, Krause G, Schöneberg T (2012) Using ortholog sequence data to predict the functional relevance of mutations in G-protein-coupled receptors. FASEB J 26:3273–3281

Crepaldi P, Cacciari B, Bonache MC, Spalluto G, Varani K, Borea PA, von Kügelgen I, Hoffmann K, Pugliano M, Razzari C, Cattaneo M (2009) 6-Amino-2-mercapto-3H-pyrimidin-4-one derivatives as new candidates for the antagonism at the P2Y12 receptors. Bioorg Med Chem 17:4612–4621

Daly ME, Dawood BB, Lester WA, Peake IR, Rodeghiero F, Goodeve AC, Makris M, Wilde JT, Mumford AD, Watson SP, Mundell SJ (2009) Identification and characterization of a novel P2Y12 variant in a patient diagnosed with type 1 von Willebrand disease in the European MCMDM-1VWD study. Blood 113:4110–4113

Diehl P, Olivier C, Halscheid C, Helbing T, Bode C, Moser M (2010) Clopidogrel affects leukocyte dependent platelet aggregation by P2Y12 expressing leukocytes. Basic Res Cardiol 105:379–387

Ding Z, Bynagari YS, Mada SR, Jakubowski JA, Kunapuli SP (2009) Studies on the role of the extracellular cysteines and oligomeric structures of the P2Y12 receptor when interacting with antagonists. J Thromb Haemost 7:232–234

Dorsam RT, Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. J Clin Invest 113:340–345

El-Tayeb A, Griessmeier KJ, Müller CE (2005) Synthesis and preliminary evaluation of [3H]PSB-0413, a selective antagonist radioligand for platelet P2Y12 receptors. Bioorg Med Chem Lett 15:5450–5452

Eyo UB, Peng J, Swiatkowski P, Mukherjee A, Bispo A, LJ W (2014) Neuronal hyperactivity recruits microglial processes via neuronal NMDA receptors and microglial P2Y12 receptors after status epilepticus. J Neurosci 34:10528–10540

Eyo UB, Gu N, De S, Dong H, Richardson JR, LJ W (2015) Modulation of microglial process convergence toward neuronal dendrites by extracellular calcium. J Neurosci 35:2417–2422

Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P (2003) Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. Circulation 108:989–995

Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ Jr, Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA, Chintala MS (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest 107:1591–1598

Gaarder A, Jonsen J, Laland S, Hellem A, Owren PA (1961) Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 192:531–532

Gachet C (2006) Regulation of platelet functions by P2 receptors. Ann Rev Pharmacol Toxicol 46:277–300

Gebremeske S, LeVatte T, Liwski RS, Johnston B, Bezuhly M (2015) The reversible P2Y12 inhibitor ticagrelor inhibits metastasis and improves survival in mouse models of cancer. Int J Cancer 136:234–240

Gelosa P, Lecca D, Fumagalli M, Wypych D, Pignieri A, Cimino M, Verderio C, Enerbäck M, Nikookhesal E, Tremoli E, Abbracchio MP, Sironi L (2014) Microglia is a key player in the reduction of stroke damage promoted by the new antithrombotic agent ticagrelor. J Cereb Blood Flow Metab 34:979–988

Gremmel T, Yanachkov IB, Yanachkova MI, Wright GE, Wider J, Undyala VVR, Michelson AD, Frelinger AL, Przyklenk K (2016) Synergistic inhibition of both P2Y1 and P2Y12 adenosine diphosphate receptors as novel approach to rapidly attenuate platelet-mediated thrombosis. Arterioscler Thromb Vasc Biol 36:501–509

Gu N, Eyo UB, Murugan M, Peng J, Matta S, Dong H, LJ W (2016) Microglial P2Y12 receptors regulate microglial activation and surveillance during neuropathic pain. Brain Behav Immun 55:82–92

Guidetti GF, Lova P, Bernardi B, Campus F, Baldanzi G, Graziani A, Balduini C, Torti M (2008) The Gi-coupled P2Y12 receptor regulates diacylglycerol-mediated signaling in human platelets. J Biol Chem 283:28795–28805

Gündüz D, Tanislav C, Sedding D, Parahuleva M, Santoso S, Troidl C, Hamm CW, Aslam M (2017a) Uridine triphosphate Thio analogues inhibit platelet P2Y12 receptor and aggregation. Int J Mol Sci 18: E269

Gündüz D, Tanislav C, Schluter KD, Schulz R, Hamm C, Aslam M (2017b) Effect of ticagrelor on endothelial calcium signalling and barrier function. Thromb Haemost 117:371–381

Hashimoto M, Sugidachi A, Isobe T, Niitsu Y, Ogawa T, Jakubowski JA, Asai F (2007) The influence of P2Y12 receptor deficiency on the platelet inhibitory activities of prasugrel in a mouse model: evidence for specific inhibition of P2Y12 receptors by prasugrel. Biochem Pharmacol 74:1003–1009

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9:1512–1519

Hechler B, Gachet C (2015) Purinergic receptors in thrombosis and inflammation. Arterioscler Thromb Vasc Biol 35:2307–2315

Herbert JM, Tissinier A, Defreyn G, Maffrand JP (1993) Inhibitory effect of clopidogrel on platelet adhesion and intimal proliferation after arterial injury in rabbits. Arterioscler Thromb 13:1171–1179

Hoffmann K, Sixel U, Di Pasquale F, von Kügelgen I (2008) Involvement of basic amino acid residues in transmembrane regions 6 and 7 in agonist and antagonist recognition of the human platelet P2Y12-receptor. Biochem Pharmacol 76:1201–1213

Hoffmann K, Baqi Y, Morena MS, Glänzel M, Müller CE, von Kügelgen I (2009) Interaction of new, very potent non-nucleotide antagonists with Arg256 of the human platelet P2Y12 receptor. J Pharmacol Exp Ther 331:648–655

Hoffmann K, Lutz DA, Straßburger J, Baqi Y, Müller CE, von Kügelgen I (2014) Competitive mode and site of interaction of ticagrelor at the human platelet P2Y12 -receptor. J Thromb Haemost 12:1898–1905

Högberg C, Gidlöf O, Deflorian F, Jacobson KA, Abdelrahman A, Müller CE, Olde B, Erlinge D (2012) Farnesyl pyrophosphate is an endogenous antagonist to ADP-stimulated P2Y12 receptor-mediated platelet aggregation. Thromb Haemost 108:119–132

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207

Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S (2001) Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. J Neurosci 21:1975–1982

Horváth G, Gölöncsér F, Csölle C, Király K, Andó RD, Baranyi M, Koványi B, Máté Z, Hoffmann K, Algaier I, Baqi Y, Müller CE, von Kügelgen I, Sperlágh B (2014) Central P2Y12 receptor blockade alleviates inflammatory and neuropathic pain and cytokine production in rodents. Neurobiol Dis 70:162–178

Idzko M, Ferrari D, Eltzschig HK (2014) Nucleotide signalling during inflammation. Nature 509:310–317

Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, Humphries RG, Leff P, Clegg JA, Smith JA, Tomlinson W (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 42:213–220

Jacobson KA, Paoletta S, Katritch V, Wu B, Gao ZG, Zhao Q, Stevens RC, Kiselev E (2015) Nucleotides acting at P2Y receptors: connecting structure and function. Mol Pharmacol 88:220–230

James S, Akerblom A, Cannon CP, Emanuelsson H, Husted S, Katus H, Skene A, Steg PG, Storey RF, Harrington R, Becker R, Wallentin L (2009) Comparison of ticagrelor, the first reversible oral P2Y12 receptor antagonist, with clopidogrel in patients with acute coronary syndromes: rationale, design, and baseline characteristics of the PLATelet inhibition and patient outcomes (PLATO) trial. Am Heart J 157:599–605

Jorgensen NR, Grove EL, Schwarz P, Vestergaard P (2012) Clopidogrel and the risk of osteoporotic fractures: a nationwide cohort study. J Intern Med 272:385–393

Kamae T, Shiraga M, Kashiwagi H, Kato H, Tadokoro S, Kurata Y, Tomiyama Y, Kanakura Y (2006) Critical role of ADP interaction with P2Y12 receptor in the maintenance of alpha(IIb)beta3 activation: association with Rap1B activation. J Thromb Haemost 4:1379–1387

Kauffenstein G, Hechler B, Cazenave JP, Gachet C (2004) Adenine triphosphate nucleotides are antagonists at the P2Y12 receptor. J Thromb Haemost 2:1980–1988

Kawaguchi A, Sato M, Kimura M, Ichinohe T, Tazaki M, Shibukaw Y (2015) Expression and function of purinergic P2Y12 receptors in rat trigeminal ganglion neurons. Neurosci Res 98:17–27

Khan A, Li D, Ibrahim S, Smyth E, Woulfe DS (2014) The physical association of the P2Y12 receptor with PAR4 regulates arrestin-mediated Akt activation. Mol Pharmacol 86:1–11

Kim S, Kunapuli SP (2011) P2Y12 Receptor in platelet activation. Platelets 22:56–60

Kirkby NS, Lundberg MH, Chan MV, Vojnovic I, Solomon AB, Emerson M, Mitchell JA, Warner TD (2013) Blockade of the purinergic P2Y12 receptor greatly increases the platelet inhibitory actions of nitric oxide. Proc Natl Acad Sci U S A 110:15782–15787

Knauer KA, Fish JE, Adkinson NF Jr, Lichtenstein LM, Peters SP, Newball HH (1981) Platelet activation in antigen-induced bronchoconstriction. N Engl J Med 305:892–893

Koizumi S, Ohsawa K, Inoue K, Kohsaka S (2013) Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. Glia 61:47–54

Kubista H, Lechner SG, Wolf AM, Boehm S (2003) Attenuation of the P2Y receptor-mediated control of neuronal Ca2+ channels in PC12 cells by antithrombotic drugs. Br J Pharmacol 138:343–350

Kulick MB, von Kügelgen I (2002) P2Y-Receptors mediating an inhibition of the evoked entry of calcium through N-type calcium channels at neuronal processes. J Pharmacol Exp Ther 303:520–526

Lecchi A, Razzari C, Paoletta S, Dupuis A, Nakamura L, Ohlmann P, Gachet C, Jacobson KA, Zieger B, Cattaneo M (2015) Identification of a new dysfunctional platelet P2Y12 receptor variant associated with bleeding diathesis. Blood 125:1006–1013

Lechner SG, Dorostkar MM, Mayer M, Edelbauer H, Pankevych H, Boehm S (2004) Autoinhibition of transmitter release from PC12 cells and sympathetic neurons through a P2Y receptor-mediated inhibition of voltage-gated Ca2+ channels. Eur J Neurosci 20:2917–2928

Liu M, Yao M, Wang H, Xu L, Zheng Y, Huang B, Ni H, Xu S, Zhou X, Lian Q (2017) P2Y12 receptor-mediated activation of spinal microglia and p38MAPK pathway contribute to cancer-induced bone pain. J Pain Res 10:417–426

Liverani E, Rico MC, Garcia AE, Kilpatrick LE, Kunapuli SP (2013) Prasugrel metabolites inhibit neutrophil functions. J Pharmacol Exp Ther 344:231–243

Liverani E, Kilpatrick LE, Tsygankov AY, Kunapuli SP (2014a) The role of P2Y12 receptor and activated platelets during inflammation. Curr Drug Targets 15:720–728

Liverani E, Rico MC, Yaratha L, Tsygankov AY, Kilpatrick LE, Kunapuli SP (2014b) LPS-induced systemic inflammation is more severe in P2Y12 null mice. J Leukoc Biol 95:313–323

Liverani E, Rico MC, Tsygankov AY, Kilpatrick LE, Kunapuli SP (2016) P2Y12 Receptor modulates sepsis-induced inflammation. Arterioscler Thromb Vasc Biol 36:961–971

Lou N, Takano T, Pei Y, Al X, Goldman SA, Nedergaard M (2016) Purinergic receptor P2RY12-dependent microglial closure of the injured blood–brain barrier. Proc Natl Acad Sci U S A 113:1074–1079

Lussana F, Di Marco F, Terraneo S, Parati M, Razzari C, Scavone M, Femia EA, Moro A, Centanni S, Cattaneo M (2015) Effect of prasugrel in patients with asthma: results of PRINA, a randomized, double-blind, placebo-controlled, cross-over study. J Thromb Haemost 13:136–141

Maffrand JP, Bernat A, Delebassee D, Defreyn G, Cazenave JP, Gordon JL (1988) ADP plays a key role in thrombogenesis in rats. Thromb Haemost 59:225–230

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM, Gonzalez NS (2003) Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 64:104–112

Mildner A, Huang H, Radke J, Stenzel W, Priller J (2017) P2Y12 receptor is expressed on human microglia under physiological conditions throughout development and is sensitive to neuroinflammatory diseases. Glia 65:375–387

Mitchell C, Syed NI, Tengah A, Gurney AM, Kennedy C (2012) Identification of contractile P2Y1, P2Y6, and P2Y12 receptors in rat intrapulmonary artery using selective ligands. J Pharmacol Exp Ther 343:755–762

Moore CS, Ase AR, Kinsara A, Rao VTS, Michell-Robinson M, Leong SY, Butovsky O, Ludwin SK, Séguéla P, Bar-Or A, Antel JP (2015) P2Y12 Expression and function in alternatively activated human microglia. Neurol Neuroimmunol Neuroinflamm 2: e80

Müller CE (2002) P2-Pyrimidinergic receptors and their ligands. Curr Pharm Des 8:2353–2369

Mundell SJ, Jones ML, Hardy AR, Barton JF, Beaucourt SM, Conley PB, Poole AW (2006) Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. Mol Pharmacol 70:1132–1142

Mundell SJ, Barton JF, Mayo-Martin MB, Hardy AR, Poole AW (2008) Rapid resensitization of purinergic receptor function in human platelets. J Thromb Haemost 6:1393–1404

Nicholas RA (2015) Insights into platelet P2Y12 receptor activation. Blood 125:893–895

Niitsu Y, Jakubowski JA, Sugidachi A, Asai F (2005) Pharmacology of CS-747 (prasugrel, LY640315), a novel, potent antiplatelet agent with in vivo P2Y12 receptor antagonist activity. Semin Thromb Hemost 31:184–194

Nisar S, Daly ME, Federici AB, Artoni A, Mumford AD, Watson SP, Mundell SJ (2011) An intact PDZ motif is essential for correct P2Y12 purinoceptor traffic in human platelets. Blood 118:5641–5651

Niu X, Pi SL, Baral S, Xia YP, He QW, Li YN, Jin HJ, Li M, Wang MD, Mao L, Hu B (2017) P2Y12 promotes migration of vascular smooth muscle cells through cofilin dephosphorylation during atherogenesis. Arterioscler Thromb Vasc Biol 37:515–524

Nylander S, Mattsson C, Ramstrom S, Lindahl TL (2003) The relative importance of the ADP receptors, P2Y12 and P2Y1, in thrombin-induced platelet activation. Thromb Res 111:65–73

Nylander S, Mattsson C, Ramström S, Lindahl TL (2004) Synergistic action between inhibition of P2Y12/P2Y1

and P2Y12/thrombin in ADP- and thrombin-induced human platelet activation. Br J Pharmacol 142:1325–1331

Ohlmann P, Lecchi A, El-Tayeb A, Müller CE, Cattaneo M, Gachet C (2013) The platelet P2Y (12) receptor under normal and pathological conditions. Assessment with the radiolabeled selective antagonist [(3)H]PSB-0413. Purinergic Signal 9:59–66

Ohsawa K, Irino Y, Sanagi T, Nakamura Y, Suzuki E, Inoue K, Kohsaka S (2010) P2Y12 receptor-mediated integrin-beta1 activation regulates microglial process extension induced by ATP. Glia 58:790–801

Olivier CB, Diehl P, Schnabel K, Weik P, Zhou Q, Bode C, Moser M (2014) Third generation P2Y12 antagonists inhibit platelet aggregation more effectively than clopidogrel in a myocardial infarction registry. Thromb Haemost 111:266–272

Ou W, He Y, Li A, Liu B, Jin L (2016) Genotype frequencies of CYP2C19, P2Y12 and GPIIIa polymorphisms in coronary heart disease patients of Han ethnicity, and their impact on Clopidogrel responsiveness. Int Heart J 57:586–592

Paoletta S, Sabbadin D, von Kügelgen I, Hinz S, Katritch V, Hoffmann K, Abdelrahman A, Straßburger J, Baqi Y, Zhao Q, Stevens RC, Moro S, Müller CE, Jacobson KA (2015) Modeling ligand recognition at the P2Y12 receptor in light of X-ray structural information. J Comput Aided Mol Des 29:737–756

Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. Nature 454:183–187

Parker WA, Bhatt DL, Prats J, Day JR, Steg PG, Stone GW, Hamm CW, Mahaffey KW, Price MJ, Gibson CM, White HD, Storey RF (2017) Characteristics of dyspnoea and associated clinical outcomes in the CHAMPION PHOENIX study. Thromb Haemost 117:1093–1100

Parlow JJ, Burney MW, Case BL, Girard TJ, Hall KA, Harris PK, Hiebsch RR, Huff RM, Lachance RM, Mischke DA, Rapp SR, Woerndle RS, Ennis MD (2010) Piperazinyl glutamate pyridines as potent orally bioavailable P2Y12 antagonists for inhibition of platelet aggregation. J Med Chem 53:2010–2037

Patel YM, Lordkipanidzé M, Lowe GC, Nisar SP, Garner K, Stockley J, Daly ME, Mitchell M, Watson SP, Austin SK, Mundell SJ (2014) A novel mutation in the P2Y12 receptor and a function-reducing polymorphism in protease-activated receptor 1 in a patient with chronic bleeding. J Thromb Haemost 12:716–725

Pedata F, Dettori I, Coppi E, Melani A, Fusco I, Corradetti R, Pugliese AM (2016) Purinergic signalling in brain ischemia. Neuropharmacology 104:105–130

Pitchford SC, Momi S, Baglioni S, Casali L, Giannini S, Rossi R, Page CP, Gresele P (2008) Allergen induces the migration of platelets to lung tissue in allergic asthma. Am J Respir Crit Care Med 177:604–612

Qin C, Zhou J, Gao Y, Lai W, Yang C, Cai Y, Chen S, Du C (2017) Critical role of P2Y12 receptor in regulation of Th17 differentiation and experimental autoimmune encephalomyelitis pathogenesis. J Immunol 199:72–81

Quintas C, Fraga S, Gonçalves J, Queiroz G (2009) The P2Y1 and P2Y12 receptors mediate autoinhibition of transmitter release in sympathetic innervated tissues. Neurochem Int 55:505–513

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

Ralevic V, Burnstock G (2003) Involvement of purinergic signaling in cardiovascular diseases. Drug News Perspect 16:133–140

Rauch BH, Rosenkranz AC, Ermler S, Böhm A, Driessen J, Fischer JW, Sugidachi A, Jakubowski JA, Schrör K (2010) Regulation of functionally active P2Y12 ADP receptors by thrombin in human smooth muscle cells and the presence of P2Y12 in carotid artery lesions. Arterioscler Thromb Vasc Biol 30:2434–2442

Remijn JA, IJsseldijk MJ, Strunk AL, Abbes AP, Engel H, Dikkeschei B, Dompeling EC, de Groot PG, Slingerland RJ (2007) Novel molecular defect in the platelet ADP receptor P2Y12 of a patient with haemorrhagic diathesis. Clin Chem Lab Med 45:187–189

Sasaki Y, Hoshi M, Akazawa C, Nakamura Y, Tsuzuki H, Inoue K, Kohsaka S (2003) Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. Glia 44:242–250

Savi P, Zachayus JL, Delesque-Touchard N, Labouret C, Hervé C, Uzabiaga MF, Pereillo JM, Culouscou JM, Bono F, Ferrara P, Herbert JM (2006) The active metabolite of clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci U S A 103:11069–11074

Scavone M, Femia EA, Cattaneo M (2017) P2Y12 Receptor gene mutations associated with bleeding. Platelets 28:421–423

Schmidt P, Ritscher L, Dong EN, Hermsdorf T, Cöster M, Wittkopf D, Meiler J, Schöneberg T (2013) Identification of determinants required for agonistic and inverse agonistic ligand properties at the ADP receptor P2Y12. Mol Pharmacol 83:256–266

Shanker G, Kontos JL, Eckman DM, Wesley-Farrington-D, Sane DC (2006) Nicotine upregulates the expression of P2Y12 on vascular cells and megakaryoblasts. J Thromb Thrombolysis 22:213–220

Siasos G, Kioufis S, Oikonomou E, Zaromitidou M, Maniatis K, Vavuranakis M, Kokkou E, Tousoulis D (2016) Impact of C34T P2Y12 ADP receptor polymorphism and smoking status on cardiovascular outcome in coronary artery disease patients receiving clopidogrel. Int J Cardiol 210:161–163

Simon J, Vigne P, Eklund KM, Michel AD, Carruthers AM, Humphrey PP, Frelin C, Barnard EA (2001) Activity of adenosine diphosphates and triphosphates

on a P2Y(T)-type receptor in brain capillary endothelial cells. Br J Pharmacol 132:173–182

Sipe GO, Lowery RL, Tremblay MÈ, Kelly EA, Lamantia CE, Majewska AK (2016) Microglial P2Y12 is necessary for synaptic plasticity in mouse visual cortex. Nat Commun 7:10905

Sirotkina OV, Laskovets AB, Andoskin PA, Emelyanov AK, Zabotina AM, Vavilova TV (2016) Increase in GP IIb-IIIa and P2Y12 receptors in activated platelets as the possible indicator of de novo protein synthesis. Mol Biol 50:111–117

Smith TH, Li JG, Dores MR, Trejo J (2017) Protease-activated receptor-4 and P2Y12 dimerize, co-internalize and activate Akt signaling via endosomal recruitment of β-arrestin. J Biol Chem 292:13867–13878. doi:10.1074

Södergren AL, Holm ACS, Ramström S, Lindström EG, Grenegård M, Öllinger K (2016) Thrombin-induced lysosomal exocytosis in human platelets is dependent on secondary activation by ADP and regulated by endothelial-derived substances. Platelets 27:86–92

Springthorpe B, Bailey A, Barton P, Birkinshaw TN, Bonnert RV, Brown RC, Chapman D, Dixon J, Guile SD, Humphries RG, Hunt SF, Ince F, Ingall AH, Kirk IP, Leeson PD, Leff P, Lewis RJ, Martin BP, McGinnity DF, Mortimore MP, Paine SW, Pairaudeau G, Patel A, Rigby AJ, Riley RJ, Teobald BJ, Tomlinson W, Webborn PJ, Willis PA (2007) From ATP to AZD6140: the discovery of an orally active reversible P2Y12 receptor antagonist for the prevention of thrombosis. Bioorg Med Chem Lett 17:6013–6018

Stefanini L, Bergmeier W (2016) RAP1-GTPase signaling and platelet function. J Mol Med (Berl) 94:13–19

Stefanini L, Paul DS, Robledo RF, Chan ER, Getz TM, Campbell RA, Kechele DO, Casari C, Piatt R, Caron KM, Mackman N, Weyrich AS, Parrott MC, Boulaftali Y, Adams MD, Peters LL, Bergmeier W (2015) RASA3 Is a critical inhibitor of RAP1-dependent platelet activation. J Clin Invest 125:1419–1432

Su X, Floyd DH, Hughes A, Xiang J, Schneider JG, Uluckan O, Heller E, Deng H, Zou W, Craft CS, Wu K, Hirbe AC, Grabowska D, Eagleton MC, Townsley S, Collins L, Piwnica-Worms D, Steinberg TH, Novack DV, Conley PB, Hurchla MA, Rogers M, Weilbaecher KN (2012) The ADP receptor P2RY12 regulates osteoclast function and pathologic bone remodeling. J Clin Invest 122:3579–3592

Sugidachi A, Asai F, Ogawa T, Inoue T, Koike H (2000) The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties. Br J Pharmacol 129:1439–1446

Sugidachi A, Mizuno M, Ohno K, Jakubowski JA, Tomizawa A (2016) The active metabolite of prasugrel, R-138727, improves cerebral blood flow and reduces cerebral infarction and neurologic deficits in a non-human primate model of acute ischaemic stroke. Eur J Pharmacol 788:132–139

Suh DH, Trinh HK, Liu JN, Phamle D, Park SM, Park HS, Shin YS (2016) P2Y12 antagonist attenuates eosinophilic inflammation and airway hyperresponsiveness in a mouse model of asthma. J Cell Mol Med 20:333–341

Sunkaria A, Bhardwaj S, Halder A, Yadav A, Sandhir R (2016) Migration and phagocytic ability of activated microglia during post-natal development is mediated by calcium-dependent purinergic signalling. Mol Neurobiol 53:944–954

Swiatkowski P, Murugan M, Eyo UB, Wang Y, Rangaraju S, SB O, LJ W (2016) Activation of microglial P2Y12 receptor is required for outward potassium currents in response to neuronal injury. Neuroscience 318:22–33

Syberg S, Brandao-Burch A, Patel JJ, Hajjawi M, Arnett TR, Schwarz P, Jorgensen NR, Orriss IR (2012) Clopidogrel (Plavix), a P2Y12 receptor antagonist, inhibits bone cell function in vitro and decreases trabecular bone in vivo. J Bone Miner Res 27:2373–2386

Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, Soga T, Matsushime H, Furuichi K (2001) Molecular cloning of the platelet P2TAC ADP receptor: pharmacological comparison with another ADP receptor, the P2Y1 receptor. Mol Pharmacol 60:432–439

Tamagawa T, Shinoda M, Honda K, Furukawa A, Kaji K, Nagashima H, Akasaka R, Chen J, Sessle BJ, Yonehara Y, Iwata K (2016) Involvement of microglial P2Y12 signaling in tongue cancer pain. J Dent Res 95:1176–1182

Tatsumi E, Yamanaka H, Kobayashi K, Yagi H, Sakagami M, Noguchi K (2015) RhoA/ROCK pathway mediates p38 MAPK activation and morphological changes downstream of P2Y12/13 receptors in spinal microglia in neuropathic pain. Glia 63:216–228

Thomas MR, Storey RF (2015) Effect of P2Y12 inhibitors on inflammation and immunity. Thromb Haemost 114:490–497

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. J Neurosci 28:4949–4956

Tozaki-Saitoh H, Miyata H, Yamashita T, Matsushita K, Tsuda M, Inoue K (2017) P2Y12 receptors in primary microglia activate nuclear factor of activated T cell signaling to induce C-C chemokine 3 expression. J Neurochem 141:100–110

Tsuda M, Inoue K (2016) Neuron-microglia interaction by purinergic signaling in neuropathic pain following neurodegeneration. Neuropharmacology 104:76–81

Uehara K, Uehara A (2011) P2Y1, P2Y6, and P2Y12 receptors in rat splenic sinus endothelial cells: an immunohistochemical and ultrastructural study. Histochem Cell Biol 136:557–567

van Giezen JJ, Nilsson L, Berntsson P, Wissing BM, Giordanetto F, Tomlinson W, Greasley PJ (2009) Ticagrelor binds to human P2Y12 independently

from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. J Thromb Haemost 7:1556–1565

von Kügelgen I (2006) Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacol Ther 110:415–432

von Kügelgen I, Harden TK (2011) Molecular pharmacology, physiology, and structure of the P2Y receptors. Adv Pharmacol 61:373–415

von Kügelgen I, Hoffmann K (2016) Pharmacology and structure of P2Y receptors. Neuropharmacology 104:50–61

von Kügelgen I, Wetter A (2000) Molecular pharmacology of P2Y-receptors. Naunyn-Schmiedebergs. Arch Pharmacol 362:310–323

Wang L, Östberg O, Wihlborg AK, Brogren H, Jern S, Erlinge D (2003) Quantification of ADP and ATP receptor expression in human platelets. J Thromb Haemost 1:330–336

Wang L, Jacobsen SE, Bengtsson A, Erlinge D (2004) P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. BMC Immunol 5:16

Webster CM, Hokari M, McManus A, Tang XN, Ma H, Kacimi R, Yenari MA (2013) Microglial P2Y12 deficiency/inhibition protects against brain ischemia. PLoS One 8:e70927

West LE, Steiner T, Judge HM, Francis SE, Storey RF (2014) Vessel wall, not platelet, P2Y12 potentiates early atherogenesis. Cardiovasc Res 102:429–435

Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T, Erlinge D (2004) ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. Arterioscler Thromb Vasc Biol 24:1810–1815

Yanachkov IB, Chang H, Yanachkova MI, Dix EJ, Berny-Lang MA, Gremmel T, Michelson AD, Wright GE, Frelinger AL (2016) New highly active antiplatelet agents with dual specificity for platelet P2Y1 and P2Y12 adenosine diphosphate receptors. Eur J Med Chem 107:204–218

Yang HH, Chen Y, Gao CY (2016) Associations of P2Y12R gene polymorphisms with susceptibility to coronary heart disease and clinical efficacy of antiplatelet treatment with clopidogrel. Cardiovasc Ther 34:460–467

Yi X, Wang Y, Lin J, Cheng W, Zhou Q, Wang C (2016) Interaction of CYP2C19, P2Y12, and GPIIIa variants associates with efficacy of clopidogrel and adverse events on patients with ischemic stroke. Clin Appl Thromb Hemost 1076029616648408

Yi X, Zhou Q, Wang C, Lin J, Liu P, Fu C (2017) Platelet receptor gene (P2Y12, P2Y1) and platelet glycoprotein gene (GPIIIa) polymorphisms are associated with antiplatelet drug responsiveness and clinical outcomes after acute minor ischemic stroke. Eur J Clin Pharmacol 73:437–443

Zech G, Hessler G, Evers A, Weiss T, Florian P, Just M, Czech J, Czechtizky W, Görlitzer J, Ruf S, Kohlmann M, Nazare M (2012) Identification of high-affinity $P2Y_{12}$ antagonists based on a phenylpyrazole glutamic acid piperazine backbone. J Med Chem 55:8615

Zetterberg F, Svensson P (2016) State of affairs: design and structure–activity relationships of reversible P2Y12 receptor antagonists. Bioorg Med Chem Lett 26:2739–2754

Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, Palmer K, Bayne M, Monsma FJ (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J Biol Chem 276:8608–8615

Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, Moss SM, Paoletta S, Kiselev E, Lu W, Fenalti G, Zhang W, Müller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014a) Structure of the human P2Y12 receptor in complex with an antithrombotic drug. Nature 509:115–118

Zhang J, Zhang K, Gao ZG, Paoletta S, Zhang D, Han GW, Li T, Ma L, Zhang W, Müller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014b) Agonist-bound structure of the human P2Y12 receptor. Nature 509:119–122

Zhang Y, Peti-Peterdi J, Müller CE, Carlson NG, Baqi Y, Strasburg DL, Heiney KM, Villanueva K, Kohan DE, Kishore BK (2015) P2Y12 receptor localizes in the renal collecting duct and its blockade augments arginine vasopressin action and alleviates nephrogenic diabetes insipidus. J Am Soc Nephrol 26:2978–2987

Zhang Z, Wu G, Wang C, Jin X, Li D, Lin K (2016) Features of reversible P2Y12 receptor antagonists based on piperazinyl-glutamate-pyridines. Med Chem Res 25:1204–1215

Zhang J, Li Z, Hu X, Su Q, He C, Liu J, Ren H, Qian M, Liu J, Cui S, Jiang W (2017) Knockout of P2Y12 aggravates experimental autoimmune encephalomyelitis in mice via increasing of IL-23 production and Th17 cell differentiation by dendritic cells. Brain Behav Immunol 62:245–255

Zhou S, Fang D, Tan S, Lin W, Wu W, Zheng K (2016) Investigating the binding mechanism of novel 6-aminonicotinate-based antagonists with P2Y12 by 3D-QSAR, docking and molecular dynamics simulations. J Biomol Struct Dyn 30:1–28

Adv Exp Med Biol - Protein Reviews (2017) 19:139–168
DOI 10.1007/5584_2017_91

Published online: 17 August 2017

An Update on $P2Y_{13}$ Receptor Signalling and Function

Raquel Pérez-Sen, Rosa Gómez-Villafuertes, Felipe Ortega, Javier Gualix, Esmerilda G. Delicado, and María Teresa Miras-Portugal

Abstract

The distribution of nucleotide P2Y receptors across different tissues suggests that they fulfil key roles in a number of physiological and pathological conditions. $P2Y_{13}$ is one of the latest P2Y receptors identified, a novel member of the Gi-coupled P2Y receptor subfamily that responds to ADP, together with $P2Y_{12}$ and $P2Y_{14}$. Pharmacological studies drew attention to this new ADP receptor, with a pharmacology that overlaps that of $P2Y_{12}$ receptors but with unique features and roles. The *P2RY12–14* genes all reside on human chromosome 3 at 3q25.1 and their strong sequence homology supports their evolutionary origin through gene duplication. Polymorphisms of $P2Y_{13}$ receptors have been reported in different human populations, yet their consequences remain unknown. The $P2Y_{13}$ receptor is versatile in its signalling, extending beyond the canonical signalling of a Gi-coupled receptor. Not only can it couple to different G proteins (Gs/Gq) but the $P2Y_{13}$ receptor can also trigger several intracellular pathways related to the activation of MAPKs (mitogen-activated protein kinases) and the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 axis. Moreover, the availability of $P2Y_{13}$ receptor knockout mice has highlighted the specific functions in which it is involved, mainly in the regulation of cholesterol and glucose

Raquel Pérez-Sen and Rosa Gómez-Villafuertes contributed equally to this work.

R. Pérez-Sen, R. Gómez-Villafuertes, F. Ortega, J. Gualix, E.G. Delicado (✉), and M.T. Miras-Portugal (✉)
Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Instituto Universitario de Investigación en Neuroquímica, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos, Universidad Complutense Madrid, 28040 Madrid, Spain
e-mail: esmerild@ucm.es; mtmiras@ucm.es

metabolism, bone homeostasis and aspects of central nervous system function like pain transmission and neuroprotection. This review summarizes our current understanding of this elusive receptor, not only at the pharmacological and molecular level but also, in terms of its signalling properties and specific functions, helping to clarify the involvement of $P2Y_{13}$ receptors in pathological situations.

Keywords
Cholesterol metabolism • GSK3 • MAP kinases • Nervous system • Neuroprotection • $P2Y_{13}$ receptor • Pain

Abbreviations

ADP	Adenosine 5′-diphosphate.
Ap_3A	P1,P3-Di(adenosine-5′) triphosphate.
Ap_4A	P1,P4-Di(adenosine-5′) triphosphate.
ATP	Adenosine 5′-triphosphate
cAMP	Adenosine 3′,5′-cyclic monophosphate.
CT1007900	(6-[1-(2-Dimethylaminopyrimidin-5-ylmethyl)-piperidin-4-yl]-2-morpholin-4-yl-pyrimidin-4-ol monohydrate).
GPCRs	G protein–coupled receptors.
GSK3	Glycogen synthase kinase 3.
HDL	High density lipoprotein.
2MeSADP	2-methylthio-adenosine 5′-diphosphate.
MAP kinases	Mitogen-activated protein kinases.
MRS2211	2-[(2-Chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde.
MRS2179	2′-Deoxy-N6-methyl adenosine 3′,5′-diphosphate.
PLC	Phospholipase C.
PI3K	Phosphatidylinositol 3-kinase.
PPADS	Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid).
RCT	Reverse cholesterol transport
SNP	Single-nucleotide polymorphism.

1 Introduction

$P2Y_{13}$ is a G-protein-coupled receptor (GPCR) that is included along with $P2Y_{12}$ and $P2Y_{14}$ in the P2Y subgroup of receptors, of which the main physiological agonist is ADP. GPCRs are a very large family of membrane proteins that account for approximately 2% of all the genes in the human genome. These receptors control a wide range of key physiological functions and they are also the pharmacological target to treat a large number of prevalent human diseases. P2Y receptors belong to the rhodopsin family, also known as Class A GPCRs, and based on sequence homology they have been included in the δ subfamily of Class A GPCRs, which also contains glycoprotein receptors, protease-activated receptors and olfactory receptors. Based on their pharmacology, signal transduction and structure, P2Y receptors are classified into two main subfamilies. The first subgroup are coupled to phospholipase C (PLC) via Gq proteins and it includes the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors. Conversely, the "$P2Y_{12}$-like" subfamily that contains the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors preferentially signal through Gi proteins, and they mainly inhibit adenylate cyclase activity or they regulate ion channel activity (von Kugelgen and Hoffmann

2016). Currently, the only crystallographic structure available for these "$P2Y_{12}$-like" receptors is that of $P2Y_{12}$ (Zhang et al. 2014a; b), confirming to a large extent its inclusion in the δ subfamily receptors, although some of their specific features do not exactly match those of the δ or α subfamilies of Class A GPCRs. Whether the structural characteristics of $P2Y_{12}$ are shared by all P2Y family members, or if they are specific to the $P2Y_{12}$-like ADP-Gi coupled receptors, will require studies of the structure of these other receptors. Notably, the $P2Y_{13}$ receptor is closely related to $P2Y_{12}$.

Historically the discovery of ADP receptors is closely associated with platelet function. There is an abundance of adenine nucleotides inside the dense granules of platelets, mainly ADP together with serotonin, and the release of the content of these granules induces platelet aggregation and clot formation. In fact, ADP was the first known aggregating agent and hence, a search began for specific ADP receptors in platelets. $P2Y_1$ was the first P2Y receptor family described in a platelet model and it was shown to be activated by ADP, changing the commonly accepted idea that ATP was its main agonist. This receptor was isolated by hybridization screening of a cDNA library generated from the embryonic chick brain (Webb et al. 1993). $P2Y_1$ is coupled to the Gq protein, activating the PLC pathway and mobilizing internal calcium stores in most cellular models, including platelets (Hechler et al. 1998; Savi et al. 1998). Platelets challenged with ADP also produce Gi-mediated inhibition of adenylyl cyclase, which lowers the cAMP available (Gachet et al. 1997). However, the specific ADP-receptor subtype coupled to this inhibition of adenylyl cyclase remained elusive until it was associated with clinical bleeding disorders, and to the absence of a response to the ADP-selective anti-aggregating drugs ticlopidine and clopidogrel (Cattaneo and Gachet 1999). Thus, the association of familial bleeding disorders with the anti-aggregant pharmacology was the key to hunt this ADP receptor.

The identification of a platelet ADP receptor targeted by antithrombotic drugs was preceded by the isolation of a functional P2 receptor that responded equally to ATP and UTP, leading to it being named P2U, currently $P2Y_2$ (Alexander et al. 2015; Lustig et al. 1993). $P2Y_{12}$ was the first nucleotide receptor to be associated with a genetic defect, a familial bleeding disorder (Hollopeter et al. 2001), and its identification facilitated the development of powerful antiplatelet aggregating agents that are among the most effective drugs in preventing cardiovascular diseases and ictus. Subsequently, the orphan GPCR SP1999 was shown to be the cognate receptor for ADP (Zhang et al. 2001), also corresponding in sequence to that described by Hollopeter and co-workers (2001). This receptor was linked to Gαi and it is expressed strongly in neural tissues and blood platelets. Since then the search for other homologous P2Y receptors became more intense.

Following a similar strategy as for $P2Y_{12}$, the orphan GPCRs GPR86 and SP174 were identified as ADP receptors and named $P2Y_{13}$ (Communi et al. 2001; Zhang et al. 2002). This new receptor couples to Gi proteins and it shares a high degree of sequence homology to $P2Y_{12}$, as well as a similar rank order of potency for ADP and analogues. The comparison of human and mouse $P2Y_{13}$ receptors demonstrated approximately 75% sequence homology and a similar pharmacological profile, although ADP and nucleotide analogues appear to act more potently on the murine receptor (Zhang et al. 2002). More extensive pharmacological characterization of the human $P2Y_{13}$ receptor has also been carried out, allowing further functional studies to discriminate the distinct P2Y-Gi-coupled receptors (Marteau et al. 2003).

The clinical and pharmacological relevance of $P2Y_{12}$ has somehow cast a shadow on the important physiological role of $P2Y_{13}$. Thus, in this review, we will try to provide an overview of this less well known family member, bringing together the data available regarding different aspects of this multi-faceted receptor. The review is organized in sections to make its content more comprehensible and accessible. The pharmacology of $P2Y_{13}$ with respect to other P2Y receptors represents a good starting point, which is followed by a comparative study of the sequence of $P2Y_{13}$ and its known single nucleotide polymorphisms (SNPs). The use of genetically

modified animals and a better understanding of the mechanisms that control its expression will be particularly useful to assign specific cellular responses to $P2Y_{13}$. This is particularly relevant when attempting to understand the signals generated by this receptor given the number of complex pathways it can activate. This signalling is clearly related to the wide range of essential functions that $P2Y_{13}$ performs in the control of relevant physiological functions, such as protection from oxidative and genotoxic stress, lipoprotein mobilization in cholesterol metabolism or pain, to mention just a few. All these examples indicate that $P2Y_{13}$ is a key receptor in the purinergic field, and that better understanding its physiology will provide us with useful tools to cope with pathophysiological situations that could be relevant to human disease.

2 Pharmacology of the $P2Y_{13}$ Receptor

Regarding the pharmacological profile of $P2Y_{13}$ receptors, they share a characteristic preference for ADP. ADP is the most potent agonist of the naturally occurring nucleotides that act on the $P2Y_{13}$ receptor, stimulating the receptor at an EC_{50} in the nanomolar range (Communi et al. 2001; Zhang et al. 2002; Marteau et al. 2003) a pharmacological feature shared with the $P2Y_{12}$ receptor (Hollopeter et al. 2001; Zhang et al. 2001). Another P2Y receptor that prefers ADP and that is potently activated by this nucleoside diphosphate is $P2Y_1$ (Leon et al. 1997). The $P2Y_{13}$ receptor also responds to adenine diphosphate analogues adenosine such as 2-methylthio-ADPβS (2MeSADP) or 5′-*O*-(2-thiodiphosphate) (Fig. 1) (Communi et al. 2001; Zhang et al. 2002; Marteau et al. 2003). In some cellular systems, 2MeSADP proved to be more potent than ADP, whereas under other experimental conditions both compounds were equipotent, possibly reflecting the distinct affinity for 2MeSADP or ADP of multiple active conformations of the $P2Y_{13}$ receptor, as well as differences in their preference for G proteins (Marteau et al. 2003). ATP and 2-methylthio-ATP appear to be partial and weak agonists of the $P2Y_{13}$ receptor (Marteau et al. 2003). Although the interaction of the $P2Y_{12}$ and $P2Y_{13}$ receptors with nucleotide analogues follows a similar pharmacological profile, inosine diphosphate is about fivefold more potent for human $P2Y_{13}$ than for $P2Y_{12}$ receptors. Moreover, inosine diphosphate acts more potently on murine $P2Y_{13}$ than human $P2Y_{13}$ and $P2Y_{12}$ receptors, with an EC_{50} of 9.2, 552 and 3180 nM, respectively (Zhang et al. 2002).

In addition to conventional mononucleotide agonists, dinucleotides have also been tested for their ability to stimulate the $P2Y_{13}$ receptor. Diadenosine triphosphate (Ap_3A) potently activates the $P2Y_{13}$ receptor, whereas higher diadenosine polyphosphate homologues (Ap_4A, Ap_5A and Ap_6A) are inactive (Zhang et al. 2002; Marteau et al. 2003). A similar profile has been observed with the $P2Y_1$ receptor (Patel et al. 2001), suggesting that selective sensitivity to Ap_3A is a common feature of ADP receptors. It has been hypothesized that dinucleoside triphosphates can structurally mimic nucleoside diphosphates (Shaver et al. 2005). Indeed, Ap_3A can be stored in secretory vesicles in neural and neuroendocrine tissues through the activity of a broad specificity vesicular nucleotide transporter capable of internalizing a wide variety of nucleotides, as well as the diadenosine polyphosphates (Gualix et al. 1997). Moreover, Ap_3A has been identified in microdialysis samples from the cerebellum of conscious, freely moving rats under basal conditions (i.e.: in the absence of any exogenously added stimuli). The extracellular concentration of Ap_3A in cerebellar interstitial fluid (10.5 nM) is double that of the other diadenosine polyphosphates detected (Ap_4A and Ap_5A) and it is in a range that allows this dinucleotide to interact with the $P2Y_{13}$ receptor (Gualix et al. 2014).

Regarding antagonists, the human $P2Y_{13}$ receptor is blocked by suramin, reactive blue-2 and high concentrations of the selective purinergic P2X antagonist, PPADS (Marteau et al. 2003). In recent years, PPADS analogues have been designed in an effort to identify more potent and/or selective $P2Y_{13}$ receptor antagonists. Among them, the 2-chloro-5-nitro

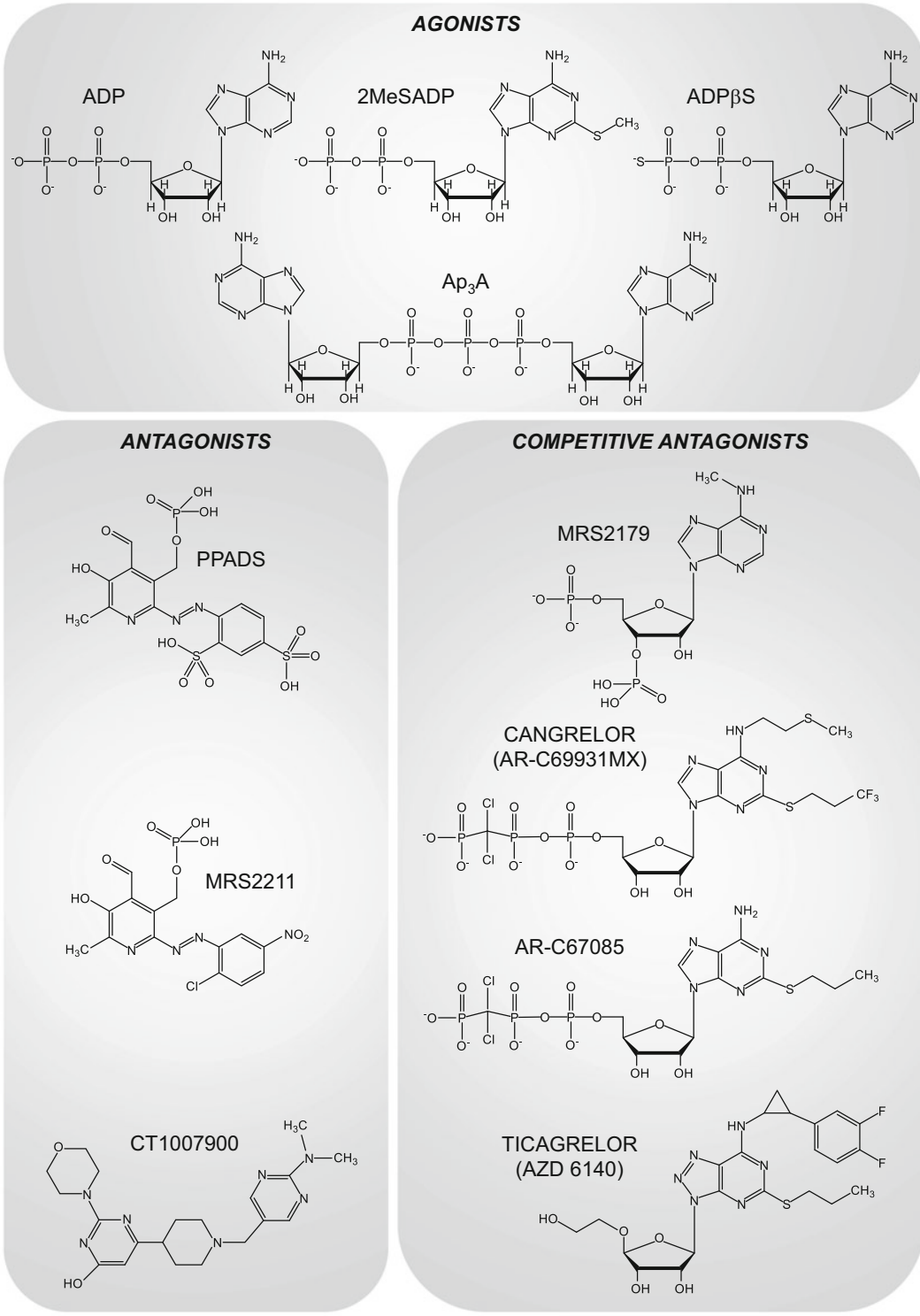

Fig. 1 Structures of agonists and antagonists of ADP receptors

analogue MRS2211 (2-[(2-Chloro-5-nitrophenyl) azo]-5-hydroxy-6-methyl-3-[(phosphonooxy) met hyl]-4-pyridinecarboxaldehyde) proved to be 45-fold more potent than PPADS as a competitive antagonist of the human $P2Y_{13}$ receptor, with a pA_2-value of 6.3 (Kim et al. 2005). Moreover, MRS2211 is >20-fold more selective as an antagonist at the $P2Y_{13}$ receptor than of the $P2Y_1$ and $P2Y_{12}$ receptors, cangrelor (Kim et al. 2005). The $P2Y_{12}$ antagonists and AR-C67085 also block the human $P2Y_{13}$ receptors, and cangrelor apparently acts 100 times more potently than AR-C67085. By contrast to its competitive interaction with the $P2Y_{12}$ receptor, cangrelor depressed the maxima of the agonist dose-response curves in studies on the recombinant human $P2Y_{13}$ receptor, compatible with a non-competitive interaction (Marteau et al. 2003). The rat $P2Y_{13}$ receptor is also blocked by nanomolar concentrations of cangrelor but not by the selective $P2Y_1$ antagonist MRS2179 (2′-Deoxy-N6-methyl adenosine 3′,5-′-diphosphate), even when used at a concentration as high as 100 μM (Fumagalli et al. 2004). However, cangrelor is a partial agonist of the mouse $P2Y_{13}$ receptor, enhancing $P2Y_{13}$-mediated high density lipoprotein (HDL) endocytosis by hepatocytes more potently than its endogenous agonist, ADP (Jacquet et al. 2005). Ticagrelor is an antagonist of the $P2Y_{12}$ receptor approved for the prevention of thromboembolic events in patients with acute coronary syndrome. Ticagrelor and its active metabolite, TAM, act as $P2Y_{13}$ antagonists in a transfected cell system *in vitro*, although they had no impact on $P2Y_{13}$-regulated pro-platelet formation by human megakaryocytes in culture (Bjorquist et al. 2016). Ap_4A has also been described as a complete antagonist of the human $P2Y_{13}$ receptor, with an IC_{50} of 216 nM (Marteau et al. 2003).

Given the important roles that $P2Y_{13}$ and $P2Y_{12}$ receptors play, it will be crucial to obtain selective ligands that can discriminate between them, capable of distinguishing the influence of $P2Y_{13}$ receptors on lipid transport and metabolism, and on bone formation, as deduced from studies on knockout animals (see Sect. 4). Due to the similarities between the $P2Y_{12}$ and $P2Y_{13}$ receptors in terms of their activation by nucleotide analogues, possibly reflecting a similarity in their agonist binding sites, allosteric effectors would be one possible approach to develop selective modulators of the $P2Y_{13}$ receptor, as has already been achieved for other GPCRs (May et al. 2007; Melancon et al. 2012).

3 Sequence Analysis of the $P2Y_{13}$ Receptor

Considering that the $P2Y_{12}$ and $P2Y_{13}$ receptors exhibit similar pharmacological features, we looked into the molecular structure of this receptor subfamily. Alignments of amino acid sequences of human $P2Y_{13}$ with either $P2Y_{12}$ or $P2Y_{14}$ receptors reveal approximately 40–45% identity (Fig. 2, Table 1). Moreover, the *P2RY12*, *P2RY13* and *P2RY14* genes reside on human chromosome 3 at 3q25.1, which would be consistent with gene duplication having led to their evolutionary origin. Conversely, Gq-coupled P2Y receptors share less than 20% identity with $P2Y_{13}$ receptors, even the $P2Y_1$ receptor that has a similar pharmacological profile and chromosomal location (the *P2RY1* gene is situated at human chromosome 3q25.2, in close proximity to the *P2RY12*, *P2RY13* and *P2RY14* genes). Notably, the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors cluster with *GPR87* in the same region of human chromosome 3, an orphan GPCR that shares 38%, 36% and 44% amino acid identity with the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors, respectively (Fig. 3). Although the protein encoded by the *GPR87* gene is still to be identified, it would not be a surprise if it were a new P2Y-like receptor, as occurred with GPR17. From a phylogenetic point of view, GPR17 lies in an apparently intermediate position between P2Y and cysteinyl leukotriene receptors, as GPR17 can bind both uracil-nucleotide sugars (UDP, UDP-galactose and UDP-glucose) and cysteinyl leukotrienes (LTD4, LTC4 and LTE4) (Marucci et al. 2016). Interestingly, inhibition of GPR17 by

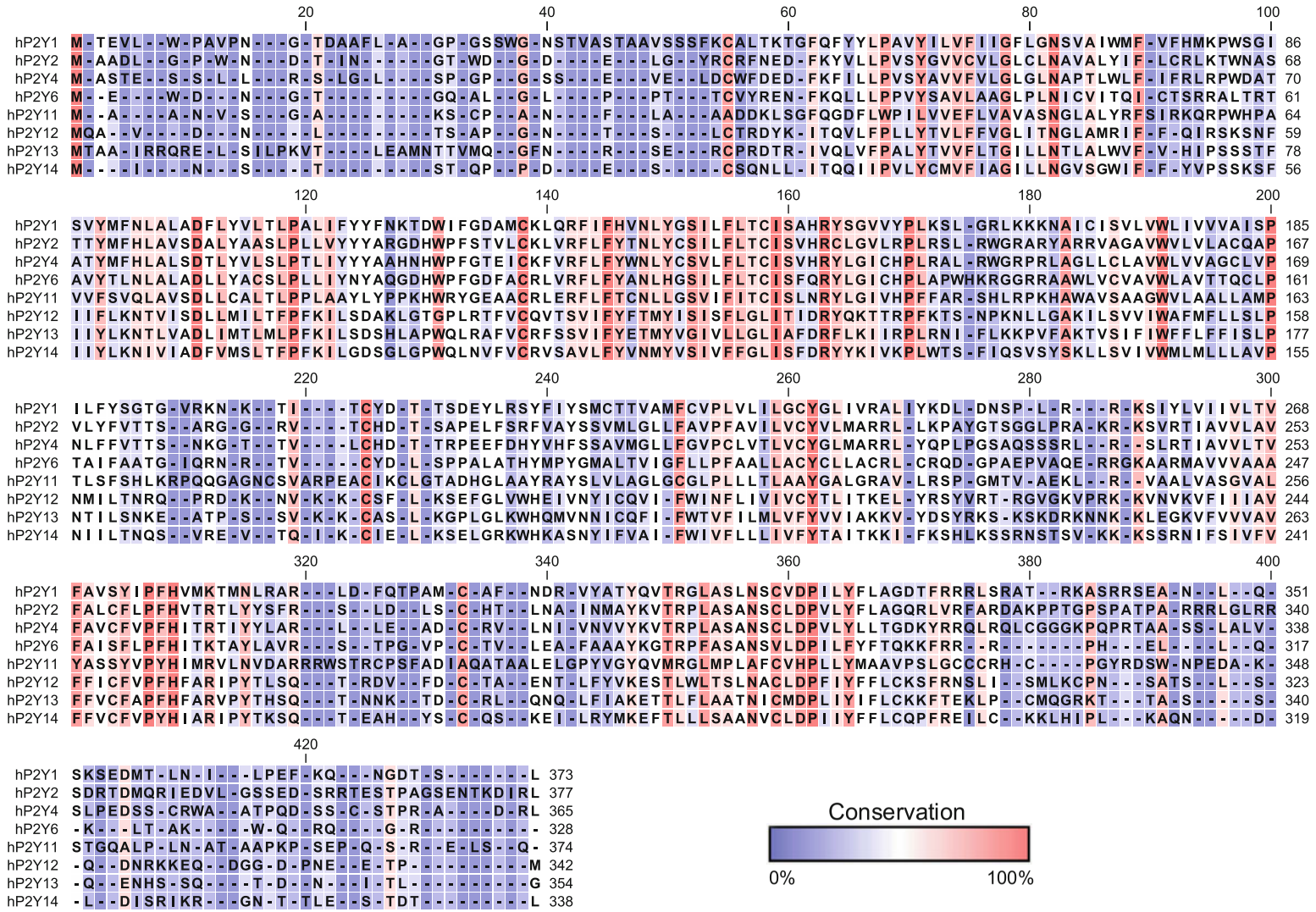

Fig. 2 Comparative alignment of the human P2Y receptor amino acid sequences

Table 1 Similarity (*above the diagonal*) and Jukes-Cantor distance (*below the diagonal*) in globally aligned human P2Y receptor amino acid sequences

	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
$P2Y_1$		33.25	37.21	32.19	25.62	19.39	19.00	20.31
$P2Y_2$	1.15		53.03	35.17	24.13	21.30	19.95	22.51
$P2Y_4$	1.03	0.65		3693	27.20	22.28	22.74	22.99
$P2Y_6$	1.19	1.09	1.04		23.48	22.03	19.73	20.86
$P2Y_{11}$	1.45	1.52	1.38	1.56		16.28	14.60	15.68
$P2Y_{12}$	1.79	1.67	1.62	1.63	2.02		45.43	45.06
$P2Y_{13}$	1.82	1.76	1.59	1.77	2.18	0.81		40.72
$P2Y_{14}$	1.73	1.61	1.58	1.70	2.08	0.82	0.93	

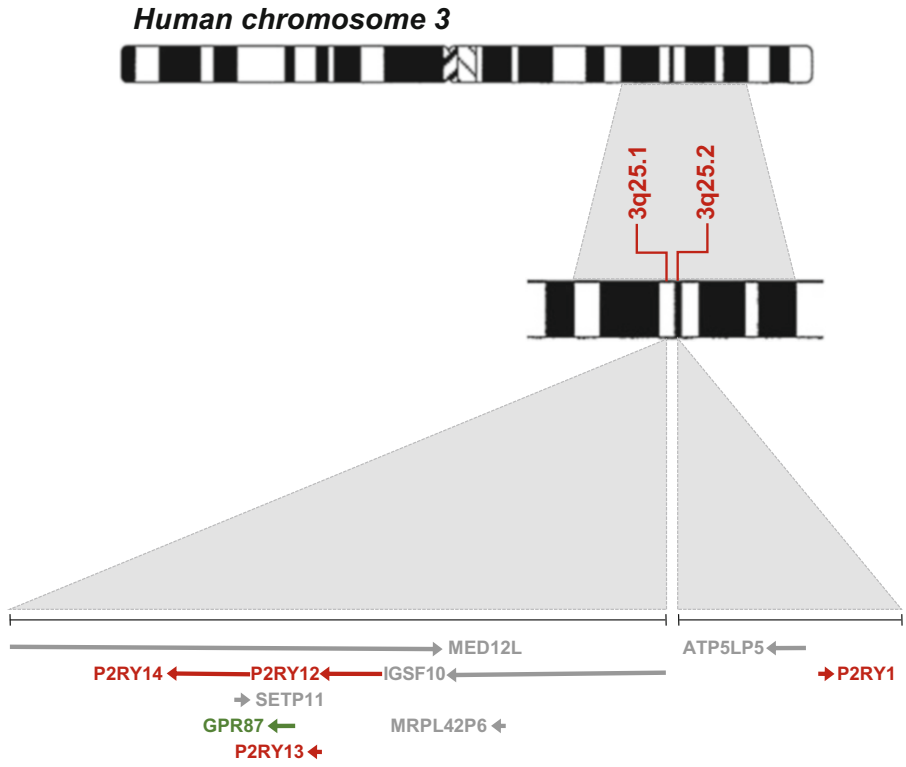

Fig. 3 Chromosomal location of the P2RY genes on human chromosome 3. *P2RY12*, *P2RY13* and *P2RY14* genes cluster on the long arm (band 25.1) of chromosome 3, together with the orphan GPCR *GPR87*. The *P2RY1* gene is also located nearby (band 25.2) (Modified from (Milewicz and Seidman 2000))

montelukast, a well-known anti-asthmatic drug that antagonizes CysLT1R, reduces neuroinflammation, it elevates hippocampal neurogenesis and it improves learning and memory in aged rats (Marschallinger et al. 2015).

Regarding interspecific variation, the alignment of $P2Y_{13}$ receptors from 13 different species reveals that both their nucleotide and amino acid sequences are highly conserved, especially in mammals (Fig. 4). Humans, great apes (chimpanzee *Pan troglodytes*, orangutan *Pongo pygmaeus*, and gorilla *Gorilla gorilla*), and old world monkeys (rhesus macaque *Macaca mulatta*) show more than 95% identity at both the nucleotide and amino acid level. Human and new world monkeys (Ma's night monkey *Aotus*

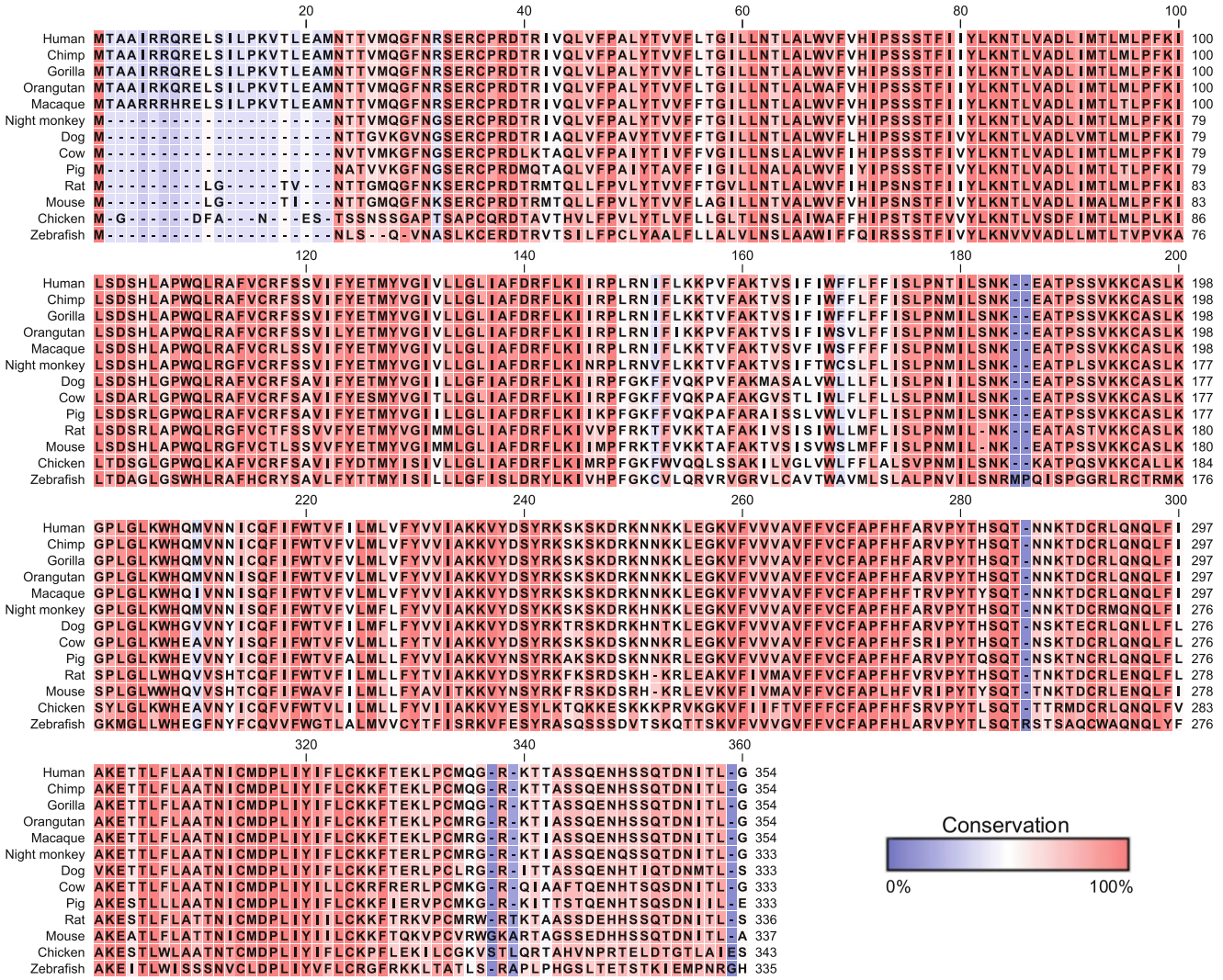

Fig. 4 Alignment of the P2Y$_{13}$ receptors from 13 different species, including mammals, birds and fish

nancymaae), rodents (rat *Rattus norvegicus* and mouse *Mus musculus*) and other mammals with diverse diets (carnivorous dog *Canis familiaris*, herbivorous cow *Bos taurus* and omnivorous pig *Sus scrofa*) show approximately 75–85% sequence identity. Finally, humans and birds (chicken *Gallus gallus*) or fish (zebrafish *Danio rerio*) share less than 60% and 45% identity, respectively (Table 2).

As mentioned previously, the first crystallographic assessment of a "$P2Y_{12}$-like" subfamily member of purinergic receptors was that of human $P2Y_{12}$ receptor, both the agonist- and antagonist-bound structures (Zhang et al. 2014a; b). Key residues involved in both 2MeSADP and AZD1283 binding were Y^{105}, F^{106}, L^{155}, N^{159}, N^{191}, R^{256}, Y^{259} and K^{280}. Other residues specifically participate in 2MeSADP binding, including R^{19}, R^{93}, C^{97}, S^{156}, T^{163}, C^{175}, K^{179}, H^{187} and Q^{263}, whereas V^{102}, Y^{109}, V^{190}, Q^{195}, F^{252} and L^{276} contribute to the interaction with AZD1283. In addition to the AZD1283 binding site (pocket 1), an analysis of the extracellular interface revealed an adjacent ligand-binding region (pocket 2). Both pockets seem to be required for Ap_4A recognition and binding to the receptor. One nucleotide may bind in pocket 1 while the second half of the dinucleotide molecule was predicted to reach pocket 2, the polyphosphate moiety occupying a highly cationic region of the binding site (Zhang et al. 2014a, b). Crystal structure data can be very helpful in optimizing receptor models for structure-guided drug design (Jacobson et al. 2015). Thus, a model of ticagrelor binding to the human $P2Y_{12}$ receptor and homology models of the human $P2Y_{14}$ receptor for ligand docking have recently been described (Kiselev et al. 2014; Paoletta et al. 2015). A similar strategy could be used to study the $P2Y_{13}$ receptor, since neither crystal structure nor homology models of this receptor are as yet available. Remarkably, alignment of the human $P2Y_{12}$ and $P2Y_{13}$ amino acid sequences reveals that most of the key residue positions implicated in agonist/antagonist binding to the $P2Y_{12}$ receptor are conserved in the $P2Y_{13}$ receptor, with the exception of $F^{106}E$, $L^{155}I$ and $T^{163}S$. It is noteworthy that the relative distances between all the residues are also conserved (Fig. 5, Table 3). It is not inconceivable that the change of these 3 amino acids is involved in the pharmacological differences observed between $P2Y_{12}$ and $P2Y_{13}$ receptors.

4 Single Nucleotide Polymorphisms (SNPs) of the Human *P2RY13* Gene

Many human diseases have a causative association with genetic components and recent advances have significantly improved our understanding of the causal effects of genetic changes associated with a growing number of diseases. There is currently a great deal of information regarding the SNPs in genes associated with disease, including those affecting the genes encoding human purinergic receptors. SNPs are widespread in genes encoding ionotropic nucleotide receptors, particularly the *P2RX7* gene (Caseley et al. 2014), variants of which are associated with altered chronic pain sensitivity, cardiovascular risk, susceptibility to affective mood disorders, multiple sclerosis, childhood febrile seizure, osteoporosis, tuberculosis, toxoplasmosis and sepsis. Furthermore, SNPs in the *P2RX2* gene are associated with susceptibility to hearing loss, and a *P2XR4* gene variant is related to high pulse pressure and age-related macular degeneration.

Concerning P2Y receptors, non-synonymous SNPs in the *P2RY1* and *P2RY12* genes are mainly associated to alterations in platelet aggregation. Thus, the A1622G mutation of the *P2RY1* gene could contribute to an inadequate platelet response to anti-coagulants, which would be associated with a higher risk of cardio- and cerebrovascular diseases (Lordkipanidze et al. 2011; Timur et al. 2012). Haplotype variants of the *P2RY12* gene have also been related to platelet aggregation, peripheral arterial disease, venous thromboembolism, myocardial infarction and cerebrovascular accidents (Lordkipanidze et al. 2011; Cavallari et al. 2007; Kim et al. 2013; Li

Table 2 Similarity (%) in globally aligned nucleotide coding sequences (CDS, *above the diagonal*) and amino acid sequences (*below the diagonal*) of the $P2Y_{13}$ proteins/genes from 13 different species

	Human	Chimp	Gorilla	Orangutan	Macaque	Night monkey	Dog	Cow	Pig	Rat	Mouse	Chicken	Zebrafish
Human		99.44	99.34	98.22	96.62	90.05	80.28	80.09	81.41	74.91	74.79	63.57	50.83
Chimp	99.15		99.53	98.22	96.81	90.23	80.09	80.09	81.60	74.72	74.60	63.48	51.11
Gorilla	98.87	99.72		98.12	96.71	90.14	80.00	80.00	81.31	74.72	74.60	63.38	50.65
Orangutan	96.89	97.18	96.89		96.15	89.86	79.81	79.53	80.85	74.44	74.51	63.29	51.02
Macaque	95.20	96.05	95.76	95.48		89.95	79.62	79.44	80.94	74.53	74.79	62.83	51.02
Night monkey	87.57	88.42	88.14	88.14	87.57		84.85	83.65	85.14	77.90	77.47	63.44	53.33
Dog	79.38	78.81	78.53	78.25	77.40	83.78		83.53	85.83	75.71	75.49	63.93	54.85
Cow	77.40	77.68	77.40	77.40	77.12	81.38	82.88		88.12	74.04	73.63	64.12	55.53
Pig	75.71	75.71	75.42	75.14	74.86	78.98	84.08	87.39		75.81	75.00	64.80	55.14
Rat	75.77	76.06	75.77	75.21	75.21	78.70	74.85	74.56	73.67		89.35	62.01	54.35
Mouse	73.31	73.60	73.31	73.03	73.60	74.93	71.68	69.91	69.91	87.54		61.44	52.56
Chicken	56.86	56.86	56.58	56.02	55.74	56.85	60.35	61.52	60.64	57.10	55.07		52.49
Zebrafish	43.45	43.45	43.18	42.90	42.90	44.67	46.15	45.86	46.75	42.69	41.40	46.82	

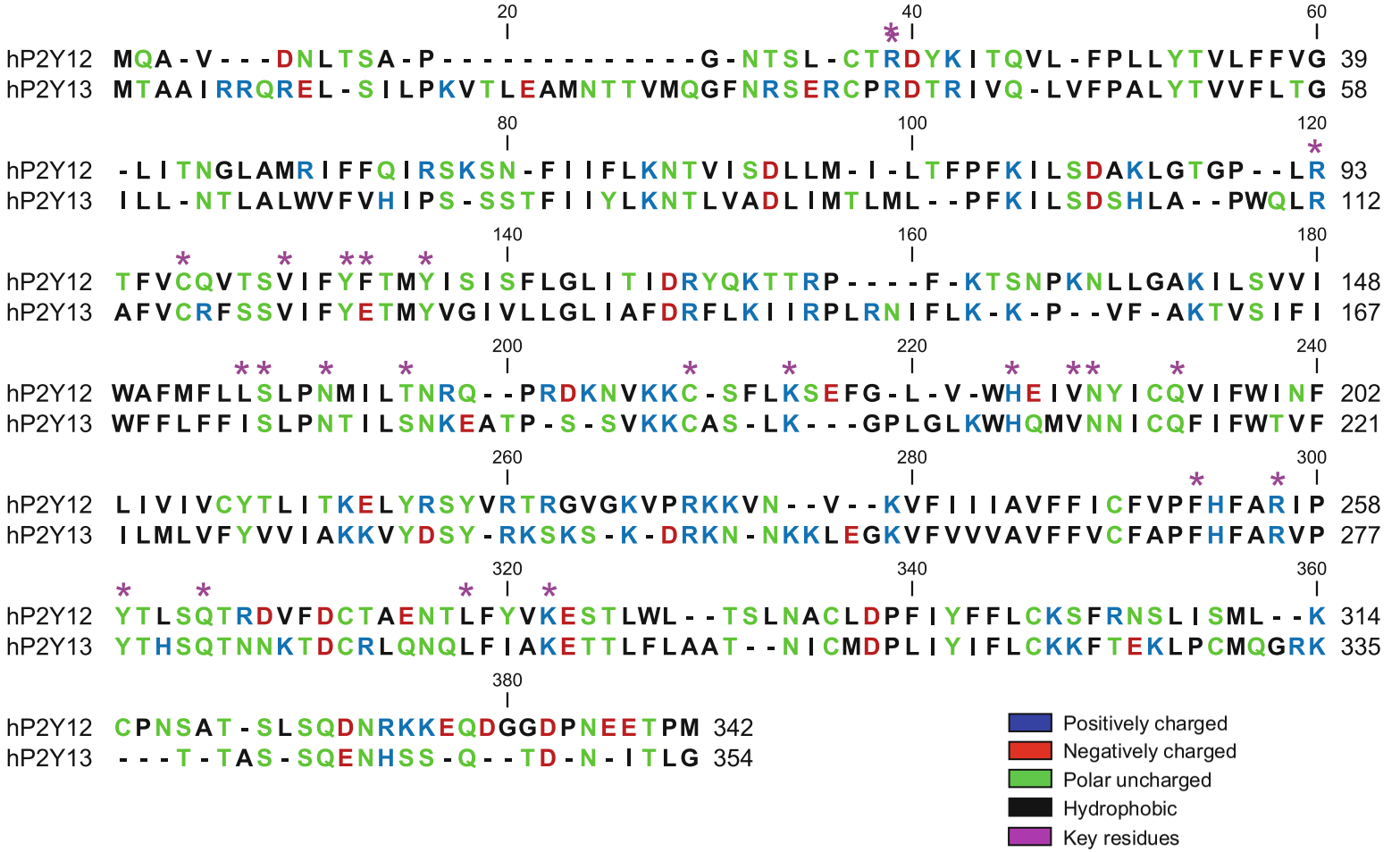

Fig. 5 Alignment of the amino acid sequences of human $P2Y_{12}$ and $P2Y_{13}$ receptors. Amino acids with similar chemical characteristics are colour-coded: R, H, K – positively charged side chains (*blue*); D, E – negatively charged chains (*red*); S, C, T, N, Q, Y – polar uncharged side chains (*green*); G, A, I, L, M, P, F, W, V – hydrophobic side chains (*black*). *Purple asterisks* indicate the location of key residues involved in agonist/antagonist binding to $P2Y_{12}$ receptor (Zhang et al. 2014a, b)

Table 3 Key amino acids that participate in 2MeSADP and AZD1283 binding to $P2Y_{12}$ receptors and their putative equivalents in $P2Y_{13}$ receptor

2MeSADP interaction		AZD1283 interaction	
hP2Y12	hP2Y13	hP2Y12	hP2Y13
R19	R38	V102	V121
R93	R112	Y105	Y124
C97	C116	***F106***	***E125***
Y105	Y124	Y109	Y128
F106	***E125***	***L155***	***I174***
L155	I174	N159	N178
S156	S175	V190	V209
N159	N178	N191	N210
T163	***S182***	Q195	Q214
C175	C194	F252	F271
K179	K198	R256	R275
H187	H206	Y259	Y278
N191	N210	L276	L295
R256	R275	K280	K299
Y259	Y278		
Q263	Q282		
K280	K299		

Non-conserved residues are in bold and italic

et al. 2015; Oestreich et al. 2014; Ou et al. 2016; Siasos et al. 2016; Zee et al. 2008). Moreover, several SNPs and haplotypes of the *P2RY12* gene are associated with lung function in patients with asthma (Bunyavanich et al. 2012). Polymorphisms in *P2RY2* gene have been related to cerebral and myocardial infarction, essential hypertension (Wang et al. 2009a, b, 2010) and decreased risk of osteoporosis (Wesselius et al. 2013). Recently, a gene-based analysis of regulatory variants identified *P2RY13* and *P2RY14* as putative asthma risk genes, and functional studies in mice demonstrated that selective agonists of both receptors can promote airway inflammation (Ferreira et al. 2016). However, the pathophysiological consequences of *P2RY13* polymorphisms in human populations remain unclear.

Over recent years, the availability of high-throughput DNA sequencing technologies has allowed whole genomes or exomes (protein-coding regions) of a large numbers of humans to be analysed. The recent generation of an extensive catalogue of human protein-coding genetic variation, the Exome Aggregation Consortium (ExAC), provides a powerful source of information about the patterns of low-frequency exome variants from nearly 70,000 individuals of diverse geographic ancestries, representing a public resource for the clinical interpretation of genetic variants observed in disease patients (Lek et al. 2016). Using the ExAC database (http://exac.broadinstitute.org; version 0.3, January 2015), we identified 184 candidate polymorphisms in the human *P2RY13* gene, 166 of which lie in exons. When only high-quality filtered variants were analysed, most (98.4%) had an allele frequency of less than 0.01% (very low-frequency variants) and 57% of them were singletons (only observed once in the data set). In terms of the mutational properties of exon variants, there are 48 synonymous (non--protein-altering) SNPs and 118 non-synonymous variants, the latter including 111 missense SNPs, 1 in-frame deletion, 1 stop lost and 3 protein-truncating variants (2 frame-shift and 1 stop gained mutations). In addition, 43 of the missense variants were predicted to be probable/possible damaging variants (Table 4).

Although the consequences of loss-of-function mutations in the human *P2RY13* receptor remain unknown, the availability of a $P2Y_{13}$ knockout mice has proved to be an invaluable resource to assess both the physiological role of this receptor and its potential involvement in specific pathologies (Fabre et al. 2010). Mice lacking the $P2Y_{13}$ receptor display altered bone remodelling, with less complex bone structures. In addition, these mice suffer a significant reduction in the osteoclast and osteoblast populations on the surfaces of the cortical bone, leading to a reduced capacity for mineralization. These osteoblasts proliferate normally *in vitro*, although their mineralization capacity is dampened. Moreover, their gene expression is altered, with an upregulation of osteoprotegerin and a downregulation of the RhoA genes. Conversely, osteoclasts maintain their function, although the number of resorbing osteoclast is decreased. However, the bone phenotype of the $P2Y_{13}$ null mice protects against the loss of bone linked to oestrogen-deficiency (Orriss et al. 2011; Wang et al. 2012).

Table 4 Polymorphisms identified in human *P2RY13* gene using ExAC database (Lek et al. 2016)

Variant	Consequence	Mutational properties	Allele count	Allele frequency
3:151047352 T/C		Upstream gene	2	0.00009252
3:151047362 T/C		Upstream gene	1	0.00004633
3:151046811 AT/A		Intron	1	0.000008935
3:151046821 A/G		Intron	1	0.000009178
3:151046822 C/G		Intron	3	0.00002757
3:151046822 CAAAAG/C		Intron	1	0.000009189
3:151046823 A/G		Intron	1	0.000009182
3:151046842 T/G		Intron	1	0.000009522
3:151047240 C/T		Intron	1	0.00004658
3:151047252 C/T (rs112766831)		Intron	254	0.01179
3:151045729 T/C		3′ UTR	4	0.00003619
3:151045733 T/C		3′ UTR	5	0.00004524
3:151045735 C/T		3′ UTR	1	0.000009049
3:151045736 G/T		3′ UTR	16	0.0001447
3:151045736 G/A		3′ UTR	9	0.00008142
3:151045744 C/T		3′ UTR	7	0.00006332
3:151045755 A/G		3′ UTR	1	0.000009038
3:151045756 G/C		3′ UTR	14	0.0001265
3:151045782 G/T	p.Gly354Gly	Synonymous	1	0.000008995
3:151045812 A/G	p.His344His	Synonymous	4	0.00003530
3:151045821 T/C	p.Gln341Gln	Synonymous	1	0.000008766
3:151045862 G/A	p.Leu328Leu	Synonymous	1	0.000008340
3:151045863 C/T	p.Lys327Lys	Synonymous	1	0.000008334
3:151045914 A/G	p.Ile310Ile	Synonymous	1	0.000008264
3:151045931 A/G	p.Leu305Leu	Synonymous	2	0.00001653
3:151045959 C/G	p.Leu295Leu	Synonymous	1	0.000008272
3:151046064 G/A	p.Val260Val	Synonymous	2	0.00001656
3:151046067 A/C	p.Val259Val	Synonymous	1	0.000008283
3:151046094 G/A	p.Asn250Asn	Synonymous	1	0.000008292
3:151046124 T/C	p.Arg240Arg	Synonymous	1	0.000008296
3:151046127 A/G	p.Tyr239Tyr	Synonymous	3	0.00002489
3:151046142 T/C	p.Lys234Lys	Synonymous	1	0.000008307
3:151046166 C/T	p.Val226Val	Synonymous	1	0.000008287
3:151046178 G/T	p.Ile222Ile	Synonymous	1	0.000008282
3:151046280 T/C	p.Pro188Pro	Synonymous	7	0.00005796
3:151046303 A/G	p.Leu181Leu	Synonymous	2	0.00001656
3:151046307 C/T	p.Thr179Thr	Synonymous	3	0.00002484
3:151046310 A/G	p.Asn178Asn	Synonymous	1	0.000008301
3:151046322 G/A	p.Ile174Ile	Synonymous	1	0.000008310
3:151046325 G/A	p.Phe173Phe	Synonymous	1	0.000008312
3:151046328 G/A	p.Phe172Phe	Synonymous	1	0.000008314
3:151046331 C/T	p.Leu171Leu	Synonymous	10	0.00008314
3:151046346 G/A	p.Phe166Phe	Synonymous	1	0.000008320
3:151046358 C/T	p.Thr162Thr	Synonymous	3	0.00002498
3:151046382 T/A	p.Leu154Leu	Synonymous	1	0.000008345
3:151046405 T/G	p.Arg147Arg	Synonymous	1	0.000008334
3:151046409 G/A (rs142736005)	p.Ile145Ile	Synonymous	23	0.0001915
3:151046445 C/T	p.Leu133Leu	Synonymous	1	0.000008289
3:151046451 G/A	p.Ile131Ile	Synonymous	1	0.000008285

(continued)

Table 4 (continued)

Variant	Consequence	Mutational properties	Allele count	Allele frequency
3:151046484 C/T	p.Ser120Ser	Synonymous	8	0.00006611
3:151046493 A/C	p.Arg117Arg	Synonymous	1	0.000008258
3:151046502 A/G	p.Phe114Phe	Synonymous	1	0.000008255
3:151046508 T/C	p.Arg112Arg	Synonymous	8	0.00006602
3:151046511 G/C	p.Leu111Leu	Synonymous	2	0.00001650
3:151046556 A/C	p.Leu96Leu	Synonymous	1	0.000008245
3:151046576 A/G (rs150522091)	p.Leu90Leu	Synonymous	4	0.00003299
3:151046580 G/A	p.Ala88Ala	Synonymous	3	0.00002474
3:151046588 A/G (rs139399025)	p.Leu86Leu	Synonymous	19	0.0001567
3:151046604 G/T (rs3732757)	p.Ile80Ile	Synonymous	6992	0.05767
3:151046607 G/A	p.Ile79Ile	Synonymous	1	0.000008246
3:151046619 G/A	p.Ser75Ser	Synonymous	1	0.000008244
3:151046646 C/A	p.Leu66Leu	Synonymous	1	0.000008243
3:151046655 A/G	p.Thr63Thr	Synonymous	2	0.00001649
3:151046673 G/A	p.Thr57Thr	Synonymous	3	0.00002473
3:151046745 A/G	p.Ser33Ser	Synonymous	1	0.000008311
3:151047308 G/A	p.Ala3Ala	Synonymous	1	0.00004622
3:151046800 T/TA	c.49-6dupT	Splice region	1	0.000008749
3:151046803 C/A	c.49-8G > T	Splice region	1	0.000008797
3:151045784 C/T	p.Gly354Ser	Missense	1	0.000008986
3:151045807 C/A	p.Ser346Ile	Missense	2	0.00001775
3:151045810 C/T	p.Ser345Asn	Missense	1	0.000008845
3:151045820 C/G	p.Glu342Gln	Missense	3	0.00002635
3:151045829 A/G	p.Ser339Pro	Missense	7	0.00006076
3:151045832 C/A (rs145063671)	p.Ala338Ser	Missense	40	0.0003462
3:151045835 T/C	p.Thr337Ala	Missense	4	0.00003443
3:151045840 T/C	p.Lys335Arg	Missense	1	0.000008563
3:151045852 A/G	p.Met331Thr	Missense	2	0.00001686
3:151045856 A/G	p.Cys330Arg	Missense	15	0.0001258
3:151045867 T/G	p.Glu326Ala	Missense	1	0.000008312
3:151045873 A/T	p.Phe324Tyr	Missense	1	0.000008292
3:151045889 A/G	p.Phe319Leu	Missense	3	0.00002481
3:151045891 A/G	p.Ile318Thr	Missense	1	0.000008269
3:151045904 G/A	p.Pro314Ser	Missense	1	0.000008266
3:151045905 A/T	p.Asp313Glu	Missense	1	0.000008265
3:151045906 T/A	p.Asp313Val	Missense	1	0.000008266
3:151045915 A/G	p.Ile310Thr	Missense	1	0.000008265
3:151045939 G/A	p.Thr302Ile	Missense	1	0.000008267
3:151045951 G/A	p.Ala298Val	Missense	1	0.000008270
3:151045952 C/T (rs148292157)	p.Ala298Thr	Missense	1	0.000008270
3:151045954 A/G	p.Ile297Thr	Missense	1	0.000008270
3:151045976 T/C (rs61736003)	p.Arg290Gly	Missense	1131	0.009355
3:151046008 G/A	p.Thr279Ile	Missense	2	0.00001654
3:151046018 C/A	p.Val276Phe	Missense	1	0.000008272
3:151046063 C/T	p.Val261Met	Missense	16	0.0001325
3:151046068 A/G	p.Val259Ala	Missense	3	0.00002485
3:151046097 G/C	p.Asn249Lys	Missense	1	0.000008294
3:151046104 C/T	p.Arg247Lys	Missense	1	0.000008295
3:151046110 T/C	p.Lys245Arg	Missense	1	0.000008294

(continued)

Table 4 (continued)

Variant	Consequence	Mutational properties	Allele count	Allele frequency
3:151046112 A/C	p.Ser244Arg	Missense	1	0.000008295
3:151046119 G/A	p.Ser242Phe	Missense	1	0.000008296
3:151046121 C/G	p.Lys241Asn	Missense	1	0.000008296
3:151046123 T/G	p.Lys241Gln	Missense	1	0.000008295
3:151046129 A/G	p.Tyr239His	Missense	9	0.00007467
3:151046146 T/G	p.Lys233Thr	Missense	7	0.00005806
3:151046163 A/C (rs150366287)	p.Phe227Leu	Missense	5	0.00004144
3:151046167 A/G	p.Val226Ala	Missense	1	0.000008285
3:151046171 G/A	p.Leu225Phe	Missense	3	0.00002485
3:151046186 C/A	p.Val220Phe	Missense	1	0.000008279
3:151046207 A/T	p.Cys213Ser	Missense	2	0.00001655
3:151046207 A/G	p.Cys213Arg	Missense	1	0.000008274
3:151046222 T/C (rs149544268)	p.Met208Val	Missense	17	0.0001407
3:151046233 T/G	p.Lys204Thr	Missense	1	0.000008278
3:151046263 C/G (rs188633801)	p.Cys194Ser	Missense	4	0.00003312
3:151046263 C/A (rs188633801)	p.Cys194Phe	Missense	1	0.000008281
3:151046275 G/C	p.Ser190Cys	Missense	2	0.00001656
3:151046278 G/A	p.Ser189Leu	Missense	1	0.000008282
3:151046279 A/T	p.Ser189Thr	Missense	1	0.000008280
3:151046281 G/A	p.Pro188Leu	Missense	1	0.000008282
3:151046297 T/C	p.Asn183Asp	Missense	1	0.000008280
3:151046299 C/T	p.Ser182Asn	Missense	1	0.000008280
3:151046305 A/T	p.Ile180Asn	Missense	1	0.000008279
3:151046306 T/C	p.Ile180Val	Missense	1	0.000008280
3:151046308 G/A (rs1466684)	p.Thr179Met	Missense	104,481	0.8636
3:151046321 A/G	p.Ser175Pro	Missense	2	0.00001662
3:151046322 G/C	p.Ile174Met	Missense	5	0.00004155
3:151046323 A/T	p.Ile174Asn	Missense	2	0.00001662
3:151046341 C/G	p.Trp168Ser	Missense	1	0.000008318
3:151046359 G/A	p.Thr162Met	Missense	3	0.00002498
3:151046374 G/T	p.Pro157His	Missense	2	0.00001668
3:151046375 G/T (rs147188000)	p.Pro157Thr	Missense	3	0.00002503
3:151046378 T/G	p.Lys156Gln	Missense	11	0.00009175
3:151046398 A/G (rs138841969)	p.Leu149Ser	Missense	9	0.00007508
3:151046406 G/C	p.Ile146Met	Missense	1	0.000008330
3:151046413 T/C	p.Lys144Arg	Missense	1	0.000008322
3:151046418 G/T	p.Phe142Leu	Missense	2	0.00001663
3:151046441 C/G (rs144496684)	p.Gly135Arg	Missense	19	0.0001575
3:151046450 C/T (rs148391906)	p.Val132Met	Missense	2	0.00001657
3:151046458 A/C	p.Val129Gly	Missense	1	0.000008278
3:151046462 A/C	p.Tyr128Asp	Missense	2	0.00001655
3:151046469 C/G	p.Glu125Asp	Missense	1	0.000008269
3:151046477 A/G	p.Phe123Leu	Missense	9	0.00007438
3:151046486 A/T	p.Ser120Thr	Missense	1	0.000008261
3:151046490 A/T (rs184462683)	p.Phe118Leu	Missense	7	0.00005781
3:151046494 C/T	p.Arg117His	Missense	3	0.00002478
3:151046495 G/T	p.Arg117Ser	Missense	8	0.00006606
3:151046502 A/C	p.Phe114Leu	Missense	10	0.00008255
3:151046506 G/T (rs144128158)	p.Ala113Asp	Missense	510	0.004209

(continued)

Table 4 (continued)

Variant	Consequence	Mutational properties	Allele count	Allele frequency
3:151046559 C/T	p.Met95Ile	Missense	1	0.000008246
3:151046573 T/C	p.Ile91Val	Missense	1	0.000008246
3:151046581 G/A	p.Ala88Val	Missense	2	0.00001649
3:151046587 A/G	p.Leu86Ser	Missense	2	0.00001650
3:151046602 T/C	p.Tyr81Cys	Missense	1	0.000008248
3:151046627 G/A	p.Pro73Ser	Missense	1	0.000008244
3:151046630 T/C	p.Ile72Val	Missense	1	0.000008244
3:151046636 C/T	p.Val70Ile	Missense	1	0.000008244
3:151046637 A/C	p.Phe69Leu	Missense	1	0.000008244
3:151046648 G/C	p.Leu66Val	Missense	1	0.000008242
3:151046690 T/C	p.Thr52Ala	Missense	1	0.000008244
3:151046696 G/C	p.Leu50Val	Missense	1	0.000008244
3:151046716 A/G	p.Val43Ala	Missense	1	0.000008254
3:151046723 G/A	p.Arg41Trp	Missense	1	0.000008260
3:151046725 G/A	p.Thr40Ile	Missense	1	0.000008263
3:151046732 T/C (rs146597143)	p.Arg38Gly	Missense	1	0.000008273
3:151046740 C/T	p.Arg35Gln	Missense	2	0.00001658
3:151046741 G/A	p.Arg35Trp	Missense	2	0.00001660
3:151046752 T/C	p.Asn31Ser	Missense	1	0.000008342
3:151046763 C/T	p.Met27Ile	Missense	3	0.00002519
3:151046768 C/T	p.Val26Met	Missense	6	0.00005059
3:151046776 T/G	p.Asn23Thr	Missense	4	0.00003395
3:151046778 C/T (rs141361811)	p.Met22Ile	Missense	1	0.000008517
3:151046783 C/T	p.Ala21Thr	Missense	2	0.00001713
3:151046785 T/C	p.Glu20Gly	Missense	1	0.000008567
3:151046785 T/A	p.Glu20Val	Missense	1	0.000008567
3:151046795 C/T	p.Val17Met	Missense	3	0.00002607
3:151047289 C/G	p.Glu10Gln	Missense	1	0.00004619
3:151047304 T/C	p.Ile5Val	Missense	1	0.00004617
3:151047307 C/T (rs139632884)	p.Ala4Thr	Missense	1	0.00004622
3:151047309 G/A	p.Ala3Val	Missense	2	0.00009235
3:151046309 T/C	p.Thr179Ala	Missense	1	0.000008302
3:151045796 TGTC/T	p.Asp349del	Inframe deletion	1	0.000008937
3:151045781 A/G	p.Ter355ArgextTer11	Stop lost	4	0.00003599
3:151045931 A/AG	p.Ala306GlyfsTer4	Frameshift	3	0.00002480
3:151046097 G/GT	p.Asn249LysfsTer38	Frameshift	1	0.000008294
3:151046230 C/T	p.Trp205Ter	Stop gained	1	0.000008277

Another major feature of the $P2Y_{13}$ knockout mice is related to lipoprotein metabolism. Even though HDL levels in plasma are normal (Fabre et al. 2010) or slightly decreased (Blom et al. 2010), hepatic uptake of HDL holo-particles is impaired in the absence of the receptor. Furthermore, mice exhibit deficient biliary cholesterol excretion and a reduced hepatic cholesterol content. Remarkably, the reverse cholesterol transport (RCT) that is crucial for the atheroprotective role of HDL proteins is also affected, with a striking reduction in macrophage-to-faeces RCT, making them more sensitive to a high cholesterol diet (Fabre et al. 2010; Lichtenstein et al. 2013). Despite the strong reduction in RCT, $P2Y_{13}$ null mice do not exhibit enhanced atherosclerosis, possibly to the notable differences between human and mouse lipoprotein metabolism. However, dual $P2Y_{13}$/apoE knockout mice develop enhanced aortic sinus lesions with more

infiltrated macrophages (Lichtenstein et al. 2015). Importantly, the atherosclerotic pathology is independent of the $P2Y_{13}$ blood cell receptors. Moreover, a high fat diet is also responsible of neuropathies that affect the enteric nervous system, crucial for the maintenance and regulation of gastrointestinal activity. Experiments performed on $P2Y_{13}$ null mice fed with a high fat diet reveal that the purinergic receptor is responsible for the myenteric neuronal loss induced by the high fat diet or palmitic acid (Voss et al. 2014).

In summary, there is strong evidence that the $P2Y_{13}$ receptor constitutes a promising therapeutic target for pathologies like osteoporosis, atherosclerosis and intestinal neuropathies. However, the role of the receptor in distinct tissues in the organism implies that its involvement in other pathologies should not be ruled out, encouraging further research into this receptor.

5 $P2Y_{13}$ Receptor Signaling

The range of signals transmitted by $P2Y_{13}$ receptors extend beyond their versatility in G protein coupling, and several intracellular pathways can be activated independently of the canonical inhibition of adenylate cyclase.

5.1 G Protein Signalling

Like the $P2Y_{12}$ receptor, the $P2Y_{13}$ receptor is an ADP receptor mainly coupled to Gi proteins. Activation of the $P2Y_{13}$ receptor with ADP or the analogue 2MeSADP leads to the inhibition of adenylate cyclase and a decrease in cAMP production triggered by forskolin, a direct adenylate cyclase activator or following Gs-coupled GPCR stimulation. The EC_{50} values for these events are in the nanomolar range (Communi et al. 2001; Zhang et al. 2002; Marteau et al. 2003; Carrasquero et al. 2005), yet such inhibition was reversed at higher agonist concentrations. From the very first studies, biphasic dose-response curves for the effect of ADP on forskolin-cAMP accumulation were described, with inhibition at nanomolar agonist concentrations and potentiation at micromolar levels, clearly demonstrating the versatility of $P2Y_{13}$ receptor coupling to different G proteins (Gi/Gs) (Communi et al. 2001; Marteau et al. 2003).

Studies with Gα16/Gαq in heterologous expression systems (Communi et al. 2001; Zhang et al. 2002; Marteau et al. 2003) and native tissues, such as immature human dendritic cells and rat cerebellar astrocytes (Carrasquero et al. 2005; Marteau et al. 2004), revealed that $P2Y_{13}$ can also couple to PLC (Fig. 6). Functional $P2Y_{13}$ receptors are present in cerebellar astrocytes as ADP elicits calcium transients that are not sensitive to the $P2Y_1$ specific antagonist, MRS2179. The presence of Gq-coupled $P2Y_{13}$ receptors is restricted to astrocytic populations other than those expressing $P2Y_1$ receptors, indicating some degree of specialization (Carrasquero et al. 2005; Jimenez et al. 1999, 2000). A link to the canonical pathway of adenylate cyclase inhibition further demonstrated the identity of the $P2Y_{13}$ receptor in this glial model. Moreover, when $P2Y_{13}$ and $P2X_7$ purinergic receptors are functionally expressed in rat cerebellar astrocytes they mediate the increase in intracellular calcium elicited by BzATP in these cells (Carrasquero et al. 2009). Moreover, some populations of rat cerebellar granule neurons exhibit 2MeSADP-mediated calcium mobilization with the pharmacological profile of a $P2Y_{13}$ receptor (Hervas et al. 2003).

5.2 MAP Kinase Activation

Downstream of the first membrane effectors, $P2Y_{13}$ receptor stimulation induces Gi-dependent Mitogen Activated Protein Kinase (MAPK) activation (Communi et al. 2001). The $P2Y_{13}$ receptor was first seen to couple to the ERK1/2 MAPK in native human dendritic cells (Marteau et al. 2004). ERK activation triggered by $P2Y_{13}$ receptor agonists was sensitive to ARC-699931MX and intracellular calcium chelation by BAPTA-AM. Again, rat cerebellar

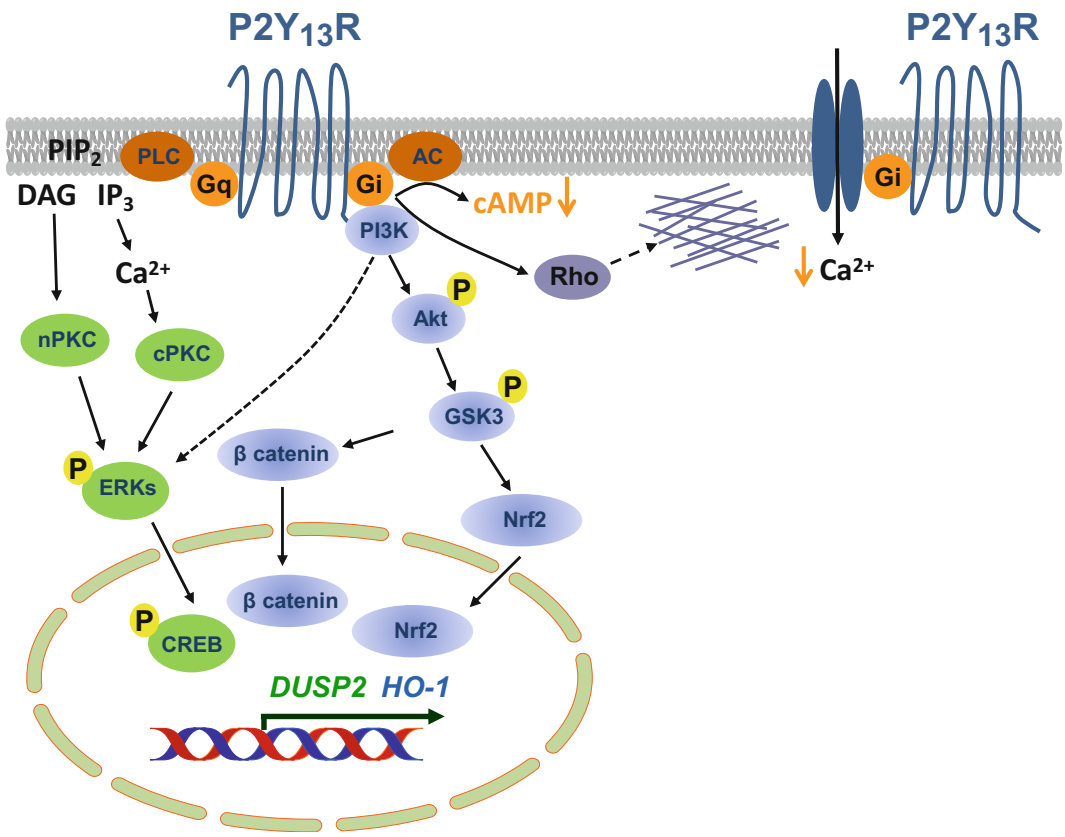

Fig. 6 Schematic representation of the intracellular signalling cascades activated by P2Y$_{13}$ receptor stimulation. P2Y$_{13}$ couples to adenylate cyclase inhibition through the canonical mechanism described for Gi-coupled receptors. P2Y$_{13}$ receptor stimulation *via* the Gi protein also triggers the PI3K/Akt/GSK3 axis. Phosphorylation of GSK3 causes enzyme inactivation and releases two key proteins, β-catenin and Nrf2, which act as transcription factors. The activation of the antioxidant Nfr2/HO-1 axis promotes cell survival in the face of oxidative stress. The P2Y$_{13}$ receptor also couples to Gq proteins in some cell types, promoting phospholipase C activation that in turn stimulates intracellular calcium mobilization and DAG production. These second messengers activate different PKC isoforms and induce ERK activation, which is required for *Dusp2* phosphatase gene transcription. DUSP2 could be responsible for the neuroprotection displayed by the P2Y$_{13}$ receptor to counteract the neurotoxic actions of cisplatin. In some cell models, ERK activation can also be achieved *via* PI3K (broken lines). The P2Y$_{13}$ receptor also couples to RhoA activation and cytoskeleton reorganization, and it may inhibit Ca^{2+} channel activity *via* the βγ subunits of the activated Gi protein and modulate neurotransmitter release

astrocytes and granule neurons provided examples of Gi-dependent 2MeSADP-mediated ERK activation, which was completely inhibited by Pertussis toxin (Carrasquero et al. 2005; Ortega et al. 2011; Perez-Sen et al. 2015). In addition, the sensitivity to the specific antagonist MRS2211, together with lack of effect of MRS2179, a P2Y$_1$ antagonist, confirmed the participation of P2Y$_{13}$ receptors in this signalling.

ERK activation by P2Y$_{13}$ receptors in cerebellar astrocytes and granule neurons resembles that of tyrosine kinase receptors, with maximal activation reached after 10–15 min. In astrocytes, the EC_{50} value of ERK activation correlated with that obtained in experiments where cAMP production was inhibited (around 40 nM). Interestingly, the activation of ERKs was dependent on nProtein Kinase C and src-like kinase activation (Carrasquero et al. 2005). Alternatively, PI3 kinase (PI3K) seemed to lie upstream of ERK activation in granule neurons, as its activity was completely abolished by the PI3K inhibitors

wortmannin and LY294002. In this neuronal model the CREB (cAMP response element-binding) transcription factor was activated in an ERK-dependent manner, conferring neuroprotection against apoptotic stimuli (Fig. 6) (Ortega et al. 2011; Perez-Sen et al. 2015).

In recent studies, new targets of ERK signalling activated by $P2Y_{13}$ receptors have been identified (Morente et al. 2014). The stimulation of granule neurons by 2MeSADP induced the $P2Y_{13}$ dependent expression of an early gene, *Dusp2*, which was sensitive to the $P2Y_{13}$ antagonist, MRS2211. DUSP2 is a dual specificity protein phosphatase that is involved in regulating MAPK activity. As described below, DUSP2 may be responsible for some neuroprotection associated to $P2Y_{13}$ receptor stimulation. Opposite effects were found in pancreatic β cells, where the $P2Y_{13}$ receptor inhibition activated ERK/Akt/CREB signalling (Tan et al. 2010).

5.3 PI3K/Akt/GSK3 Activation

Another interesting feature of the $P2Y_{13}$ receptor is its specific coupling to the PI3K/Akt/GSK3 axis in granule neurons. Accordingly, 2MeSADP induces Thr^{308} phosphorylation and activation of Akt in these cells, which is sensitive to Pertussis toxin and PI3K inhibition. This mechanism connects $P2Y_{13}$ receptors to important intracellular pathways in granule neurons. As such, 2MeSADP induces the rapid and transient phosphorylation of one of the main Akt targets, GSK3 (Ser^{21} and Ser^{9} residues of the α and β isoforms, respectively), which inhibits its catalytic activity. The EC_{50} value for 2MeSADP is close to 20 nM, and both the $P2Y_{13}$ antagonist MRS2211 and the PI3K inhibitors wortmaninn and LY294002, prevent this effect (Ortega et al. 2008).

The signalling triggered by $P2Y_{13}$ receptors that inhibits GSK3 deserves some attention, particularly as some key GSK3 substrates normally retained in the cytosol escape from GSK3-mediated phosphorylation and their subsequent proteasomal degradation (Fig. 6). The nuclear translocation of these GSK3 substrates occurs very rapidly in granule neurons, after a 10–30 min stimulation with 1 μM 2MeSADP, such as for the transcriptional regulator β-catenin (Ortega et al. 2008). Likewise, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates the expression of antioxidant genes, is also regulated by GSK3, and a new mechanism by which GSK3 can phosphorylate Nrf2 to promote its further degradation has been demonstrated (Cuadrado 2015; Rada et al. 2012; Rojo et al. 2008). The inhibition of GSK3 induced by $P2Y_{13}$ receptors lies upstream of the increase in Nrf2, and it accumulates in the nucleus of granule neurons after a 6 h stimulation with 2MeSADP, which also turned out to provide neuroprotection (see below). Furthermore, GSK3 has a key role in the formation of amyloid plaques and neurofibrillary tangles, being one of the most relevant therapeutic targets for Alzheimer's disease (Diaz-Hernandez et al. 2012; Maqbool et al. 2016).

The activation of the PI3K/Akt signalling pathway is not exclusive to $P2Y_{13}$ receptors, as it is also induced by the Gi-coupled $P2Y_{12}$ receptor. In C6 glioma cells, the intracellular cascade triggered by 2MeSADP to induce proliferation involves Rap1 activation by Gβγ subunits, which leads to PI3K-dependent Akt activation. In this pathway, Ca^{2+} mobilization and assembly of the PyK2/Src/PLD2 complex is required to achieve the final effect, and this signalling contributes to the proliferation elicited by $P2Y_{12}$ receptors in glioma cells (Van Kolen et al. 2006; Van Kolen and Slegers 2004).

As described for other GPCRs, including the $P2Y_{12}$ receptor, $P2Y_{13}$ stimulation also couples to Rho signalling and subsequent cytoskeleton reorganization. The $P2Y_{13}$ receptor activates RhoA and ROCK I in hepatocytes and osteoblasts, inducing relevant physiological effects (as discussed below). The mechanism of action for RhoA activation is still not completely understood, although it could involve Gi subunits binding to Rho specific guanine exchange factors (Rho-GEF) (Wang et al. 2012; Malaval et al. 2009).

5.4 Molecular Mechanisms Regulating $P2Y_{13}$ Receptor Expression

It is well known that basal ubiquitination and deubiquitination are important to control the cell surface expression of several GPCRs (Dores and Trejo 2012). In terms of the $P2Y_{13}$ receptor, constitutive ubiquitination of its C- terminus tail is directly modulated in the endoplasmic reticulum. Consequently, the $P2Y_{13}$ receptor is degraded through the proteasome pathway, thereby controlling the density of functional receptors at the cell surface and cellular responsiveness (Pons et al. 2014). In this context, the discovery of deubiquitinating enzymes that specifically regulate $P2Y_{13}$ receptor ubiquitination might provide the basis for a novel therapeutic approach to improve hepatic HDL uptake and bile acid secretion, for instance preventing or impairing the development of atherosclerosis (Serhan et al. 2013).

6 Physiological Relevance of $P2Y_{13}$ Receptors

Given the widespread distribution of the purinergic $P2Y_{13}$ receptor, its signalling could be involved in regulating multiple activities in different tissues and organs. The $P2Y_{13}$ receptor is mainly expressed in the spleen, bone marrow cells, peripheral leukocytes, brain, liver, pancreas and heart. Here, we will summarize the direct evidence for the physiological roles of the $P2Y_{13}$ receptor.

6.1 Metabolic Disorders: Atherosclerosis and Diabetes

One of the most promising activities of $P2Y_{13}$ receptor is related to its influence on atherosclerosis. The $P2Y_{13}$ receptor plays a pivotal role in HDL metabolism, which transports cholesterol from peripheral tissues to the liver for elimination. The identification of a new pathway in the liver involving the F1-ATPase and the $P2Y_{13}$ receptor, which regulates the removal of HDL-cholesterol (HDL-c), has enhanced our understanding of HDL metabolism (Jacquet et al. 2005). HDL endocytosis triggered by the $P2Y_{13}$ receptor is dependent on activation of the small GTPase RhoA and ROCK1, producing the cytoskeletal arrangements that drive endocytosis. This step is followed by an increase in biliary lipid secretion (Malaval et al. 2009). The participation of the $P2Y_{13}$ receptor is clearly evident when the effects of its agonists are studied, such as cangrelor and the new available drug, CT1007900 (6-[1-(2-Dimethylaminopyrimidin-5-ylmethyl)-piperidin-4-yl]-2-morpholin-4-yl-pyrimidin-4-ol monohydrate). Acute administration of these compounds increases RCT in the same way as ADP, and in the case of cangrelor, its effect was greater than that found with the physiological agonist. This was unexpected for a compound initially designed as a $P2Y_{12}$ and $P2Y_{13}$ antagonist, and that turned out to act as a partial agonist for $P2Y_{13}$ receptors (Jacquet et al. 2005; Serhan et al. 2013; Goffinet et al. 2014). The fact that impaired HDL clearance occurred in $P2Y_{13}$ null mouse as well as in-loss-of function experiments (using $P2Y_{13}$-shRNA) favours a central role of the $P2Y_{13}$ receptor in RCT (Fabre et al. 2010; Lichtenstein et al. 2013).

The therapeutic potential of $P2Y_{13}$ agonists was also tested in long-term studies carried out through their continuous delivery, which efficiently induced the clearance of circulating cholesterol in the form of HDL particles and its elimination in the form of bile acid secreted by the liver. Moreover, after 1 month of oral treatment with the new agonist CT1007900, HDL particle size was reduced and fewer atherosclerotic plaques were deposited in a mouse model of atherosclerotic pathology (Lichtenstein et al. 2015; Goffinet et al. 2014). These studies provided clues that the $P2Y_{13}$ receptor is a promising therapeutic target for the treatment of atherosclerosis, in mice at least.

Therefore, in clinical trials to assess cangrelor as an anti-aggregating therapy, it should be borne in mind that it may act on hepatic $P2Y_{13}$ receptors and reduce the size of HDL particles. Indeed, it

seems that the same effects as those observed in mice will take place in humans. However, while treatment with cangrelor and the $P2Y_{13}$ agonist CT1007900 exerts a significant effect on circulating HDL, it does not substantially affect plasma HDL-C levels (Martinez et al. 2015). In addition, the $P2Y_{13}$ receptor may also be involved in insulin secretion as $P2Y_1$ and $P2Y_{13}$ receptors modulate insulin release from pancreatic β cells. The activation of $P2Y_1$ Gq-coupled receptors increases intracellular calcium levels and induces insulin release from isolated pancreatic β cells, whereas $P2Y_{13}$ receptor activation in these cells had the opposite effect. Administration of MRS2211, a $P2Y_{13}$ antagonist during glucose injection in mice results in both increased insulin secretion and reduced glucose levels (Amisten et al. 2010), providing a therapeutic opportunity for $P2Y_{13}$ receptor antagonists in the treatment of diabetes. Similarly, blocking $P2Y_{13}$ receptors protects pancreatic beta cells from apoptosis (Tan et al. 2013).

6.2 Bone Homeostasis

The $P2Y_{13}$ receptor also contributes to bone formation and remodelling, a new and important function for this receptor. The first clue regarding this activity of $P2Y_{13}$ came from the striking bone phenotype and altered bone turnover in $P2Y_{13}$ null mice (Wang et al. 2012). The lack of the $P2Y_{13}$ receptor mainly affected the osteogenic response, since activation of this receptor was crucial to obtain adequate differentiation of bone marrow cells into osteoblasts (Biver et al. 2013) due to the activation of RhoA/ROCK (see below) (Wang et al. 2012). These changes in bone phenotype were age-dependent and while $P2Y_{13}$ receptors modulate bone remodelling in mature animals, at younger ages this receptor affects hormonal regulators of phosphate homeostasis. Indeed, in young mice, the increase in trabecular bone formation is correlated to higher serum phosphate levels and increased FGF23 production (Wang et al. 2014).

Another important aspect of $P2Y_{13}$ receptor activity in bone homeostasis is associated to its coordinated activity with other nucleotide receptors. In red blood cells, the $P2Y_{13}$ receptor provides negative feedback modulation of ATP release and thus, increased ATP production in $P2Y_{13}$ null mice may facilitate an osteogenic response of osteoblasts upon mechanical stimulation. The enhanced osteogenic response together with the protective role of the $P2Y_{13}$ receptor in conditions of oestrogen deprivation, suggest that specific inactivation of this receptor could have therapeutic applications in preventing bone loss in diseases like osteoporosis (Wang et al. 2013).

6.3 The Nervous System and Neurological Implications

Prior to the cloning of Gi coupled ADP receptors, several studies reported the inhibitory effect of P2Y-like receptors in modulating neurotransmitter release in both the peripheral sympathetic and central nervous system (Koch et al. 1997; von Kugelgen et al. 1994). Later on, the identification of the $P2Y_{12}$ and $P2Y_{13}$ receptors, and specific antagonists, helped to identify $P2Y_{13}$ as the receptor responsible for inhibiting noradrenaline release in the rat vas deferens and in rat brain hippocampal slices (Csolle et al. 2008; Queiroz et al. 2003). Moreover, $P2Y_{13}$ receptors were also involved in fine-tuning cholinergic transmission at mammalian neuromuscular junctions, where a specific $P2Y_{13}$ antagonist abolishes the effect of 2MeSADP, inhibiting spontaneous and evoked presynaptic acetylcholine release (Guarracino et al. 2016). Indeed, direct negative modulation of N-type Ca^{2+} channels by Gi protein subunits may underlie these effects (Wirkner et al. 2004).

6.3.1 Pain Transmission

$P2Y_{13}$ receptors have also been implicated in pain transmission, and both pro-nociceptive and anti-nociceptive actions have been described in different experimental pain models. In peripheral sensory neurons, the expression of several types of P2Y receptors responding to ADP following

peripheral nerve injury and they modulate nociceptive signalling in different ways. While $P2Y_1$ is pro-nociceptive and facilitates pain transmission, the stimulation of a Gi-coupled ADP receptor with the pharmacological profile of $P2Y_{13}$ produces analgesic effects. Indeed, ADP and other $P2Y_{13}$ agonists reduce the magnitude of depolarization-evoked Ca^{2+} transients in dorsal root ganglia (DRG) sensory neurons, and this effect was also reproduced in $P2Y_1$ knockout mice (Malin and Molliver 2010). Notably, $P2Y_1$ was required for the full expression of inflammatory hyperalgesia after peripheral nerve injury, and when antagonized it occluded the action of Gi-coupled ADP receptors. Only after $P2Y_1$ blockade was the anti-nociceptive action of $P2Y_{13}$ fully revealed. The integration of these opposing signals adjusts nociceptor sensitivity. However, $P2Y_1$ receptors may exert anti-nociceptive effects in other pain models (Ando et al. 2010; Selden et al. 2007) and the selective $P2Y_1$ receptor agonist MRS2365 has potent analgesic activity against neuropathic and acute pain.

In line with the anti-nociceptive effects of ADP, new roles for $P2Y_1$ and $P2Y_{13}$ receptors have been described in the regulation of inhibitory glycinergic neurotransmission in the spinal cord. Interestingly, $P2Y_{13}$ acts in conjunction with $P2Y_1$ receptors to regulate glycine transporters in primary neuronal cultures of the spinal cord and in brainstem preparations (Jimenez et al. 2011). They reduced the activity of the neuronal glycine transporter, GLYT2, to increase the levels of inhibitory glycine neurotransmitter in the synaptic cleft. Conversely, both receptors, along with the $P2Y_{12}$ receptor, activate the glial GLYT1 transporter, reducing glycine levels at glycinergic and glutamatergic synapses, and thereby decreasing glycine concentrations in the NMDA receptor milieu. This produced a net increase in the inhibitory over the excitatory pathways that may contribute to anti-nociception.

The intracellular mechanisms triggered by $P2Y_1$/$P2Y_{13}$ receptor stimulation involved PLC/PKC activation, nitric oxide (NO) release and paracrine PKG-I activation. Regulation of GLYT activity by this pathway was corroborated in heterologous COS cell systems expressing recombinant glycine transporters and the $P2Y_1$ receptor. In fact, the regulatory activity of $P2Y_1$ receptors was lost after siRNA knockdown of NO synthase activity. This common mechanism of action reflects how PKG mediates the phosphorylation of key residues in either glycine transporters or adaptor proteins, which explains the contrasting changes in transport activity reported. These studies reveal a paracrine regulation of GLYT1 and GLYT2 by ADP-P2Y receptors in the spinal cord that contributes to the processing of nociceptive information. This paracrine regulatory mechanism extends the role of ADP receptors to the regulation of nociceptive signalling, an activity involving the modulation of the glycine levels that influences both excitatory and inhibitory neurotransmission at spinal cord synapses. Indeed, GLYT2 pharmacological blockade in the spinal cord produces pain relief in models of acute pain (Dohi et al. 2009).

In comparison to the anti-nociceptive action of $P2Y_{13}$ receptors in sensory neurons, $P2Y_{12}$ receptors expressed in spinal cord microglia also participate in the establishment of neuropathic pain and mechanical allodynia following peripheral nerve injury. Activation of p38 signalling by $P2Y_{12}$ receptors contributes to the generation and maintenance of hyperalgesia (Kobayashi et al. 2008; Tatsumi et al. 2015; Tozaki-Saitoh et al. 2008). Although $P2Y_{13}$ receptors may be expressed sporadically in microglial cells, several P2Y receptors are up-regulated in response to nerve injury, including $P2Y_{13}$, which can then contribute to the development of neuropathic pain (Kobayashi et al. 2012). In this respect, the increases in calcium promoted by Gq-coupling of $P2Y_{13}$ receptors in dorsal spinal cord microglia contribute to the early phase of pain hyper-sensitization and to the changes in size of microglia (Kobayashi et al. 2013; Zheng et al. 2014).

All these findings indicate that purinergic receptors fine tune different populations of sensory neurons and glial cells in the spinal cord and dorsal horn to modulate nociceptive sensitivity. Before ADP formation and activation of P2Y receptors, ATP can exert a direct and acute

stimulation of nociceptive signalling through the activation of ionotropic P2X receptors, mainly P2X2/3 receptors present in DRG neurons (Lewis et al. 1995). In addition, P2X4 receptors participate in the maintenance of neuropathic pain by acting on microglial cells (Inoue and Tsuda 2012). A similar interaction between $P2Y_1$ and $P2Y_{13}$ receptors occurs at the axonal growth cone, where both receptors modulate intracellular signalling triggered by P2X7 receptors to control axonal elongation and sprouting (del Puerto et al. 2012). Moreover, the $P2Y_{13}$ receptor antagonist MRS2211 accelerates neurite outgrowth in PC12, Neuro2a and MEB5 cells (Yano et al. 2012).

6.3.2 Cell Survival and Neuroprotection

In contrast to the pro-apoptotic role of $P2Y_{13}$ receptors in enteric neurons and pancreatic β cells, these receptors play a predominant survival role in the central nervous system. $P2Y_{13}$ receptors promoted the survival of cerebellar astrocytes and granule neurons, both neural models in which $P2Y_{13}$ is co-expressed with $P2Y_1$ receptors, and where specialized functions are elicited by their coupling to different signalling targets. In granule neurons, $P2Y_{13}$ receptors provide neuroprotection against different types of apoptotic stimuli and their activation protects granule neurons from oxidative stress induced by hydrogen peroxide. Indeed, both the production of reactive oxygen species and cell death induced by H_2O_2 treatment diminish after a 2 h pre-treatment with the $P2Y_{13}$ agonist, 2MeSADP. The intracellular mechanism responsible for this neuroprotective effect involves activation of the antioxidant axis Nrf2/heme oxygenase-1 (HO-1). Nrf2 transcriptional activity induces the expression of HO-1, whose levels increase 6 h after $P2Y_{13}$ receptor activation. Both HO-1 expression and survival in response to oxidative stress are prevented in cultured neurons obtained from Nrf2 knockout mice, demonstrating that the antioxidant Nrf2/HO-1 axis is functional in granule neurons and that it is regulated by $P2Y_{13}$ receptors (Perez-Sen et al. 2015; Espada et al. 2010). Similar neuroprotection against oxidative stress is elicited by ADP-P2Y receptors in astrocytes (unpublished results).

Along similar lines, $P2Y_{13}$ receptors also prevent cell death induced by toxic extracellular concentrations of glutamate. The neuroprotection elicited by 2MeSADP is not as potent as that exerted by well-known trophic factors in granule neurons, such as the neurotrophin BDNF (brain derived neurotrophic factor), although it shares a similar mechanism of action. Indeed, a 2 h pre-treatment with both 2MeSADP and BDNF prevents caspase-3 activation in a manner dependent on the activation of the ERK/CREB pathway. The increase in neuron survival promoted by 2MeSADP is also abolished by the antagonist MRS2211 and the PI3K inhibitor wortmannin, again validating the contribution of a Gi-coupled PI3K activity triggered by the $P2Y_{13}$ receptor (Ortega et al. 2011).

$P2Y_{13}$ receptors also protect against other kinds of stress. Genotoxic stress induced by both UV exposure and cisplatin, the cytotoxic drug employed in chemotherapy, also produces apoptotic cell death of granule neurons. Again, $P2Y_{13}$ receptor activation enhances cell survival in these conditions and this protection was abolished by MRS2211 (Morente et al. 2014). In addition, to the PI3K and ERK signalling activated by $P2Y_{13}$ receptors, other signalling mechanisms appear to contribute to the increase in cell survival. Interestingly, these stress conditions promote MAPK p38 over-activation and long-term accumulation of phosphorylated p38 in the nucleus. 2MeSADP pre-treatment before exposure to the cytotoxic stimuli decreases nuclear p38 phosphorylation towards basal levels, indicative of an activation of protein phosphatase activity. In particular, the induction of the expression of an immediate early gene, *Dusp2*, is promoted by 2MeSADP-mediated ERK1,2 signalling in granule neurons (Morente et al. 2014). DUSP2 is a dual specificity phosphatase with a nuclear localization that is selective for p38 and JNK MAPK. Therefore, $P2Y_{13}$ receptors participate in homeostatic mechanisms

in granule neurons contributing to bidirectional regulation of MAPK signalling cascades (Perez-Sen et al. 2015; Marin-Garcia et al. 2009).

7 Concluding Remarks

The data available regarding the $P2Y_{13}$ receptor reflects the great efforts and advances made by scientists working in this area. It is currently accepted that the protein sequence of the $P2Y_{13}$ receptor is very similar to that of the $P2Y_{12}$ receptor, for which a crystallographic structure is available. Thus, plausible explanations for the similarities in their pharmacological properties can be drawn, which in turn make their clear and unequivocal characterization more difficult. However, the presence of two pockets in the receptor structure could explain the agonistic effect of the Ap_3A diadenosine triphosphate, and the possible existence of an allosteric modulatory site near to the orthosteric site where the endogenous agonistic ligand, ADP, binds. Hence, the development of so-called biotopic orthosteric/allosteric ligands could define a functionally selective ligand able to specifically discriminate the members of this receptor subfamily, mainly $P2Y_{12}$, although this field remains unexplored. Nevertheless, such biotopic GPCR agonists and antagonists have recently been reported for the muscarinic receptor M2 and A1 adenosine receptor (Keov et al. 2011). Beyond the similarities between the $P2Y_{13}$ and $P2Y_{12}$ receptor, their expression does not seem to overlap in different tissues, which favours their specialized functions. In addition, while they share some signalling properties, $P2Y_{13}$ is distinct in its ability to switch to Gq proteins and calcium mobilization pathways, as well as to activate signalling cascades that seem to be exclusive to them.

On the other hand, there are no data available concerning the formation of homo- or heterodimers of $P2Y_{13}$, even though this receptor coexists in most cells with other P2Y receptors and GPCRs. Indeed, the complexity of the signalling cascades highlights the possibility of a broad crosstalk among these receptors. The co-expression of $P2Y_{13}$ together with $P2Y_1$ receptors in many cellular models seems to be a general rule of particular interest. The best-known example of interactions between two P2Y ADP-responding receptors is that previously described in platelets for $P2Y_1$ and $P2Y_{12}$ receptors. Both these receptors can act in a coordinated way to regulate complementary functions, or even to behave in an antagonistic fashion. Thus, $P2Y_{13}$ appears to interact with the $P2Y_1$ receptor in the axonal growth cone, modulating the signalling of the ionotropic P2X7 receptor and resulting in axonal elongation, just one such example among many (del Puerto et al. 2012). These interactions testify to the complexity and broad possibilities of the signalling cascades that regulate metabolism. It is noteworthy that beyond the classical pathways regulated by protein kinases, $P2Y_{13}$ can also induce the expression of different genes involved in protection against oxidative stress. Likewise, it can drive the expression of protein phosphatases like the dual phosphatases family member, DUSP, which can restore the steady cell state and allow the cell to respond to new agonist challenges.

In the near future, more precise and specific pharmacological agonists and antagonists may become available, as well as molecular tools. Thus, the pharmacological potential of $P2Y_{13}$ receptors as a target in pathophysiological situations will be revealed in full, advancing the biomedical horizons for this purinergic receptor.

Acknowledgments We are grateful to Dr. Mark Sefton for the english copy-editing of the manuscript. The work in the authors' laboratory is funded by the Spanish Ministerio de Economia y Competitividad (MINECO, BFU 2014-53654-P) and Red de excelencia Consolider-Ingenio Spanish Ion Channel Initiative" (BFU2015-70067REDC); by the Comunidad de Madrid (BRADE-CM S2013/ICE-2958), UCM-Santander (PR26/16-18B-3) and by the Fundación Ramón Areces Grant (PR2018/16-02). F. Ortega is a recipient of a Ramón y Cajal contract (RYC-2013-13290).

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical Approvals This article does not contain any studies with human participants or animals performed by any of the authors.

References

Alexander SP, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Southan C, Davies JA, Collaborators C (2015) The concise guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. Br J Pharmacol 172 (24):5744–5869

Amisten S, Meidute-Abaraviciene S, Tan C, Olde B, Lundquist I, Salehi A, Erlinge D (2010) ADP mediates inhibition of insulin secretion by activation of P2Y13 receptors in mice. Diabetologia 53(9):1927–1934

Ando RD, Mehesz B, Gyires K, Illes P, Sperlagh B (2010) A comparative analysis of the activity of ligands acting at P2X and P2Y receptor subtypes in models of neuropathic, acute and inflammatory pain. Br J Pharmacol 159(5):1106–1117

Biver G, Wang N, Gartland A, Orriss I, Arnett TR, Boeynaems JM, Robaye B (2013) Role of the P2Y13 receptor in the differentiation of bone marrow stromal cells into osteoblasts and adipocytes. Stem Cells 31 (12):2747–2758

Bjorquist A, Di Buduo CA, Femia EA, Storey RF, Becker RC, Balduini A, Nylander S, Cattaneo M (2016) Studies of the interaction of ticagrelor with the P2Y13 receptor and with P2Y13-dependent pro-platelet formation by human megakaryocytes. Thromb Haemost 116(6):1079–1088

Blom D, Yamin TT, Champy MF, Selloum M, Bedu E, Carballo-Jane E, Gerckens L, Luell S, Meurer R, Chin J, Mudgett J, Puig O (2010) Altered lipoprotein metabolism in P2Y(13) knockout mice. Biochim Biophys Acta 1801(12):1349–1360

Bunyavanich S, Boyce JA, Raby BA, Weiss ST (2012) Gene-by-environment effect of house dust mite on purinergic receptor P2Y12 (P2RY12) and lung function in children with asthma. Clin Exp Allergy 42 (2):229–237

Carrasquero LM, Delicado EG, Jimenez AI, Perez-Sen R, Miras-Portugal MT (2005) Cerebellar astrocytes co-express several ADP receptors. Presence of functional P2Y(13)-like receptors. Purinergic Signal 1 (2):153–159

Carrasquero LM, Delicado EG, Bustillo D, Gutierrez-Martin Y, Artalejo AR, Miras-Portugal MT (2009) P2X7 and P2Y13 purinergic receptors mediate intracellular calcium responses to BzATP in rat cerebellar astrocytes. J Neurochem 110(3):879–889

Caseley EA, Muench SP, Roger S, Mao HJ, Baldwin SA, Jiang LH (2014) Non-synonymous single nucleotide polymorphisms in the P2X receptor genes: association with diseases, impact on receptor functions and potential use as diagnosis biomarkers. Int J Mol Sci 15(8):13344–13371

Cattaneo M, Gachet C (1999) ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 19 (10):2281–2285

Cavallari U, Trabetti E, Malerba G, Biscuola M, Girelli D, Olivieri O, Martinelli N, Angiolillo DJ, Corrocher R, Pignatti PF (2007) Gene sequence variations of the platelet P2Y12 receptor are associated with coronary artery disease. BMC Med Genet 8:59

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM (2001) Identification of a novel human ADP receptor coupled to G(i). J Biol Chem 276(44):41479–41485

Csolle C, Heinrich A, Kittel A, Sperlagh B (2008) P2Y receptor mediated inhibitory modulation of noradrenaline release in response to electrical field stimulation and ischemic conditions in superfused rat hippocampus slices. J Neurochem 106(1):347–360

Cuadrado A (2015) Structural and functional characterization of Nrf2 degradation by glycogen synthase kinase 3/beta-TrCP. Free Radic Biol Med 88 (Pt B):147–157

del Puerto A, Diaz-Hernandez JI, Tapia M, Gomez-Villafuertes R, Benitez MJ, Zhang J, Miras-Portugal MT, Wandosell F, Diaz-Hernandez M, Garrido JJ (2012) Adenylate cyclase 5 coordinates the action of ADP, P2Y1, P2Y13 and ATP-gated P2X7 receptors on axonal elongation. J Cell Sci 125(Pt 1):176–188

Diaz-Hernandez JI, Gomez-Villafuertes R, Leon-Otegui-M, Hontecillas-Prieto L, Del Puerto A, Trejo JL, Lucas JJ, Garrido JJ, Gualix J, Miras-Portugal MT, Diaz-Hernandez M (2012) In vivo P2X7 inhibition reduces amyloid plaques in Alzheimer's disease through GSK3beta and secretases. Neurobiol Aging 33(8):1816–1828

Dohi T, Morita K, Kitayama T, Motoyama N, Morioka N (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. Pharmacol Ther 123(1):54–79

Dores MR, Trejo J (2012) Ubiquitination of G protein-coupled receptors: functional implications and drug discovery. Mol Pharmacol 82(4):563–570

Espada S, Ortega F, Molina-Jijon E, Rojo AI, Perez-Sen R, Pedraza-Chaverri J, Miras-Portugal MT, Cuadrado A (2010) The purinergic P2Y(13) receptor activates the Nrf2/HO-1 axis and protects against oxidative stress-induced neuronal death. Free Radic Biol Med 49(3):416–426

Fabre AC, Malaval C, Ben Addi A, Verdier C, Pons V, Serhan N, Lichtenstein L, Combes G, Huby T, Briand F, Collet X, Nijstad N, Tietge UJ, Robaye B, Perret B, Boeynaems JM, Martinez LO (2010) P2Y13 receptor is critical for reverse cholesterol transport. Hepatology 52(4):1477–1483

Ferreira MA, Jansen R, Willemsen G, Penninx B, Bain LM, Vicente CT, Revez JA, Matheson MC, Hui J, Tung JY, Baltic S, Le Souef P, Montgomery GW, Martin NG, Robertson CF, James A, Thompson PJ,

Boomsma DI, Hopper JL, Hinds DA, Werder RB, Phipps S, Australian Asthma Genetics Consortium C (2016) Gene-based analysis of regulatory variants identifies 4 putative novel asthma risk genes related to nucleotide synthesis and signaling. J Allergy Clin Immunol 139(4):1148–1157

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio MP (2004) Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. Biochem Pharmacol 68 (1):113–124

Gachet C, Hechler B, Leon C, Vial C, Leray C, Ohlmann P, Cazenave JP (1997) Activation of ADP receptors and platelet function. Thromb Haemost 78 (1):271–275

Goffinet M, Tardy C, Boubekeur N, Cholez G, Bluteau A, Oniciu DC, Lalwani ND, Dasseux JL, Barbaras R, Baron R (2014) P2Y13 receptor regulates HDL metabolism and atherosclerosis in vivo. PLoS One 9 (4):e95807

Gualix J, Fideu MD, Pintor J, Rotllan P, Garcia-Carmona F, Miras-Portugal MT (1997) Characterization of diadenosine polyphosphate transport into chromaffin granules from adrenal medulla. FASEB J 11 (12):981–990

Gualix J, Gomez-Villafuertes R, Pintor J, Llansola M, Felipo V, Miras-Portugal MT (2014) Presence of diadenosine polyphosphates in microdialysis samples from rat cerebellum in vivo: effect of mild hyperammonemia on their receptors. Purinergic Signal 10(2):349–356

Guarracino JF, Cinalli AR, Fernandez V, Roquel LI, Losavio AS (2016) P2Y13 receptors mediate presynaptic inhibition of acetylcholine release induced by adenine nucleotides at the mouse neuromuscular junction. Neuroscience 326:31–44

Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP, Gachet C (1998) The P2Y1 receptor is necessary for adenosine 5′-diphosphate-induced platelet aggregation. Blood 92(1):152–159

Hervas C, Perez-Sen R, Miras-Portugal MT (2003) Coexpression of functional P2X and P2Y nucleotide receptors in single cerebellar granule cells. J Neurosci Res 73(3):384–399

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409(6817):202–207

Inoue K, Tsuda M (2012) P2X4 receptors of microglia in neuropathic pain. CNS Neurol Disord Drug Targets 11 (6):699–704

Jacobson KA, Gao ZG, Paoletta S, Kiselev E, Chakraborty S, Jayasekara PS, Balasubramanian R, Tosh DK (2015) John Daly lecture: structure-guided drug design for adenosine and P2Y receptors. Comput Struct Biotechnol J 13:286–298

Jacquet S, Malaval C, Martinez LO, Sak K, Rolland C, Perez C, Nauze M, Champagne E, Terce F, Gachet C, Perret B, Collet X, Boeynaems JM, Barbaras R (2005) The nucleotide receptor P2Y13 is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis. Cell Mol Life Sci 62(21):2508–2515

Jimenez AI, Castro E, Mirabet M, Franco R, Delicado EG, Miras-Portugal MT (1999) Potentiation of ATP calcium responses by A2B receptor stimulation and other signals coupled to Gs proteins in type-1 cerebellar astrocytes. Glia 26(2):119–128

Jimenez AI, Castro E, Communi D, Boeynaems JM, Delicado EG, Miras-Portugal MT (2000) Coexpression of several types of metabotropic nucleotide receptors in single cerebellar astrocytes. J Neurochem 75(5):2071–2079

Jimenez E, Zafra F, Perez-Sen R, Delicado EG, Miras-Portugal MT, Aragon C, Lopez-Corcuera B (2011) P2Y purinergic regulation of the glycine neurotransmitter transporters. J Biol Chem 286 (12):10712–10724

Keov P, Sexton PM, Christopoulos A (2011) Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. Neuropharmacology 60 (1):24–35

Kim YC, Lee JS, Sak K, Marteau F, Mamedova L, Boeynaems JM, Jacobson KA (2005) Synthesis of pyridoxal phosphate derivatives with antagonist activity at the P2Y13 receptor. Biochem Pharmacol 70 (2):266–274

Kim KA, Song WG, Lee HM, Joo HJ, Park JY (2013) Effect of P2Y1 and P2Y12 genetic polymorphisms on the ADP-induced platelet aggregation in a Korean population. Thromb Res 132(2):221–226

Kiselev E, Barrett MO, Katritch V, Paoletta S, Weitzer CD, Brown KA, Hammes E, Yin AL, Zhao Q, Stevens RC, Harden TK, Jacobson KA (2014) Exploring a 2-naphthoic acid template for the structure-based design of P2Y14 receptor antagonist molecular probes. ACS Chem Biol 9(12):2833–2842

Kobayashi K, Yamanaka H, Fukuoka T, Dai Y, Obata K, Noguchi K (2008) P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. J Neurosci 28(11):2892–2902

Kobayashi K, Yamanaka H, Yanamoto F, Okubo M, Noguchi K (2012) Multiple P2Y subtypes in spinal microglia are involved in neuropathic pain after peripheral nerve injury. Glia 60(10):1529–1539

Kobayashi K, Yamanaka H, Noguchi K (2013) Expression of ATP receptors in the rat dorsal root ganglion and spinal cord. Anat Sci Int 88(1):10–16

Koch H, von Kugelgen I, Starke K (1997) P2-receptor-mediated inhibition of noradrenaline release in the rat hippocampus. Naunyn Schmiedeberg's Arch Pharmacol 355(6):707–715

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M,

Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG, Exome Aggregation C (2016) Analysis of protein-coding genetic variation in 60,706 humans. Nature 536(7616):285–291

Leon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C (1997) The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. FEBS Lett 403(1):26–30

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377 (6548):432–435

Li MP, Tang J, Wen ZP, Zhang YJ, Zhang W, Zhou HH, Zhang ZL, Chen XP (2015) Influence of P2Y12 polymorphisms on platelet activity but not ex-vivo antiplatelet effect of ticagrelor in healthy Chinese male subjects. Blood Coagul Fibrinolysis 26 (8):874–881

Lichtenstein L, Serhan N, Annema W, Combes G, Robaye B, Boeynaems JM, Perret B, Tietge UJ, Laffargue M, Martinez LO (2013) Lack of P2Y13 in mice fed a high cholesterol diet results in decreased hepatic cholesterol content, biliary lipid secretion and reverse cholesterol transport. Nutr Metab (Lond) 10 (1):67

Lichtenstein L, Serhan N, Espinosa-Delgado S, Fabre A, Annema W, Tietge UJ, Robaye B, Boeynaems JM, Laffargue M, Perret B, Martinez LO (2015) Increased atherosclerosis in P2Y13/apolipoprotein E double-knockout mice: contribution of P2Y13 to reverse cholesterol transport. Cardiovasc Res 106(2):314–323

Lordkipanidze M, Pharand C, Schampaert E, Palisaitis DA, Diodati JG (2011) Heterogeneity in platelet cyclooxygenase inhibition by aspirin in coronary artery disease. Int J Cardiol 150(1):39–44

Lustig KD, Shiau AK, Brake AJ, Julius D (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci U S A 90 (11):5113–5117

Malaval C, Laffargue M, Barbaras R, Rolland C, Peres C, Champagne E, Perret B, Terce F, Collet X, Martinez LO (2009) RhoA/ROCK I signalling downstream of the P2Y13 ADP-receptor controls HDL endocytosis in human hepatocytes. Cell Signal 21(1):120–127

Malin SA, Molliver DC (2010) Gi- and Gq-coupled ADP (P2Y) receptors act in opposition to modulate nociceptive signaling and inflammatory pain behavior. Mol Pain 6:21

Maqbool M, Mobashir M, Hoda N (2016) Pivotal role of glycogen synthase kinase-3: a therapeutic target for Alzheimer's disease. Eur J Med Chem 107:63–81

Marin-Garcia P, Sanchez-Nogueiro J, Diez A, Leon-Otegui M, Linares M, Garcia-Palencia P, Bautista JM, Miras-Portugal MT (2009) Altered nucleotide receptor expression in a murine model of cerebral malaria. J Infect Dis 200(8):1279–1288

Marschallinger J, Schaffner I, Klein B, Gelfert R, Rivera FJ, Illes S, Grassner L, Janssen M, Rotheneichner P, Schmuckermair C, Coras R, Boccazzi M, Chishty M, Lagler FB, Renic M, Bauer HC, Singewald N, Blumcke I, Bogdahn U, Couillard-Despres S, Lie DC, Abbracchio MP, Aigner L (2015) Structural and functional rejuvenation of the aged brain by an approved anti-asthmatic drug. Nat Commun 6:8466

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM, Gonzalez NS (2003) Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 64(1):104–112

Marteau F, Communi D, Boeynaems JM, Suarez Gonzalez N (2004) Involvement of multiple P2Y receptors and signaling pathways in the action of adenine nucleotides diphosphates on human monocyte-derived dendritic cells. J Leukoc Biol 76 (4):796–803

Martinez LO, Najib S, Perret B, Cabou C, Lichtenstein L (2015) Ecto-F1-ATPase/P2Y pathways in metabolic and vascular functions of high density lipoproteins. Atherosclerosis 238(1):89–100

Marucci G, Dal Ben D, Lambertucci C, Santinelli C, Spinaci A, Thomas A, Volpini R, Buccioni M (2016) The G protein-coupled receptor GPR17: overview and update. ChemMedChem 11(23):2567–2574

May LT, Leach K, Sexton PM, Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. Annu Rev Pharmacol Toxicol 47:1–51

Melancon BJ, Hopkins CR, Wood MR, Emmitte KA, Niswender CM, Christopoulos A, Conn PJ, Lindsley CW (2012) Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. J Med Chem 55(4):1445–1464

Milewicz DM, Seidman CE (2000) Genetics of cardiovascular disease. Circulation 102(20 Suppl 4):IV103–IV111

Morente V, Perez-Sen R, Ortega F, Huerta-Cepas J, Delicado EG, Miras-Portugal MT (2014) Neuroprotection elicited by P2Y13 receptors against genotoxic stress by inducing DUSP2 expression and MAPK signaling recovery. Biochim Biophys Acta 1843(9):1886–1898

Oestreich JH, Steinhubl SR, Ferraris SP, Loftin CD, Akers WS (2014) Effect of genetic variation in P2Y12 on TRAP-stimulated platelet response in healthy subjects. J Thromb Thrombolysis 38 (3):372–379

Orriss I, Syberg S, Wang N, Robaye B, Gartland A, Jorgensen N, Arnett T, Boeynaems JM (2011) Bone phenotypes of P2 receptor knockout mice. Front Biosci (Schol Ed) 3:1038–1046

Ortega F, Perez-Sen R, Miras-Portugal MT (2008) Gi-coupled P2Y-ADP receptor mediates GSK-3 phosphorylation and beta-catenin nuclear translocation in granule neurons. J Neurochem 104(1):62–73

Ortega F, Perez-Sen R, Delicado EG, Teresa Miras-Portugal M (2011) ERK1/2 activation is involved in the neuroprotective action of P2Y13 and P2X7 receptors against glutamate excitotoxicity in cerebellar granule neurons. Neuropharmacology 61 (8):1210–1221

Ou W, He Y, Li A, Liu B, Jin L (2016) Genotype frequencies of CYP2C19, P2Y12 and GPIIIa polymorphisms in coronary heart disease patients of Han ethnicity, and their impact on Clopidogrel responsiveness. Int Heart J 57(5):586–592

Paoletta S, Sabbadin D, von Kugelgen I, Hinz S, Katritch V, Hoffmann K, Abdelrahman A, Strassburger J, Baqi Y, Zhao Q, Stevens RC, Moro S, Muller CE, Jacobson KA (2015) Modeling ligand recognition at the P2Y12 receptor in light of X-ray structural information. J Comput Aided Mol Des 29(8):737–756

Patel K, Barnes A, Camacho J, Paterson C, Boughtflower R, Cousens D, Marshall F (2001) Activity of diadenosine polyphosphates at P2Y receptors stably expressed in 1321N1 cells. Eur J Pharmacol 430(2–3):203–210

Perez-Sen R, Queipo MJ, Morente V, Ortega F, Delicado EG, Miras-Portugal MT (2015) Neuroprotection mediated by P2Y13 nucleotide receptors in neurons. Comput Struct Biotechnol J 13:160–168

Pons V, Serhan N, Gayral S, Malaval C, Nauze M, Malet N, Laffargue M, Gales C, Martinez LO (2014) Role of the ubiquitin-proteasome system in the regulation of P2Y13 receptor expression: impact on hepatic HDL uptake. Cell Mol Life Sci 71 (9):1775–1788

Queiroz G, Talaia C, Goncalves J (2003) ATP modulates noradrenaline release by activation of inhibitory P2Y receptors and facilitatory P2X receptors in the rat vas deferens. J Pharmacol Exp Ther 307(2):809–815

Rada P, Rojo AI, Evrard-Todeschi N, Innamorato NG, Cotte A, Jaworski T, Tobon-Velasco JC, Devijver H, Garcia-Mayoral MF, Van Leuven F, Hayes JD, Bertho G, Cuadrado A (2012) Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. Mol Cell Biol 32(17):3486–3499

Rojo AI, Rada P, Egea J, Rosa AO, Lopez MG, Cuadrado A (2008) Functional interference between glycogen synthase kinase-3 beta and the transcription factor Nrf2 in protection against kainate-induced hippocampal cell death. Mol Cell Neurosci 39(1):125–132

Savi P, Beauverger P, Labouret C, Delfaud M, Salel V, Kaghad M, Herbert JM (1998) Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 422(3):291–295

Selden NR, Carlson JD, Cetas J, Close LN, Heinricher MM (2007) Purinergic actions on neurons that modulate nociception in the rostral ventromedial medulla. Neuroscience 146(4):1808–1816

Serhan N, Cabou C, Verdier C, Lichtenstein L, Malet N, Perret B, Laffargue M, Martinez LO (2013) Chronic pharmacological activation of P2Y13 receptor in mice decreases HDL-cholesterol level by increasing hepatic HDL uptake and bile acid secretion. Biochim Biophys Acta 1831(4):719–725

Shaver SR, Rideout JL, Pendergast W, Douglass JG, Brown EG, Boyer JL, Patel RI, Redick CC, Jones AC, Picher M, Yerxa BR (2005) Structure-activity relationships of dinucleotides: potent and selective agonists of P2Y receptors. Purinergic Signal 1 (2):183–191

Siasos G, Kioufis S, Oikonomou E, Zaromitidou M, Maniatis K, Vavuranakis M, Kokkou E, Tousoulis D (2016) Impact of C34T P2Y12 ADP receptor polymorphism and smoking status on cardiovascular outcome in coronary artery disease patients receiving clopidogrel. Int J Cardiol 210:161–163

Tan C, Salehi A, Svensson S, Olde B, Erlinge D (2010) ADP receptor P2Y(13) induce apoptosis in pancreatic beta-cells. Cell Mol Life Sci 67(3):445–453

Tan C, Voss U, Svensson S, Erlinge D, Olde B (2013) High glucose and free fatty acids induce beta cell apoptosis via autocrine effects of ADP acting on the P2Y(13) receptor. Purinergic Signal 9(1):67–79

Tatsumi E, Yamanaka H, Kobayashi K, Yagi H, Sakagami M, Noguchi K (2015) RhoA/ROCK pathway mediates p38 MAPK activation and morphological changes downstream of P2Y12/13 receptors in spinal microglia in neuropathic pain. Glia 63(2):216–228

Timur AA, Murugesan G, Zhang L, Aung PP, Barnard J, Wang QK, Gaussem P, Silverstein RL, Bhatt DL, Kottke-Marchant K (2012) P2RY1 and P2RY12 polymorphisms and on-aspirin platelet reactivity in patients with coronary artery disease. Int J Lab Hematol 34(5):473–483

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. J Neurosci 28(19):4949–4956

Van Kolen K, Slegers H (2004) P2Y12 receptor stimulation inhibits beta-adrenergic receptor-induced differentiation by reversing the cyclic AMP-dependent inhibition of protein kinase B. J Neurochem 89 (2):442–453

Van Kolen K, Gilany K, Moens L, Esmans EL, Slegers H (2006) P2Y12 receptor signalling towards PKB proceeds through IGF-I receptor cross-talk and requires activation of Src, Pyk2 and Rap1. Cell Signal 18 (8):1169–1181

von Kugelgen I, Hoffmann K (2016) Pharmacology and structure of P2Y receptors. Neuropharmacology 104:50–61

von Kugelgen I, Spath L, Starke K (1994) Evidence for P2-purinoceptor-mediated inhibition of noradrenaline release in rat brain cortex. Br J Pharmacol 113 (3):815–822

Voss U, Turesson MF, Robaye B, Boeynaems JM, Olde B, Erlinge D, Ekblad E (2014) The enteric nervous system of P2Y13 receptor null mice is resistant against high-fat-diet- and palmitic-acid-induced neuronal loss. Purinergic Signal 10(3):455–464

Wang Z, Nakayama T, Sato N, Yamaguchi M, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Ozawa Y, Ma Y, Doba N, Hinohara S (2009a) Purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene is associated with cerebral infarction in Japanese subjects. Hypertens Res 32(11):989–996

Wang ZX, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Matsumoto K, Ozawa Y, Ma YT, Doba N, Hinohara S (2009b) Association of the purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene with myocardial infarction in Japanese men. Circ J 73(12):2322–2329

Wang Z, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Ohta M, Soma M, Aoi N, Ozawa Y, Ma Y (2010) The purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene associated with essential hypertension in Japanese men. J Hum Hypertens 24 (5):327–335

Wang N, Robaye B, Agrawal A, Skerry TM, Boeynaems JM, Gartland A (2012) Reduced bone turnover in mice lacking the P2Y13 receptor of ADP. Mol Endocrinol 26(1):142–152

Wang N, Rumney RM, Yang L, Robaye B, Boeynaems JM, Skerry TM, Gartland A (2013) The P2Y13 receptor regulates extracellular ATP metabolism and the osteogenic response to mechanical loading. J Bone Miner Res 28(6):1446–1456

Wang N, Robaye B, Gossiel F, Boeynaems JM, Gartland A (2014) The P2Y13 receptor regulates phosphate metabolism and FGF-23 secretion with effects on skeletal development. FASEB J 28(5):2249–2259

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, Barnard EA (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett 324(2):219–225

Wesselius A, Bours MJ, Henriksen Z, Syberg S, Petersen S, Schwarz P, Jorgensen NR, van Helden S, Dagnelie PC (2013) Association of P2Y(2) receptor SNPs with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients. Purinergic Signal 9(1):41–49

Wirkner K, Schweigel J, Gerevich Z, Franke H, Allgaier C, Barsoumian EL, Draheim H, Illes P (2004) Adenine nucleotides inhibit recombinant N-type calcium channels via G protein-coupled mechanisms in HEK 293 cells; involvement of the P2Y13 receptor-type. Br J Pharmacol 141 (1):141–151

Yano S, Tsukimoto M, Harada H, Kojima S (2012) Involvement of P2Y13 receptor in suppression of neuronal differentiation. Neurosci Lett 518(1):5–9

Zee RY, Michaud SE, Diehl KA, Chasman DI, Emmerich J, Gaussem P, Aiach M, Ridker PM (2008) Purinergic receptor P2Y, G-protein coupled, 12 gene variants and risk of incident ischemic stroke, myocardial infarction, and venous thromboembolism. Atherosclerosis 197(2):694–699

Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao XD, Liu YH, Chen GD, Pramanik B, Laz TM, Palmer K, Bayne M, Monsma FJ (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J Biol Chem 276(11):8608–8615

Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma F Jr (2002) P2Y (13): identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. J Pharmacol Exp Ther 301(2):705–713

Zhang J, Zhang K, Gao ZG, Paoletta S, Zhang D, Han GW, Li T, Ma L, Zhang W, Muller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014a) Agonist-bound structure of the human P2Y12 receptor. Nature 509 (7498):119–122

Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, Moss SM, Paoletta S, Kiselev E, Lu W, Fenalti G, Zhang W, Muller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014b) Structure of the human P2Y12 receptor in complex with an antithrombotic drug. Nature 509 (7498):115–118

Zheng X, Liang Y, Kang A, Ma SJ, Xing L, Zhou YY, Dai C, Xie H, Xie L, Wang GJ, Hao HP (2014) Peripheral immunomodulation with ginsenoside Rg1 ameliorates neuroinflammation-induced behavioral deficits in rats. Neuroscience 256:210–222

Adv Exp Med Biol - Protein Reviews (2017) 19:169–192
DOI 10.1007/5584_2017_92

Published online: 22 August 2017

Pharmacological Properties and Biological Functions of the GPR17 Receptor, a Potential Target for Neuro-Regenerative Medicine

Marta Fumagalli, Davide Lecca, Giusy T. Coppolino, Chiara Parravicini, and Maria P. Abbracchio

Abstract

In 2006, cells heterologously expressing the "orphan" receptor GPR17 were shown to acquire responses to both uracil nucleotides and cysteinyl-leukotrienes, two families of signaling molecules accumulating in brain or heart as a result of hypoxic/traumatic injuries. In subsequent years, evidence of GPR17 key role in oligodendrogenesis and myelination has highlighted it as a "model receptor" for new therapies in demyelinating and neurodegenerative diseases. The apparently contrasting evidence in the literature about the role of GPR17 in promoting or inhibiting myelination can be due to its transient expression in the intermediate stages of differentiation, exerting a pro-differentiating function in early oligodendrocyte precursor cells (OPCs), and an inhibitory role in late stage maturing cells. Meanwhile, several papers extended the initial data on GPR17 pharmacology, highlighting a "promiscuous" behavior of this receptor; indeed, GPR17 is able to respond to other emergency signals like oxysterols or the pro-inflammatory cytokine SDF-1, underlying GPR17 ability to adapt its responses to changes of the surrounding extracellular milieu, including damage conditions. Here, we analyze the available literature on GPR17, in an attempt to summarize its emerging biological roles and pharmacological properties.

Marta Fumagalli and Davide Lecca contributed equally to this work.

M. Fumagalli, D. Lecca, G.T. Coppolino, C. Parravicini, and M.P. Abbracchio (✉)
Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy
e-mail: mariapia.abbracchio@unimi.it

Keywords
Differentiation • GPCR • Multiple sclerosis • Myelination • Oligodendrocyte precursor cells

Abbreviations

CNS	central nervous system
cysLT	cysteinyl-leukotrienes
EAE	experimental autoimmune encephalomyelitis
ERK1/2	extracellular signal–regulated kinases 1 and 2
FACS-MS	frontal affinity chromatography-mass spectrometry
GPCRs	G-protein coupled receptors
HM	homology modeling
Lys	lysolecithin
MCAo	middle cerebral artery occlusion
MS	multiple sclerosis
NC-IUPHAR	Nomenclature Committee of the International Union of Pharmacology
OLs	oligodendrocytes
OPCs	oligodendrocyte precursor cells
MBP	myelin basic protein

1 Introduction: The History of GPR17

In 2006, a paper was published where it was demonstrated that cells heterologously expressing the "orphan" receptor GPR17 (i.e., a molecularly identified, 339 amino acid-long G_i-protein-coupled receptor that still lacked a defined ligand) acquired responses to both uracil nucleotides (such as UDP, UDP-glucose, UDP-galactose) and cysteinyl-leukotrienes (cysLTs, like LTC_4 and LTD_4) (Ciana et al. 2006), two chemically unrelated families of signaling molecules that are known to massively accumulate in organs like the brain or the heart as a result of hypoxic/traumatic injuries. Uracil nucleotides and cysLTs were already known to exert multiple biological effects via the activation of separate G-protein-coupled receptors (GPCRs): the eight recognized P2Y receptor subtypes (the $P2Y_{1,2,4,6,11,12,13,14}$ receptors, (Abbracchio et al. 2006) and the two CysLT1 and CysLT2 receptors. Interestingly, the GPR17 sequence had been originally described as the result of a cloning strategy based on the use of RT-PCR degenerate oligonucleotide primers designed on the sequences of the $P2Y_1$ and $P2Y_2$ receptors, with the final aim of identifying new members of this receptor family (Blasius et al. 1998). GPR17 was later found to be at an intermediate structural and phylogenetic position between already known P2Y and CysLT receptors, and GPR99, recently proposed as the third CysLT receptor (also known as 2-oxoglutarate receptor 1, OXGR1) (Kanaoka et al. 2013) (Fig. 1), in the so called "purine receptor cluster" of class A GPCRs (Fredriksson et al. 2003). GPR17 also emerged as representing the closest receptor to a common ancestor that, during evolution, could have generated both P2Y and CysLT receptors (Ciana et al. 2006; Parravicini et al. 2008; Parravicini et al. 2010). To further highlight GPR17 structural similarity to the other members of the P2Y family, a partial sequence of the rat receptor was initially identified from rat striatum by employing oligonucleotide primers specifically designed on the sequence of human $P2Y_{11}$ (Lecca and Abbracchio 2008). Of note, a human GPR17 long splice variant encoding a receptor with a 28-amino acid longer NH2 terminal (for a total of 367 amino acids instead of 339) had been also identified in very early studies aimed at discovering new members of the chemokine receptor family (in this respect, see also Sect. 2.1) (Blasius et al. 1998). Genomic analysis revealed a three-exon structure of the hGPR17 gene, with two putative open reading frames. While the "short isoform" derives from splicing of the

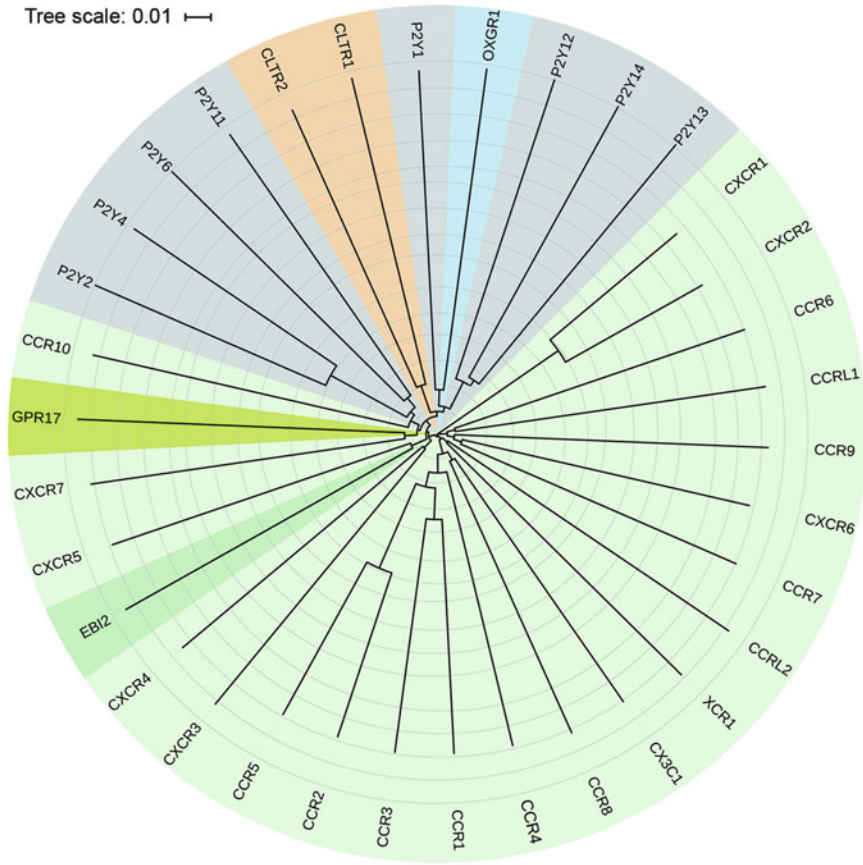

Fig. 1 Phylogenetic tree illustrating the relationship of GPR17 to selected structurally related class-A GPCRs. The evolutionary relationship analysis is based on a multiple sequence alignment performed on homologous GPCR sequences using TM-Coffee, a module of the T-Coffee package optimized for transmembrane proteins (Chang et al. 2012). Receptors belonging to the same family are clustered according to the following color code: *grey* for purinergic receptors (P2Y), *orange* for cysteinyl-leukotriene receptors (CysLT), *light green* for chemokine receptors (CXCRn, CCRn, XCRn), *emerald green* for Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2), *sky blue* for 2-Oxoglutarate receptor 1 (OXGR1/GPR99), *apple green* for GPR17

second exon, the "long one" contains all three exons of the hGPR17 gene, leading to a transcript which is 1104 bp in length (Blasius et al. 1998; Pugliese et al. 2009). Interestingly, quantitative gene expression studies revealed that GPR17 short isoform is expressed more abundantly in the brain than the long one (a tenfold increase), whereas the opposite was observed in heart and kidney. Pharmacological profile of the long isoform also showed that some differences exist between the two GPR17 receptor isoforms (Pugliese et al. 2009; Benned-Jensen and Rosenkilde 2010).

In 2006, there were already papers reporting functional interactions between "classical" P2Y and CysLT receptors. For example, under some conditions, the CysLT1 receptor antagonist montelukast effectively antagonized the responses evoked by purinergic P2Y receptors (Capra et al. 2005; Mamedova et al. 2005). Conversely, the $P2Y_{12}$ receptor had been reported to be also activated by LTE_4 (Paruchuri et al. 2009), suggesting the existence of some kind of ligand/receptor promiscuity between the P2Y and CysLT receptor families. On this basis, the identification of GPR17 as the first dual member of

the "purine receptor cluster" able to respond to both purinergic and cysLT ligands (Ciana et al. 2006) represented the demonstration of a further level of interaction between these two chemically unrelated, but functionally interconnected, systems. Later studies extended the response profile of this receptor to other classes of endogenous "emergency" molecules connected to oxidative stress, neuroinflammation and neurodegeneration, i.e., oxysterols and chemokine stromal derived factor-1 (SDF-1) (Parravicini et al. 2016), further highlighting the promiscuous behaviour of GPR17. Of note, a phylogenetic analysis among structurally related class-A GPCRs (Parravicini et al. 2008, 2010, 2016; Sensi et al. 2014), suggests that, besides P2Y and CysLT receptors, GPR17 holds a tight evolutionary relationship also with chemokine receptors and Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) (Fig. 1). The possibility that GPR17 can be activated by diverse family of ligands underlines the relevance of a new transversal signaling mechanism that synchronizes all these emergency molecules and their receptors under specific neurodegenerative conditions. Such a high promiscuity in receptor behaviour is often found in receptors involved in immunological responses and may, at least in part, depend on GPR17 ability to form dimers with other related receptors, thus widening the array of pharmacological responses (see also Sect. 2.2.1).

In subsequent years, new data have revealed a key role for GPR17 in oligodendrogenesis and myelination (Lecca et al. 2008; Chen et al. 2009). However, while some authors have provided evidence for a stimulatory role of GPR17 in the specification and maturation of oligodendrocyte precursor cells (OPCs), some others have proposed an inhibitory role. Here, we aim at analyzing all the available literature on GPR17 in an attempt to provide an overview of the different biological and pharmacological data emerged from all these papers.

2 GPR17 Characterization

2.1 Receptor Structure, Amino Acid Homology with Phylogenetically Related GPCRs and Binding Sites

GPR17 displays the typical 7-transmembrane (TM) domain topology of GPCRs, with an amino acid identity with the known P2Y and CysLT receptors between 21 and 48% (Abbracchio et al. 2006; Lecca and Abbracchio 2008). All these receptors show partial or complete conservation of a H-X-X-R/K amino acid motif in TM6 (and also of a K-E-X-X-L motif in TM7, in the case of $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) that are important for ligand recognition and have been proposed to represent specific molecular signatures for these receptors (Lecca and Abbracchio 2008) (see also below). Homology Modelling (HM) studies combined with other *in silico* tools have been performed to raise hypothesis on the molecular interaction between GPR17 and its putative endogenous ligands (Parravicini et al. 2008, 2010; Calleri et al. 2010), as well as to identify new potential ligands (Eberini et al. 2011) (see also Sect. 2.2.2). In these studies, *in silico* receptor modeling was performed using different templates, according to the progressive availability of new high-resolution GPCR structures. Starting from bovine rhodopsin, that, since 2000, has represented for many years the only atomistic scaffold for the structural investigation of GPCRs, the recent explosion in the resolution of GPCR crystal structures has given access to detailed structural information previously unavailable, allowing the construction of more and more accurate GPR17 models (Fig. 2).

For example, in 2010, crystallization of human CXCR4 (Wu et al. 2010) provided a significant improvement in the accuracy of GPR17 modelling because this structure enabled to reliably describe the extracellular regions of the receptor, especially extracellular loop 2 (ECL2) and the disulphide bridge linking the N-terminal to ECL3, known to be crucial in ligand molecular recognition (Wheatley et al. 2012), but for which none of the earlier templates was suitable. More

Fig. 2 Three-dimensional homology model of the human GPR17. Topological domains are represented as ribbon and coloured according to their secondary structure: *magenta* for alpha-helices; *yellow* for beta-sheets; white for loops and grey for turns

recently, modeling of GPR17 has been further improved thanks to the atomic resolution of the structures of two members of the P2Y receptor family: the human $P2Y_{12}$ (Zhang et al. 2014) and $P2Y_1$ (Zhang et al. 2015).

Globally, all the *in silico* results on the short isoform of GPR17 suggest that its nucleotide binding pocket is similar to that described for the other P2Y receptors (including the TM6 HXXR/K motif designated to accommodate the phosphate moieties of nucleotide ligands (Parravicini et al. 2008; Jiang et al. 1997), and that this site is also shared by other small molecules identified as GPR17 ligands, including oxysterols and new synthetic compounds (Sensi et al. 2014; Eberini et al. 2011). According to these studies, also the nucleotide-derivative antagonist cangrelor binds to the same binding pocket, behaving as a competitive antagonist for orthosteric ligands.

In both P2Y and CysLT1 and CysLT2 receptors, ligand binding is critically dependent on the basic arginine residue belonging to the conserved TM6 motif (Parravicini et al. 2010; Temporini et al. 2009). Computational studies suggested that this also holds true for Arg255 of GPR17 (Parravicini et al. 2008). To assess the actual role of this residue in receptor binding, this basic amino acid was mutated to isoleucine, and an *in silico* mutant GPR17 receptor (R255I) was generated. Using steered molecular dynamics simulations (SMD), forced unbinding of the endogenous ligand UDP from both wild type (WT) and R255I receptor models of GPR17 was modeled *in silico*. The energy required to unbind UDP from the nucleotide binding pocket of GPR17 was higher for the WT than for the mutated R255I receptor, and the exit of the ligand from its intracellular cavities occurred earlier in the R255I model compared to the WT receptor. Generation and expression of the mutated receptor in 1321N1 cells confirmed also *in vitro* that the mutation was not silent (Calleri et al. 2010).

Besides the orthosteric binding site, *in silico* studies suggested that GPR17 also possesses an "accessory" binding site in a region formed by extracellular loops ECL2, ECL3 and the N-terminal, which also faces the extracellular space. This external accessory binding site could guide small agonist ligands to the deeper principal binding site in a multistep mechanism of activation. Thanks to further *in silico* investigations, that showed the possibility of GPR17 to be stimulated also by a large peptide ligand such as SDF-1, the extracellular recognition site has been extensively characterized and GPR17 recognition mechanism has been compared to those of some peptide receptors (Parravicini et al. 2016), in which a two-step model of receptor activation, passing through both an extracellular and a TM binding site, has been proposed (Rajagopalan and Rajarathnam 2004).

Due to the intrinsic inaccuracy of the standard template-based HM techniques in predicting conformations of highly flexible and unaligned loop sequences in absence of adequate templates, no modeling studies are yet available for the long isoform of GPR17. Nevertheless, we can speculate that the N-terminal may influence the binding affinity of nucleotide agonists via a different

conformation of the external accessory binding site, resulting in a slightly different pharmacological profile of the long isoform with respect to the short one (Pugliese et al. 2009).

2.2 Pharmacology and Signaling Pathways

2.2.1 Putative Endogenous Ligands and Transduction Systems

In the initial studies, only GPR17 short isoform has been characterized, and both the human, rat (Ciana et al. 2006) and the previously unidentified mouse GPR17 receptors (Lecca and Ceruti 2008) were shown to respond to UDP, UDP-glucose, UDP-galactose and LTC_4 and LTD_4, with comparable profiles that were highly conserved across species (the chemical structures of UDP-glucose and LTD_4 are reported in Fig. 3). Interestingly, the concentration ranges at which uracil nucleotides and cysLTs activated GPR17 (i.e., μM and nM ranges, respectively) were fully consistent with those necessary for these endogenous ligands to activate their already known cognate P2Y and CysLT receptors (Abbracchio et al. 2006; Brink et al. 2003). Very similar agonist responses were detected in a number of different cell lines (1321N1, CHO, COS-7, HEK-293 cells). The 1321N1 cells was the most appropriate cells to test GPR17 responses, since they are one of the few cell lines that do not endogenously express any functional purinergic or CysLT receptors. Responses were highly specific, since no response was ever found in cells transfected with the empty vector. The antagonist response profile of GPR17 was also rather peculiar. Activation by uracil nucleotides was reversed by some typical purinergic antagonists like the $P2Y_1$ antagonist MRS2179 or the $P2Y_{12}$ antagonist cangrelor (Fig. 3). Conversely, responses to cysLTs were inhibited by typical CysLT receptor antagonists like the already marketed drug montelukast (Fig. 3) and pranlukast (Ciana et al. 2006).

GPR17 responses were demonstrated by using [^{35}S]GTPγS binding, a typical functional assay for agonists acting at G_i coupled receptors (Kotani et al. 2001; Marteau et al. 2003; Fumagalli et al. 2004). Under some circumstances, activation of GPR17 could also increase intracellular calcium levels via a phospholipase C mediated pathway; however, this effect occurred only in about 30% of 1321N1 transfected cells, suggesting preferential coupling to the adenylyl cyclase pathway (Ciana et al. 2006). In subsequent studies, GPR17 peculiar profile was confirmed in many other distinct assays independently performed in different laboratories. Concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase was also shown in oligodendendrocyte precursor cells (OPCs), the cell type natively expressing GPR17 at highest levels (Fumagalli et al. 2011a) (Table 1). Inhibition of cAMP was fully counteracted by the same antagonists utilized in the [^{35}S]GTPγS binding. In 2009, another paper appeared where, in GPR17 expressing 1321N1 cells, enhancement of an outward rectifying K^+ current was shown upon addition of either uracil nucleotides or cysLTs (Pugliese et al. 2009). These effects were blocked by MRS2179, cangrelor and montelukast. A few years later, these same authors showed that similar delayed rectifier K^+ currents were stimulated in a concentration dependent manner by GPR17 ligands in a subpopulation of OPCs and pre-oligodendrocytes, but not in terminally mature cells, fully in line with the transient expression of GPR17 during OPC specification (in this respect, see also Sect. 3.1.1) (Coppi et al. 2013). This effect was blocked by MRS2179 and cangrelor and sensitive to the K^+ channel blocker tetraethyl-ammonium. Importantly, the latter also inhibited oligodendrocyte maturation, to support previous literature data on the importance of these currents in OPC differentiation.

Fewer studies are available on hGPR17 long isoform. In the electrophysiological study already mentioned above, no significant differences between the short and long isoforms were detected (Pugliese et al. 2009). In 2010, Benned-Jensen and Rosenkilde independently confirmed the ability of heterologously expressed GPR17 to respond to uracil nucleotides in a

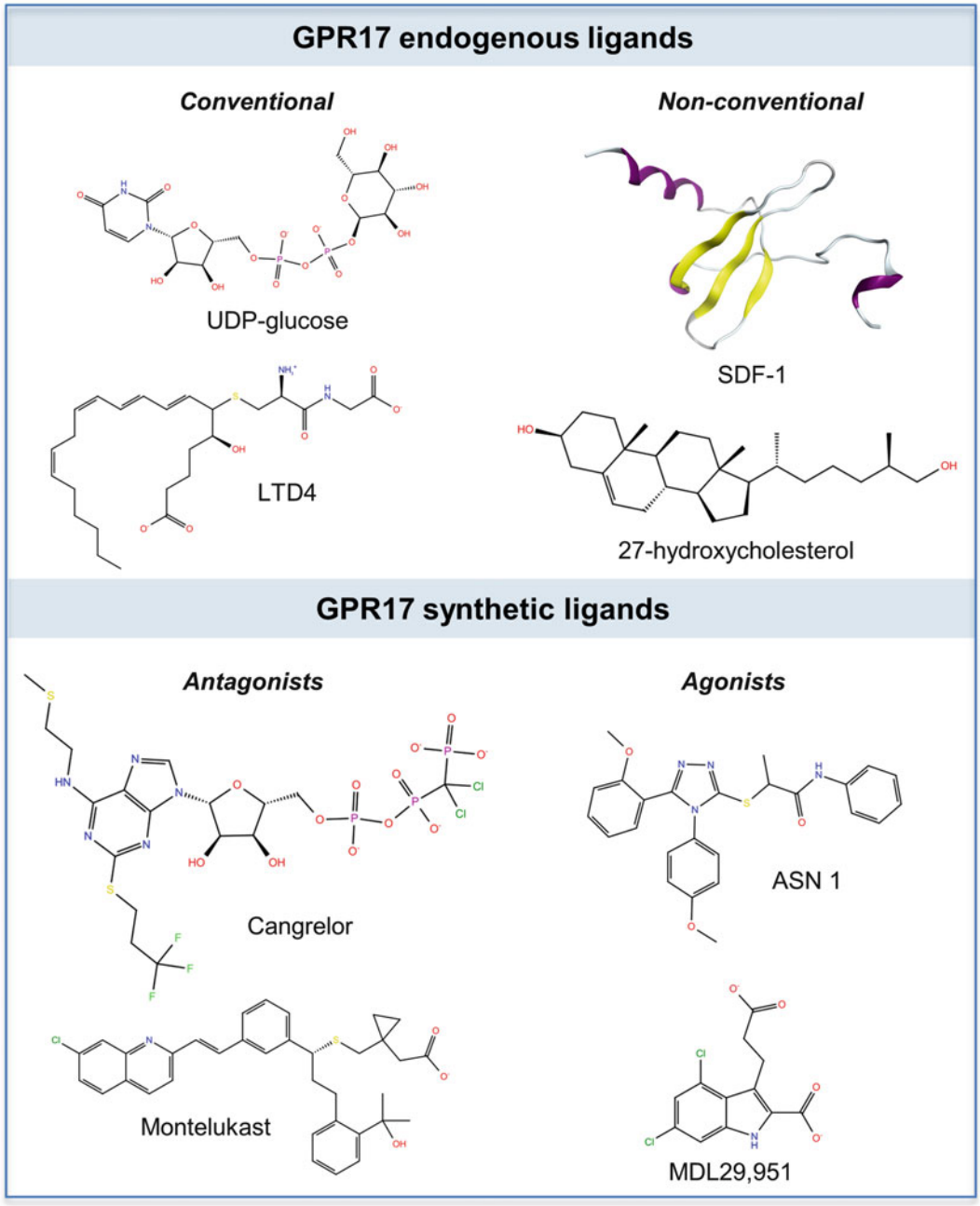

Fig. 3 Chemical structures of endogenous and synthetic compounds reported to bind GPR17. CAS Registry number of synthetic ligands are the following: MDL29,951 #130798–51-5; ASN-1: #483283–39-2. For SDF-1, a representative X-ray structure deposited in the Protein Data Bank is reported (Pdb code: 1QG7)

cAMP response element binding (CREB) trans-reporter luciferase assay in HEK293 cells (Benned-Jensen and Rosenkilde 2010). Both UDP, UDP-glucose and UDP-galactose activated GPR17 short isoform with EC_{50} values exactly in the same μM range that had been previously reported in both the [^{35}S]GTPγS binding (Ciana et al. 2006; Lecca et al. 2008) and in

Table 1 GPR17 signaling in native systems and relevant pharmacology

Tested ligand	Type of cells	Signaling	EC_{50}/IC_{50} values	Reference
UDP-glucose	Rat primary OPCs	Inhibition of cAMP production	IC_{50}: 424.7 ± 125 nM	Fumagalli et al. (2011a)
	Rat primary OPCs	Outward K^+ currents	EC_{50}: 4.6 μM	Coppi et al. (2013)
	Rat primary OPCs	Association to GRK5	N.A.	Daniele et al. (2014)
	Rat primary OPCs	β-arrestin dependent ERK1/2 activation	N.A.	Daniele et al. (2014)
	Oli-neu cells	Clathrin-mediated endocytosis	N.A.	Fratangeli et al. (2013)
	PC12 cells	ERK1/2 and p38 phosphorylation	N.A.	Daniele et al. (2010)
UDP LTD_4	Rat primary OPCs	Inhibition of cAMP production	IC_{50}: 1.29 ± 0.07 μM	(Fumagalli et al. 2011a)
	Rat primary OPCs	Inhibition of cAMP production	IC_{50}: 2.85 ± 0.89 nM	Fumagalli et al. (2011a)
	Rat primary OPCs	Association to GRK2	N.A.	Daniele et al. (2014)
	Rat primary OPCs	CREB activation	N.A.	Daniele et al. (2014)
	PC12 cells	ERK1/2 and p38 phosphorylation	N.A.	Daniele et al. (2010)
	Oli-neu cells	Clathrin-mediated endocytosis	N.A.	Fratangeli et al. (2013)
LTE_4	Rat primary OPCs	Inhibition of cAMP production	IC_{50}: 51.8 ± 6.6 pM	Fumagalli et al. (2011a)
MDL29,951	Rat primary OPCs	Inhibition of cAMP production	N.A.	Hennen et al. (2013)
	Rat primary OPCs	$Ca^{2+}{}_i$ increase	N.A.	Hennen et al. (2013)

N.A.: Not available

frontal affinity chromatography-mass spectrometry (FAC-MS) studies (Calleri et al. 2010; Temporini et al. 2009). Much lower potencies to uracil nucleotides were observed for the long receptor isoform, with a 50–170 fold increase in EC_{50} (Benned-Jensen and Rosenkilde 2010). Moreover, no responses to cysLTs were detected either on the short or long isoform, nor were cysLTs able to induce GPR17 removal from the membrane and internalization. This is in contrast with subsequent studies on cells natively expressing the receptor (Fratangeli et al. 2013) (see below). This may depend on differences in the conformation/ability of the recombinant receptor to respond to agonists compared to the native one, as well as on the fact that, in heterologously expressing systems, constitutive activity of transfected receptors may significantly alter ligand behavior (Kenakin 2001; Im 2013) (see also Conclusions). In this respect, Benned-Jensen and Rosenkilde indeed reported a notable constitutive activation of recombinant GPR17 resulting in potent inhibition of forskolin stimulated adenylyl cyclase in the absence of any endogenous ligand (Benned-Jensen and Rosenkilde 2010).

At the same time, another paper suggested GPR17 as a negative regulator of the CysLT1 receptor (Maekawa et al. 2009). This effect was proposed to depend on the formation of a CysLT1-GPR17 heteromer, as suggested by co-immunoprecipitation studies in CHO cells. The interaction between GPR17 and CysLT1 was further confirmed in primary human monocyte cells and in a rodent knock out GPR17 model, thus extending to GPR17 the previously

reported interaction and promiscuity between different members of the "purine receptor cluster". These data indicate that, besides working on its own, GPR17 may also modify the function of other related receptors by the formation of heteromers.

In 2010, the first paper describing the characteristics of GPR17 in a *native* system (rat pheocromocytoma PC12 cells) was published (Daniele et al. 2010) (Table 1). GPR17 was not expressed in undifferentiated PC12 cells but was specifically induced by a 10-day NGF treatment, suggesting a role in the control of neuronal differentiation. Both UDP-glucose and LTD_4 induced a significant pro-survival effect on PC12 cells. By *in vitro* silencing experiments with small interfering RNAs and by using receptor antagonists, these effects were confirmed to be mediated by the selective activation of GPR17. In differentiated PC12 cells, UDP-glucose and LTD_4 caused a significant increase in extracellular signal–regulated kinases 1 and 2 (ERK1/2) phosphorylation. ERK activation induced by the two agonists occurred with different kinetics: LTD_4 induced a transient ERK activation that returned to basal value within 120 min. In contrast, ERK phosphorylation induced by UDP-glucose was maintained over basal values for 120 min and the activation kinetics appeared to be biphasic with two peaks, one at 15 and the other one at 120 min. In addition, incubation of cells with the purinergic antagonist cangrelor completely counteracted UDP-glucose effects at all tested incubation times (Daniele et al. 2010). These data confirmed the responses to uracil nucleotides and cysLTs already seen on the recombinant receptors, and suggested, for the first time, that endogenous GPR17 ligands can couple to distinct G proteins and intracellular pathways, a finding that was later confirmed by other studies (Hennen et al. 2013; Daniele et al. 2014). The signaling pathways of native GPR17 are summarized in Table 1.

In 2011, in another study that independently confirmed the purinergic component of GPR17, Buccioni and coworkers (Buccioni et al. 2011) exploited an innovative and non-radioactive functional cAMP assay to monitor GPR17 activation (and the effects of various ligands) through changes in intracellular cAMP concentrations by using a mutant form of Photinus pyralis luciferase into which a cAMP-binding protein moiety had been inserted (GloSensor cAMP reagent). In HEK293 cells stably transfected with the GloSensor reagent, transient expression of hGPR17 resulted in the appearance of highly specific concentration-dependent responses to both UDP, UDP-glucose and UDP-galactose and to a series of UDP and ATP derivatives that behaved as either agonist or antagonists, with EC_{50} values that were very similar to those obtained in parallel on [^{35}S]GTPγS binding. In this system, cysLTs were not tested, due to the high constitutive expression of traditional CysLT receptors in the HEK293 cells (Ciana et al. 2006; Buccioni et al. 2011).

2.2.2 GPR17 Non-conventional Ligands

In the last years, the increasing number of class-A GPCR solved structures allowed the scientific community to recognize some common features that are crucial for their operability (Levit et al. 2014); however, these studies also revealed an unexpected heterogeneity and complexity in GPCR recognition, challenging the classical pharmacology paradigms of the 'monogamous' interaction between a specific class of natural ligands and a single GPCR (Haupt et al. 2013). In line with the growing promiscuity of GPCRs, ligand dependent transactivation has been demonstrated for GPR17, already known as a "dual" receptor: similarly to EBI2 (Hannedouche et al. 2011; Liu et al. 2011a), and the CXC chemokine receptor 2 (CXCR2) (Raccosta et al. 2013). Specifically, it was shown that, GPR17 could act as a molecular target for oxysterols, oxidized derivative of cholesterol that, in the CNS, are involved in activities not strictly associated with cholesterol metabolism. Of note, these activities are particularly relevant for neurodegenerative disorders, including demyelinating diseases (Raccosta et al. 2013;

Garenc et al. 2010). More in detail, three selected oxysterols (27-Hydroxycholesterol, 7 α-Hydroxycholesterol and 22R–Hydroxycholesterol) were tested in 1321N1 cells stably expressing GPR17, showing that all the tested compounds were able to stimulate GTPγS binding, in a concentration-dependent manner, with EC_{50} values of 4.99 ± 0.78 nM, 0.70 ± 0.09 nM and 0.21 ± 0.03 nM, for 27-Hydroxycholesterol, 7α-Hydroxycholesterol and 22R–Hydroxycholesterol, respectively.

Stimulation of cell membranes with different oxysterol concentrations after treatment with the purinergic ligand UDP-glucose showed a left-shift of the concentration-response curves or an enhancement of their maximal [^{35}S]GTPγS binding stimulation, suggesting that these ligands may cooperate under neuroinflammatory conditions.

In parallel, the effect of different concentration of the GPR17 receptor antagonist cangrelor on oxysterol-stimulated [^{35}S]GTPγS binding was evaluated, demonstrating that cangrelor can counteract GPR17 activation by oxysterols through a competitive mechanism, with IC_{50} values in a sub-nM range. These results are also in agreement with *in silico* data suggesting a common orthosteric molecular recognition mechanism for oxysterols and other small GPR17 ligands, despite different local arrangements in the TM binding site (Sensi et al. 2014).

Among other non-conventional ligands, further evidence showed that SDF-1, historically known as the endogenous ligand for CXCR4 and CXCR7 receptors, is able to transactivate GPR17 *in vitro*, specifically increasing the [^{35}S]GTPγS binding to membrane of GPR17-expressing cells, with affinity constant values of 0.14 ± 0.03 nM. The effect of SDF-1 in modulating GPR17 responses *in vitro* was further assessed in primary OPC cultures natively expressing GPR17. In this model, treatment with physiological concentrations of SDF-1 significantly increased the number of cells expressing the Myelin Basic Protein MBP compared to control, thus accelerating OPC differentiation towards a mature phenotype. The specific involvement of GPR17 in these effects was unequivocally demonstrated by further experiments showing that, in presence of the GPR17 antagonist cangrelor, SDF-1 induced no increases of either [^{35}S]GTPγS binding to cell membranes, or MBP Expression in OPC cultures. Moreover, the mechanism by which GPR17 and SDF-1 can directly interact to each other has been predicted and extensively characterized *in silico* through molecular modeling (Parravicini et al. 2016).

These results are in line with literature data, that propose a role of SDF-1 in orchestrating OPC differentiation and maturation also via CXCR4/CXCR7-axis (Li et al. 2012; Patel et al. 2010; Carbajal et al. 2011).

Interestingly, not only GPR17, CXCR4 and CXCR7, but also others chemokine receptors, like CXCR2, have demonstrated roles in regulating OPCs. As previously mentioned (see Sect. 2.1), besides sharing the same ligands, GPR17 and chemokine receptors are phylogenetically related to each other, and all participate to CNS reparative responses. This raises the hypothesis that, under neurodegenerative demyelinating conditions, oxysterols and other pro-inflammatory ligands, such as SDF-1, act as non-conventional molecules with a transversal regulatory role, representing a conserved, "unspecific" signaling mechanism, by which emergency molecules synchronize multiple receptors involved in inflammatory/immune responses.

2.2.3 New GPR17 Synthetic Ligands

In 2009 and 2010, two papers reported the development of a new FAC-MS binding method for the analysis of GPCRs (Calleri et al. 2010; Temporini et al. 2009). In this assay, UDP was found to bind to GPR17 with a Kd value of 1612.0 ± 708 nM that was very similar to the Kd value (1140.0 nM) obtained by Ciana et al. and Lecca et al. in the [^{35}S]GTPγS-binding (Ciana et al. 2006; Lecca and Ceruti 2008). This paper also unveiled a number of previously unreported GPR17 ligands, some of which were able to increase [^{35}S]GTPγS binding, with

potency values in the μM and sub-nM range. For example, the ATP analogue 2-Phenylethynyladenosine-5′-monophosphate Compound N. 4 behaved as a very potent agonist with an EC_{50} value of 36 pM. In contrast, other ligands (e.g.: N^6-Benzoyl-2′-deoxyadenosine 3′,5′-Bis phosphate, referred by the authors as Compound N. 12) did not induce any increase in [^{35}S]GTPγS binding, but counteracted stimulation induced by UDP-glucose with an antagonist profile and an affinity constant in the nM range comparable to that reported for its analogue derivative MRS2179. Both the newly identified agonists and antagonists displayed similar behavior in the FAC-MS binding assay (Calleri et al. 2010). A comparison between these data and [^{35}S]GTPγS binding results have been also reported in a recent review article on GPR17 (Marucci et al. 2016).

In the same year, an advanced *in silico* HM procedure combined with high-efficiency virtual screening of more than 120,000 compounds from the Asinex Platinum Collection (http://www.asinex.com/), a lead-like structural library, on the modeled receptor led to the selection of 5 chemically diverse molecules (the ASINEX compounds, see Fig. 3 for the chemical structure of one representative compound, 2-[[5-(2-Methoxyphenyl)-4-(4-methoxyphenyl)-4H-1,2,4-triazol-3-yl]thio]-N-phenylpropanamide, also referred as ASN 1), that were completely unrelated to already known ligands. These compounds were tested *in vitro* in the [^{35}S]GTPγS binding assay, revealing a sub-nM potency for GPR17 (Eberini et al. 2011) (see also below). None of these compounds could have been expected 'a priori' to act on GPR17, and all of them behaved as much more potent ligands than GPR17 endogenous activators (Eberini et al. 2011). Finally, in 2013, MDL29,951 was reported as an additional small molecule agonist at GPR17 (Hennen et al. 2013) (Fig. 3). In a variety of different heterologous expression systems, MDL29,951-stimulated GPR17 engaged the entire set of intracellular adaptor proteins for GPCRs: G proteins of the Gαi, Gαs, and Gαq subfamily, as well as β-arrestins. This was visualized as alterations in the concentrations of cyclic adenosine monophosphate and inositol phosphate, increased Ca^{2+} flux, phosphorylation of ERK1/2, as well as multifeatured cell activation recorded with label-free dynamic mass redistribution and impedance biosensors. pEC_{50} values for MDL29,951 at GPR17 ranged between 5 and 8.80, depending upon the transfected cell type and the used read out. MDL29,951-stimulated GPR17 effects were counteracted in a concentration-dependent manner by pranlukast and, to a lesser extent, by montelukast. This is fully in line with the activities of these antagonists on recombinant GPR17 in previous studies, in which pranlukast was significantly more potent than montelukast in antagonizing LTD4-stimulation of GPR17 (Ciana et al. 2006). In OPCs, MDL29,951 rapidly mobilized intracellular Ca^{2+} in a concentration-dependent manner and engaged both $G_{\alpha i}$ and $G_{\alpha q}$, but not $G_{\alpha s}$ signaling pathways, further suggesting differences in GPR17 responses between transfected and native systems (see also Conclusions). This is at variance from previous studies reporting $G_{\alpha i}$ coupling and decreases of intracellular cAMP as a primary transduction pathway of GPR17 in OPCs (Daniele et al. 2014; Fumagalli et al. 2011b). However, it has to be emphasized that, despite being selective for GPR17 inside the "purine receptor cluster" (Hennen et al. 2013), MDL29,951 also significantly interacts with the glycinergic site of the glutamate NMDA receptor (Salituro et al. 1992). This may be at the basis of the ability of MDL29,951 to activate multiple signaling pathways in both transfected cells and in OPCs, and of the data reported for this compound on myelination (see also Sect. 3.1.1).

2.2.4 Agonist-Induced Desensitization and Internalization

In 2011, the first complete agonist-induced GPR17 desensitization/resensitization study was published (Daniele et al. 2011). By using [^{35}S]GTPγS binding and cAMP measurements in 1321N1 cells expressing hGPR17, both UDP-glucose and LTD_4 were shown to induce a time- and concentration-dependent loss of

GPR17 response (homologous desensitization). GPR17 homologous desensitization was accompanied by internalization of receptors inside cells, as assessed by biotin labeling of cell surface receptors. Desensitization occurred in a time-dependent manner, with similar kinetics for both agonists. Upon agonist removal, receptor resensitization occurred with the typical kinetics of GPCRs. Finally, activation of GPR17 by UDP-glucose induced a partial heterologous desensitization of LTD_4-mediated responses (but not *vice versa*), suggesting that nucleotides have a hierarchy in producing desensitizing signals.

The pattern of GPR17 desensitization and internalization was fully confirmed and further expanded in differentiated oligodendroglial Oli-neu cells that natively express GPR17 (Fratangeli et al. 2013) (Table 1). Agonist-induced internalization, intracellular trafficking and membrane recycling of GPR17 were analyzed by biochemical and immunofluorescence assays using an *ad hoc*-developed new antibody against the extracellular N-terminal of GPR17. Both UDP-glucose and LTD_4 increased GPR17 internalization, although with different efficiency. At early time points, internalized GPR17 co-localized with transferrin receptor, whereas at later times it partially co-localized with the lysosomal marker Lamp1, suggesting that a portion of GPR17 is targeted to lysosomes upon ligand binding. Internalization of GPR17 occurred via clathrin-dependent endocytosis (Fratangeli et al. 2013). Analysis of receptor recycling and degradation demonstrated that a significant fraction of GPR17 is recycled to the cell surface. These results provided the first data on the agonist-induced trafficking of native GPR17 in oligodendroglial cells and may have implications in fine-tuning cell responses to demyelinating and inflammatory conditions when these ligands accumulate at lesion sites (see also Sect. 3.1.2). More recently, GPR17 downregulation by uracil nucleotides and cysLTs was confirmed in primary cultured OPCs, and the role of the GRK/β-arrestin machinery in receptor desensitization and intracellular signaling was also extensively investigated (Daniele et al. 2014). It was shown that, following OPCs treatment with the two classes of purinergic and cysLT ligands, different GRK isoforms were recruited. Specifically, cysLT-mediated GPR17 desensitization mainly involved GRK2 via a G protein-dependent mechanism (Daniele et al. 2014). This kinase promoted transient binding of the receptor to β-arrestins, rapid ERK phosphorylation and sustained nuclear CREB activation. Furthermore, GRK2, whose expression paralleled that of the receptor during the differentiation process, was required for cysLT-mediated OPCs maturation (see also Sect. 3.2.). On the other hand, purinergic ligands exclusively recruited GRK5 via a G protein-independent/β-arrestin-dependent mechanism. This kinase induced a stable association between the receptor and β-arrestin, followed by slower and sustained ERK stimulation and marginal CREB activation (Daniele et al. 2014). These results show that, through activation of GPR17 and recruitment of specific GRK isoforms, purinergic and cysLT ligands engage distinct intracellular pathways.

Recently GPR17 desensitization (and its relationship to terminal OPC maturation) has been linked to activation of mTOR (the "mammalian target of rapamycin"), which has long been known to be involved in myelination. During OPC differentiation, mTOR regulates GRK-mediated desensitization of GPR17 by promoting the nuclear translocation of the ubiquitin ligase MDM2, which had been previously only involved in cancer via regulation of p53 activity and now emerges as a new interesting actor in oligodendrogenesis (Fumagalli et al. 2015). Specifically, treatment of OPCs with either the mTOR inhibitor rapamycin, or with nutlin-3, a small molecule inhibitor of Mdm2-p53 interactions, was shown to keep MDM2 in the cytosol, where it could bind to GRK2 and sustain its degradation, thus impairing the physiological desensitization of GPR17 (Fumagalli et al. 2015). Importantly, prevention of GPR17 desensitization was also associated to a defect of OPC maturation, confirming that aberrantly elevated GPR17 levels in late stage OPCs blocks cells at immature stages (Fumagalli et al. 2015).

In another study, GPR17 plasma membrane recycling and stability was shown to be also modulated by SNX27, a recently identified protein of the endosome-associated retromer complex, whose functions in oligodendrocytes had never been studied. It was found that, after endocytosis, GPR17 is either sorted into lysosomes for degradation or recycled to the plasma membrane. Balance between degradation and recycling was important for modulation of receptor levels at the cell surface, and thus for the silencing or maintenance of GPR17-signaling pathways, that, in turn, affect OPC differentiation (see also Sect. 3.2). The endocytic trafficking of GPR17 was mediated by interaction of SNX27 with a type I PDZ-binding motif located at the C-terminus of the receptor. Of note, SNX27 knock-down reduced GPR17 plasma membrane recycling in differentiating oligodendrocytes while accelerating terminal cell maturation. Interestingly, trisomy-linked down-regulation of SNX27 in the brain of Ts65Dn mice, a model of Down syndrome, correlated with a dysfunction in $GPR17^{+}$ cells and an increase in mature oligodendrocytes, which, however, failed in reaching full maturation, eventually leading to hypomyelination (Meraviglia et al. 2016). Thus, disruption of SNX27/GPR17 interactions leading to alterations of GPR17 membrane trafficking might contribute to pathological oligodendrocyte differentiation and myelination defects present in Down syndrome (Meraviglia et al. 2016).

3 Role of GPR17 in Central Nervous System Pathophysiology

3.1 GPR17 Specific Roles in Oligodendroglial Functions and Myelination

3.1.1 Physiological Roles

In the healthy intact brain, GPR17 expression is predominantly in oligodendrocyte (OL) cells. The very first demonstration that, in the adult brain, GPR17 is highly expressed by a sub-population of endogenous quiescent parenchymal OPCs dates back to 2008 (Lecca et al. 2008) and has sparked a lot of interest on GPR17 role in CNS myelination. Specifically, GPR17 was shown to be present in ramified early neural cell precursors dispersed throughout brain's gray and white matter that also positively stained for typical early OPC markers. Since then, increasing evidence has progressively accumulated to show a pivotal role of GPR17 in OPC maturation, with different and apparently paradoxical effects during different phases of the maturation process (Chen et al. 2009; Fumagalli et al. 2011a) (see also below).

In vitro studies on purified rat postnatal OPC cultures showed that GPR17 expression coincides with a specific temporal window of the OL differentiation process. It covers two distinct phases: a first phase, during which early differentiation markers like NG2, A2B5, PDGF receptor-alpha and the immature PLP isoform DM-20 are still present (early stage 2 OPCs in Fig. 4), and a subsequent phase characterized by more ramified, still immature pre-oligodendrocytes (stages 3 and 4 in Fig. 4), where NG2 has been downregulated and more advanced markers like O4, O1 and the proteolipid myelin protein PLP are present (Fumagalli et al. 2011a). Based on these data, GPR17 is currently utilized by other independent scientists to specifically label pre-immature OLs at these two transition stages (Mitew et al. 2013; Nakatani et al. 2013; Crociara et al. 2013; Ferrara et al. 2016).

Of note, GPR17 expression progressively increases during the transition of OPCs to pre-OLs (when it is maximally expressed in cellular processes), but is then gradually silenced and never found in fully morphologically mature OLs (Fumagalli et al. 2011a) (see Fig. 4). Accordingly, *in vivo*, GPR17 is present in a subset of NG2/Olig2-positive OPCs expressing the first myelin proteins, but not in more mature cells expressing myelin basic protein (MBP). Also during rodent brain development, GPR17 expression in OPCs precedes myelin production. Interestingly, GPR17 immunoreactivity appears first

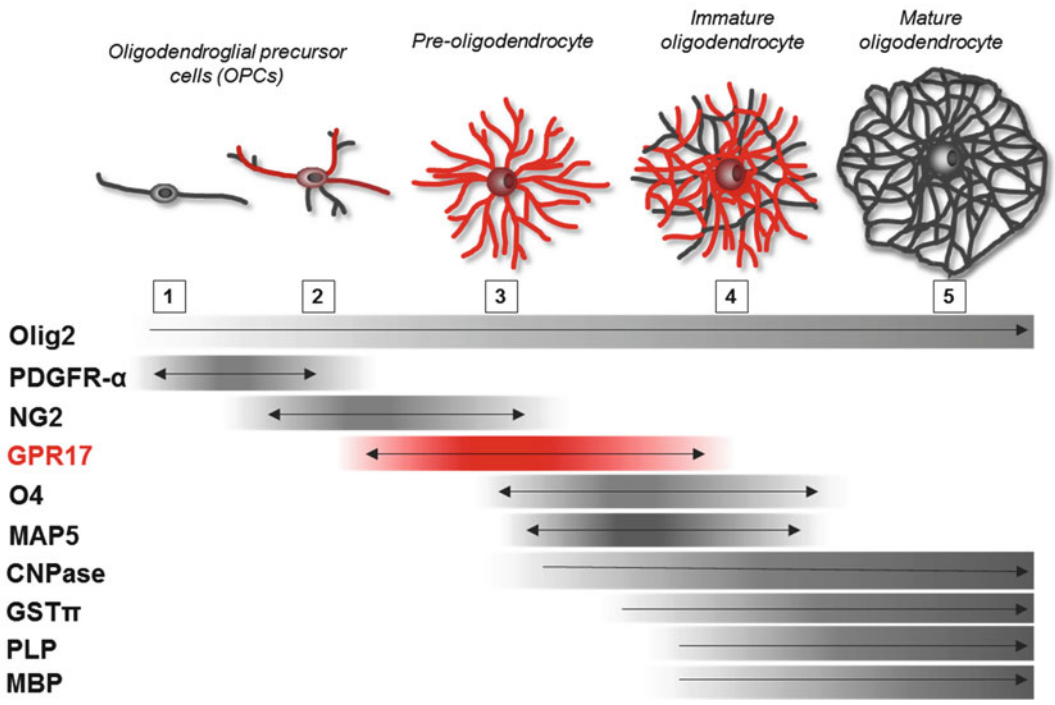

Fig. 4 Transient GPR17 expression during oligodendroglial differentiation. The expression pattern of GPR17 (in *red*) during oligodendroglial differentiation is shown in parallel to other known oligodendroglial markers (other colours). Progressive differentiation stages are indicated with numbers from 1 to 5. From a functional point of view, GPR17 exerts opposing stage-specific roles: a positive role for differentiation in early OPCs and a negative function for OL maturation in late OPCs. In late OPCs, gradual silencing of GPR17 is needed to allow OPCs to complete their maturation (see text for more details)

in the cell body, partially coinciding with markers of the Golgi apparatus, and then gradually extends to cellular processes (Boda et al. 2011). Early after birth, the expression of the receptor is low, but progressively expands to cover the 80% of OPCs at the end of the third week of life. Afterwards, GPR17 is down-regulated while myelination proceeds (Boda et al. 2011).

The transient nature of GPR17 expression in OPCs suggests that the receptor may display stage-specific roles during OL development. Intriguingly, as already reported for the Wnt/β-catenin pathway (Fancy et al. 2009; Ye et al. 2009) and more recently proposed for the transcription factor Olig2 (Mei et al. 2013), GPR17 exhibits opposing functions on OL differentiation in relation to its expression stage. In cultured cortical postnatal rat OPCs, early receptor obliteration with small interfering RNAs profoundly affected their ability to generate mature OLs, suggesting that cells are retained at a less differentiated stage (Fumagalli et al. 2011a) (Fig. 4). Although the molecular mechanisms at the basis of these events have not been yet investigated, these data highlight a pivotal role of GPR17 in the initial phases of the differentiation process. They support the hypothesis that, at these stages, GPR17 may be important to keep cells at an immature state which may, in turn, be necessary to prepare them for myelination (Fumagalli et al. 2011a). In contrast, cultured cortical progenitors from GPR17 knock-out E15.5 mouse embryos differentiated earlier toward mature OLs compared to control cells (Chen et al. 2009). The reasons for these discrepancies remain unknown, although it may be hypothesized that compensatory mechanisms are activated as a result of early embryonic GPR17 knock out. Of course, the generation of

conditional transgenic mice in which deletion of GPR17 in OPCs could be induced under controlled conditions at specific ages will help clarifying this issue.

Lecca and coworkers also clearly showed that GPR17 is no longer present in morphologically mature MBP-positive cells (Lecca et al. 2008), raising for the first time the possibility that loss of GPR17 at advanced differentiation stages is a prerequisite to allow cells to complete terminal maturation. Subsequent *in vivo* data showed that myelinogenesis is indeed defective in transgenic mice overexpressing GPR17 under the promoter of 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase), a relatively advanced OL marker (Chen et al. 2009). These animals exhibited motor disabilities, tremors and precocious death within the second week of life. The forced and un-timely expression of GPR17 at a maturation stage (i.e., in $CNPase^+$ cells), at which GPR17 is normally already downregulated, might have created conflicting signals leading to defective terminal maturation. Thus, interference with the stage-restricted expression of GPR17 resulting in un-programmed receptor expression in late OPCs completely alters the differentiation program of these cells. This hypothesis is fully in line with the demonstration that OPCs incorporating a vector for GPR17 overexpression maintained an immature morphological phenotype and never expressed the mature marker CNPase, and with data showing that, under conditions where terminal OPC maturation is impaired, such as demyelinating diseases (see Sect. 3.1.2) or treatment with the mTOR inhibitor rapamycin, that reduces OPC maturation, GPR17 is markedly up-regulated (Fumagalli et al. 2015; Tyler et al. 2011).

Both intrinsic and extrinsic mechanisms could contribute to GPR17 stage-specific functions during oligodendrocyte differentiation. GPR17 can be extrinsically regulated by physiological ligands accumulating in the extracellular milieu: activation of early OPCs (stages 2 and 3 in Fig. 4) with GPR17 endogenous putative ligands (i.e., UDP-glucose or LTD_4) indeed promoted conversion to more mature cells expressing myelin markers (Lecca et al. 2008; Fumagalli et al. 2011a; Ceruti et al. 2011). Consistent with these data, GPR17 antagonists like cangrelor (Fig. 3) delayed the ability to generate mature cells (Lecca et al. 2008; Fumagalli et al. 2011a), suggesting that GPR17 endogenous ligands are basally released in culture and are responsible for the observed spontaneous OPC *in vitro* maturation. In another independent study, while not modifying the potential of adult multipotent neural stem cells, montelukast, which also acts as a GPR17 antagonist (Ciana et al. 2006; Benned-Jensen and Rosenkilde 2010; Lecca et al. 2008), markedly increased their proliferation rate, suggesting that GPR17 antagonism induces retention of cells at a more undifferentiated stage (Huber et al. 2011).

As already mentioned, besides cAMP inhibition, GPR17 has been also shown to specifically mediate activation of delayed rectifier K^+ currents (Table 1) in a sub-population of OPCs and $O4^+$ pre-OLs, but not in mature OLs. This effect was shown to contribute to the terminal maturation of OPCs and to their migratory abilities.

In contrast with the above studies, MDL29,951, the new putative GPR17 agonist mentioned above, was reported to inhibit, rather than stimulate, OL maturation (Hennen et al. 2013). However, it is worth to note that, due to the *transient* expression of GPR17 in culture, the timing of OPC manipulation and treatment is crucial for obtaining comparable results. On the other hand, as already mentioned, MDL29,951 is not a selective ligand for this receptor, and independent effects could be due to its antagonistic activity at the glycinergic site of the glutamate NMDA receptor (Salituro et al. 1992), which has been indeed reported to promote OPC differentiation (Li et al. 2013).

Globally, these findings suggest that GPR17 exerts opposing stage-specific roles: a positive role for differentiation in early OPCs and a negative function for OL maturation in late OPCs. They also suggest that, in late OPCs, physiological GPR17 silencing is needed to allow cells to complete their maturation program. The latter

may occur via either GPR17 desensitization/internalization by endogenous agonists or by GPR17-mediated engagement of intracellular pathways culminating in nuclear events, or both. Blockade of GPR17 mRNA translation into the receptor protein a specific microRNA has been also recently reported to contribute to GPR17 regulation during OPC maturation (Lecca et al. 2016).

3.1.2 Dysregulation in Demyelinating Neurodegenerative Diseases

The demonstration that levels of endogenous nucleotides and cysLTs are massively increased upon CNS trauma and ischemia and their hypothesized roles as danger signals after injury (Davalos et al. 2005; Haynes et al. 2006) has raised the hypothesis that GPR17 may act as a crucial mediator of reactivity to acute injury. While physiologically GPR17 is mostly an oligodendroglial receptor, after acute injury, GPR17 is sequentially induced in dying neurons inside and at the borders of the ischemic/traumatic lesion, in infiltrating microglia/macrophages and in activated parenchymal OPCs in the lesion's surrounding areas, with similar expression patterns in different models of pathology. In more detail, in both rats and mice, 24 h after permanent middle cerebral artery occlusion (MCAo), GPR17 is up-regulated in neurons damaged by the ischemic insult inside the ischemic core (Ciana et al. 2006; Lecca et al. 2008). When the penumbra area is well visible and most of the neurons in the core are dead, GPR17 appears on highly activated microglia and blood-borne macrophages at the borders of the lesion (Lecca et al. 2008). This has been independently confirmed to also occur in a transient MCAo rodent model, where the number of GPR17 expressing cells was significantly upregulated in two distinct phases, 24 h and 7 days after reperfusion, consistent with an early acute neuronal injury followed by a late microgliosis (Zhao et al. 2012). It is known that OPCs are extremely sensitive to the pathophysiological state of the brain, and that they react to many different types of experimentally induced insults. Starting from 72 h after the insult, in the regions surrounding the ischemic area and in the ipsilateral corpus striatum of MCAo mice, a higher number of GPR17-expressing OPCs was indeed found compared to contralateral hemisphere (Lecca et al. 2008), suggesting an increased proliferation rate in response to demyelination.

Dysregulated expression of GPR17 has been described also after traumatic injury, in both brain (Boda et al. 2011) and spinal cord (Ceruti et al. 2009). In stab wound, a model of cortical trauma, early after lesion, the density of GPR17-expressing OPCs in gray matter was reduced compared to contralateral cortex, consistent with a global oligodendroglial loss. At later times, $GPR17^{+}$ cells increased significantly in number around the lesion in both gray and white matter, likely due to the expansion of the NG2 cell pool, which, in turn, reflects an attempt to replace dead OPCs. This reactivity lasted up to 7 days and then declined over time, going back to basal levels 14 days after lesion. This pattern has been confirmed in human samples from patients with traumatic brain injury (Franke et al. 2013). In both neurosurgical and autopsy specimens, GPR17 expression was evident inside the contused core and progressively declined distally according to a spatio-temporal gradient. Inside and around the core, GPR17 labeled dying neurons, reactive astrocytes, and activated microglia/macrophages. In peri-contused parenchyma, GPR17 was found on OPCs, some of which had proliferated, indicating re-myelination attempts. In agreement with the above data, in a double transgenic model of Alzheimer's disease (the APPPS1 mouse) a high number of GPR17-positive cells accumulated close to amyloid plaques in gray matter, revealing receptor up-regulation as a feature of oligodendroglial reactivity also in this pathological condition (Boda et al. 2011).

Similar GPR17 changes have been reported also in typically de-myelinating diseases such as in models of multiple sclerosis (MS). In this disease, remyelination occurs after the initial myelin damage, but it fails after multiple demyelination episodes, which eventually leads to axonal degeneration and progressive disability

(Franklin and Ffrench-Constant 2008). Interestingly, synthesis of cysLTs is increased in MS plaques and in the spinal cord of mice subjected to experimental autoimmune encephalomyelitis (EAE), an immune-mediated model of demyelination (Whitney et al. 2001). Of note, montelukast, an antagonist at both CysLT1 and GPR17, attenuated CNS infiltration of inflammatory cells and the clinical symptoms of EAE (Wang et al. 2011). However, the exact contribution of GPR17 to these effects has not been investigated in detail. Overexpression of the GPR17 transcript has been observed in both EAE mice and in a cohort of human MS tissues (Chen et al. 2009). GPR17 expression was significantly increased in MS plaques as compared with white matter from non-neurological donor samples and normal-appearing white matter from MS donors. In a similar way, acute damage to myelin induced by lysolecithin (Lys) injection in corpus callosum induced a strong overexpression of GPR17 at the lesion site 10 days after injury (Boda et al. 2011). Thus, independently of the original cause, GPR17 is abnormally up-regulated in MS and some models of neurodegenerative conditions characterized by myelin disruption (Fumagalli et al. 2016).

On this basis, it could be hypothesized that, after damage, GPR17 is initially induced to promote the growth and differentiation of OPCs; however, at later stages, due to lack of appropriate environmental stimuli, presence of inflammatory signals and/or intrinsic factors, physiological GPR17 downregulation is impeded, thus freezing cells at a stand-by stage, where they are neither proliferating nor differentiating. When this happens, interventions targeting GPR17 may help bypassing this checkpoint and facilitate terminal maturation. Since GPR17 is a *membrane* receptor that, at variance from other intrinsic regulators of oligodendrogenesis, can be easily targeted and manipulated with pharmacological agents, it is envisaged that agents counteracting GPR17 aberrant expression under these conditions could induce OPCs to resume myelination and promote neurorepair. To support this hypothesis, in MCAo animals, administration of GPR17 antagonists such as cangrelor or montelukast (Ciana et al. 2006; Lecca et al. 2008), or GPR17 silencing due to *in vivo* delivery of specific antisense oligonucleotides (Ciana et al. 2006; Lecca et al. 2008) or small interfering RNAs (Zhao et al. 2012) resulted in a significant reduction in brain's ischemic volume. Use of GPR17 anti-sense oligonucleotides also reduced damage and improved functional recovery in a model of spinal cord injury, in line with the hypothesis that GPR17 is aberrantly overexpressed as a consequence of damage (Ceruti et al. 2009).

In contrast to what observed in MCAo, in a rat *neonatal* model of ischemic periventricular leukomalacia (PVL), a common cerebral white matter injury, the GPR17 agonist UDP-glucose (and not an antagonist) significantly contributed to myelin sheaths recovery and improved motor functions, learning and coordination in PVL pups (Mao et al. 2012). The reason for this discrepancy may reside in the different outcome of the ischemic insult in neonatal brain compared to adults. It could be hypothesized that, in neonatal pups, existing OPCs, which are very sensitive to ischemic death, are immediately killed by the ischemic insult, with no obvious GPR17 upregulation; conversely, being these cells generated at distinct waves during the first weeks of life at much higher rates compared to adulthood, a GPR17 agonist (instead of an antagonist) would allow to properly activate newborn OPCs, thus favouring the formation of myelin sheaths and neurological recovery.

Several of the still obscure aspects of GPR17 pathophysiology have been linked to the difficulty of establishing a causal relationship between GPR17 expression and myelination *in vivo*. Since GPR17 is no longer expressed in mature myelinating OLs (Lecca et al. 2008; Fumagalli et al. 2015), it was impossible to demonstrate that cells that have expressed GPR17 in their earlier life can indeed myelinate. Only recently, the generation of the first GPR17iCreER^{T2}-GFP reporter mouse line for fate mapping studies has allowed to follow the final destiny of GPR17^{+} cells during both physiological differentiation and in disease, thanks to

the inducible expression of the green fluorescence protein (GFP). In these mice, upon tamoxifen induced recombination, OPCs expressing GPR17 at that very specific moment, become green and can be traced as such for the entire animal's life. Use of these mice has allowed to show that, in normal brain, GFP^+ cells differentiate very slowly (needing about 3 months to reach maturity), but after acute insults, they rapidly reacted to damage with proliferation and migration toward the injured site, thus representing a 'reserve pool' of adult quiescent progenitors maintained for repair purposes (Vigano et al. 2016). A full characterization of the long-term events occurring in the brain of ischemic MCAo GPR17iCreER^{T2}-GFP mice has shown that, despite massive recruitment of GFP^+ green OPCs at the ischemic site, only a few percentage of these cells become mature myelinating OLs, likely due to local unfavourable inflammatory milieu (Vigano et al. 2016; Bonfanti et al. 2017).

More recently, it has been demonstrated that GPR17 over-activation inhibited oligodendrocyte survival by reducing intracellular cAMP levels and inducing expression of the pro-apoptotic gene *Xaf1*. GPR17 overactivation also negatively regulated protein kinase A signaling pathway and expression of the transcription factor c-Fos. In line with these data, in the lysolecithin-mediated demyelination injury model, the pharmacological inhibition of GPR17 with pranlukast increased oligodendrocyte survival and promoted immature oligodendrocyte differentiation through the upregulation of Epac1, the exchange factor directly activated by cAMP (Ou et al. 2016). These data are fully consistent with our results in other injury models characterized by demyelination and abnormal GPR17 upregulation (summarized in Fumagalli et al. 2016), suggesting that under these conditions GPR17 inhibition has potential for treatment of demyelinating diseases (Ou et al. 2016).

3.2 GPR17 in Brain Rejuvenation

A recent report has investigated the roles of GPR17 in age-associated cognitive decline (Marschallinger et al. 2015). Authors have first shown that oral administration of montelukast (an antagonist of both CysLT1 receptor and GPR17, see above), for 6 weeks to moderately old rats (20 months old) resulted in structural and functional rejuvenation of aged brains, as demonstrated by restoration of blood brain barrier integrity, reduced microglia activation in the brain, increased levels of hippocampal neurogenesis and significantly improved learning and memory tasks. Importantly, montelukast had no effects on the behaviour and cognitive abilities of young animals, suggesting that its actions specifically target an aging associated defect (see also below). Regression and correlation analyses showed that montelukast-induced learning improvement in the old animals was independent of the changes in microglia morphology, but rather depended on the rate of neurogenesis measured as increased number of proliferating neuroblasts in hippocampal dentate gyrus. Interestingly, authors also provided immunohistochemical evidence for the presence of GPR17, but not CysLT1 receptor, in a subset of doublecortin $(DCX)^+$ newborn neurons in hippocampal dentate gyrus, suggesting a role in the proliferation and specification of these cells. Studies on neurospheres obtained from mice lacking FOXO1, a GPR17 regulating transcription factor, and from GPR17$^{-/-}$ mice indeed confirmed montelukast effects be due to action on GPR17/DCX^+ neuroblasts in hippocampal dentate gyrus, leading to increased neurogenesis. Globally these data suggest that, under normal conditions, GPR17 exerts a negative control on the proliferation of neural progenitors in the hippocampus; in aged animals, due to the overall decrease of neurogenesis, GPR17 inhibition of proliferation becomes detrimental and contributes to memory impairment. Under such pathological conditions, montelukast can restore neurogenesis by alleviating GPR17 inhibitory effect (Marschallinger et al. 2015).

3.3 GPR17 in Gliomas

OL markers such as Olig2, PDGFRα and NG2 are often expressed in glioma cells. Little is known about the origin of these tumors, but it is possible that they arise from dysregulated OPCs (Liu et al. 2011b). Considering that OPCs are the only proliferating population in the adult brain, defects in differentiation mechanisms favouring cell proliferation could be a primary cause of gliomas. A complementary strategy for tumor treatment is to promote pathways for maintaining quiescence and/or driving terminal differentiation of the tumoral progenitors. In this respect, a recent microarray analysis of mouse and human gliomas aimed at unveiling new candidates promoting differentiation or quiescence has highlighted GPR17 as a new potential target (Dougherty et al. 2012). In glioma cells, treatment with UDP, UDP-glucose or LTD_4 indeed reduced the formation of glioma spheres suggesting that GPR17 stimulation can represent a good strategy to drive the differentiation of highly proliferative uncommitted tumor cells to the oligodendroglial fate, negatively affecting both tumor cell proliferation and self-renewal (Dougherty et al. 2012). These data are in line with the fact that most of the OPCs expressing GPR17 in brain are quiescent (Lecca et al. 2008), and support the pro-differentiative effects of its putative endogenous ligands (see also Sects. 2.1 and 3.2).

4 Conclusions

GPR17 has emerged as a new GPCR of great interest for drug development. It is almost exclusive localization to OPCs, the myelin forming cells and the only (slowly) proliferating cell population in the intact brain, has highlighted GPR17 as a novel pharmacological target for demyelinating diseases. At variance from other myelinating genes, GPR17 is a membrane receptor, thus amenable for pharmacological modulation, which has attracted a lot of interest for the development of new therapeutic approaches to MS and other neurodegenerative diseases characterized by myelin disruption. The recent demonstration that GPR17 is also expressed by a subset of hippocampal neural progenitors involved in cognitive functions does not detract from the potential interest of GPR17 ligands in neurodegenerative diseases, since, as demonstrated by the montelukast study (Marschallinger et al. 2015), these ligands may be active only when specific pathological GPR17 changes are present.

The recent studies on GPR17 revealed its transient expression in OPCs and a more complex role than expected: a pro-differentiating role in early OPCs and a negative function on maturation in late stage OPCs. Thus, the apparently contrasting *in vitro* data obtained with different GPR17 stimulatory agents (Lecca et al. 2008; Fumagalli et al. 2011a; Hennen et al. 2013) may depend on the specific differentiation stage at which these compounds have been added to cultured OPCs. It may well be that the function of GPR17 is different in the intact and diseased brain, based on the availability of its endogenous ligands. If uracil nucleotides, cysLTs, oxysterols and chemokines like SDF-1 are indeed among the signaling molecules able to activate GPR17 *in vivo* (see also below), we envisage that their role would be more likely unveiled under pathological conditions, where these ligands massively accumulate at lesion sites inside the CNS.

Experiments in a wide variety of rodent models of neurodegeneration have shown that, independently of the nature of the insult (ischemic, traumatic or toxic) and of the presence of any concomitant neuronal pathology, demyelinating conditions invariably led to GPR17 upregulation. We believe that this dysregulation reflects an initial attempt to repair the lesion by stimulating OPCs differentiation via GPR17, but that this attempt is later invalidated by the inability of maturing cells to downregulate/internalize the receptor, which, in turn, leads a differentiation blockade. On this basis, it is envisaged that *GPR17 antagonists* would be useful in MS and neurodegenerative diseases. By counteracting GPR17 aberrant dysfunction, antagonists would help OPCs to complete their maturation, thus

re-establishing endogenous remyelination, as recently also confirmed (Ou et al. 2016).

Due to the still ambiguous state of the pharmacology for this receptor, the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) has not yet officially de-orphanized this GPCR (Davenport et al. 2013). However, as also emphasized by NC-IUPHAR, much of the work in this area has been based on recombinant expression systems using different host cells and transfection methodologies compared to data derived from native cells. In recombinant "artificial" cell systems, activity tests are highly dependent on the experimental conditions utilized and subject to several artifacts, especially in the case of receptors' constitutive activation, a typical feature of several GPCRs including GPR17 (Benned-Jensen and Rosenkilde 2010; Maekawa et al. 2009; Qi et al. 2013; Eggerickx et al. 1995; Uhlenbrock et al. 2002; Rosenkilde et al. 2006; Qin et al. 2011; Im 2004) that can profoundly alter ligand behavior (Kenakin 2001; Davenport et al. 2013).

In terms of drug development, neither uracil nucleotides nor CysLTs are suitable to this purpose, because neither ligand class is competent to discriminate between the functions of purinergic receptors, CysLT receptors, and GPR17 *in vivo*, where multiple receptors are often co-expressed. Nevertheless, the already available *in vivo* rodent data reporting positive neuro-reparative effects induced by commercially available montelukast or pranlukast (Yu et al. 2005a, b), which are potent (although non selective) GPR17 antagonists, foster the search for further GPR17 ligands (Eberini et al. 2011; Hennen et al. 2013) and may represent an important advancement for patients with neurodegenerative diseases.

Acknowledgements Authors are deeply grateful to the Italian Multiple Sclerosis (FISM) for financial support (Projects N. 2013/R1 to MPA) and to Cariplo Foundation (Projects 2014-1207 to DL and 2015-0910 to MF).

Conflicts of Interest The authors declare no conflicts of interest.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58(3):281–341

Benned-Jensen T, Rosenkilde MM (2010) Distinct expression and ligand-binding profiles of two constitutively active GPR17 splice variants. Br J Pharmacol 159(5):1092–1105

Blasius R, Weber RG, Lichter P, Ogilvie A (1998) A novel orphan G protein-coupled receptor primarily expressed in the brain is localized on human chromosomal band 2q21. J Neurochem 70(4):1357–1365

Boda E, Vigano F, Rosa P, Fumagalli M, Labat-Gest V, Tempia F, Abbracchio MP, Dimou L, Buffo A (2011) The GPR17 receptor in NG2 expressing cells: focus on in vivo cell maturation and participation in acute trauma and chronic damage. Glia 59(12):1958–1973

Bonfanti E, Gelosa P, Fumagalli M, Dimou L, Viganò F, Tremoli E, Cimino M, Sironi L, Abbracchio MP (2017) The role of oligodendrocyte precursor cells expressing the GPR17 receptor in brain remodelling after stroke. Cell Death Dis [in press]

Brink C, Dahlen SE, Drazen J, Evans JF, Hay DW, Nicosia S, Serhan CN, Shimizu T, Yokomizo T (2003) International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. Pharmacol Rev 55(1):195–227

Buccioni M, Marucci G, Dal Ben D, Giacobbe D, Lambertucci C, Soverchia L, Thomas A, Volpini R, Cristalli G (2011) Innovative functional cAMP assay for studying G protein-coupled receptors: application to the pharmacological characterization of GPR17. Purinergic Signal 7(4):463–468

Calleri E, Ceruti S, Cristalli G, Martini C, Temporini C, Parravicini C, Volpini R, Daniele S, Caccialanza G, Lecca D, Lambertucci C, Trincavelli ML, Marucci G, Wainer IW, Ranghino G, Fantucci P, Abbracchio MP, Massolini G (2010) Frontal affinity chromatography-mass spectrometry useful for characterization of new ligands for GPR17 receptor. J Med Chem 53 (9):3489–3501

Capra V, Ravasi S, Accomazzo MR, Citro S, Grimoldi M, Abbracchio MP, Rovati GE (2005) CysLT1 receptor is a target for extracellular nucleotide-induced heterologous desensitization: a possible feedback mechanism in inflammation. J Cell Sci 118(Pt 23):5625–5636

Carbajal KS, Miranda JL, Tsukamoto MR, Lane TE (2011) CXCR4 signaling regulates remyelination by endogenous oligodendrocyte progenitor cells in a viral model of demyelination. Glia 59(12):1813–1821

Ceruti S, Villa G, Genovese T, Mazzon E, Longhi R, Rosa P, Bramanti P, Cuzzocrea S, Abbracchio MP (2009) The P2Y-like receptor GPR17 as a sensor of

damage and a new potential target in spinal cord injury. Brain 132(Pt 8):2206–2218

Ceruti S, Vigano F, Boda E, Ferrario S, Magni G, Boccazzi M, Rosa P, Buffo A, Abbracchio MP (2011) Expression of the new P2Y-like receptor GPR17 during oligodendrocyte precursor cell maturation regulates sensitivity to ATP-induced death. Glia 59(3):363–378

Chang JM, Di Tommaso P, Taly JF, Notredame C (2012) Accurate multiple sequence alignment of transmembrane proteins with PSI-Coffee. BMC Bioinf 13(Suppl 4):S1

Chen Y, Wu H, Wang S, Koito H, Li J, Ye F, Hoang J, Escobar SS, Gow A, Arnett HA, Trapp BD, Karandikar NJ, Hsieh J, Lu QR (2009) The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. Nat Neurosci 12(11):1398–1406

Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, Ferrario S, Parravicini C, Capra V, Gelosa P, Guerrini U, Belcredito S, Cimino M, Sironi L, Tremoli E, Rovati GE, Martini C, Abbracchio MP (2006) The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J 25(19):4615–4627

Coppi E, Maraula G, Fumagalli M, Failli P, Cellai L, Bonfanti E, Mazzoni L, Coppini R, Abbracchio MP, Pedata F, Pugliese AM (2013) UDP-glucose enhances outward K(+) currents necessary for cell differentiation and stimulates cell migration by activating the GPR17 receptor in oligodendrocyte precursors. Glia 61(7):1155–1171

Crociara P, Parolisi R, Conte D, Fumagalli M, Bonfanti L (2013) Cellular and molecular characterization of multipolar Map5-expressing cells: a subset of newly generated, stage-specific parenchymal cells in the mammalian central nervous system. PLoS One 8(5): e63258

Daniele S, Lecca D, Trincavelli ML, Ciampi O, Abbracchio MP, Martini C (2010) Regulation of PC12 cell survival and differentiation by the new P2Y-like receptor GPR17. Cell Signal 22(4):697–706

Daniele S, Trincavelli ML, Gabelloni P, Lecca D, Rosa P, Abbracchio MP, Martini C (2011) Agonist-induced desensitization/resensitization of human G protein-coupled receptor 17: a functional cross-talk between purinergic and cysteinyl-leukotriene ligands. J Pharmacol Exp Ther 338(2):559–567

Daniele S, Trincavelli ML, Fumagalli M, Zappelli E, Lecca D, Bonfanti E, Campiglia P, Abbracchio MP, Martini C (2014) Does GRK-beta arrestin machinery work as a "switch on" for GPR17-mediated activation of intracellular signaling pathways? Cell Signal 26 (6):1310–1325

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8(6):752–758

Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE, Monaghan AE, Liew WC, Mpamhanga CP, Bonner TI, Neubig RR, Pin JP, Spedding M, Harmar AJ (2013) International union of basic and clinical pharmacology. LXXXVIII G protein-coupled receptor list: recommendations for new pairings with cognate ligands. Pharmacol Rev 65(3):967–986

Dougherty JD, Fomchenko EI, Akuffo AA, Schmidt E, Helmy KY, Bazzoli E, Brennan CW, Holland EC, Milosevic A (2012) Candidate pathways for promoting differentiation or quiescence of oligodendrocyte progenitor-like cells in glioma. Cancer Res 72 (18):4856–4868

Eberini I, Daniele S, Parravicini C, Sensi C, Trincavelli ML, Martini C, Abbracchio MP (2011) In silico identification of new ligands for GPR17: a promising therapeutic target for neurodegenerative diseases. J Comput Aided Mol Des 25(8):743–752

Eggerickx D, Denef JF, Labbe O, Hayashi Y, Refetoff S, Vassart G, Parmentier M, Libert F (1995) Molecular cloning of an orphan G-protein-coupled receptor that constitutively activates adenylate cyclase. Biochem J 309(Pt 3):837–843

Fancy SP, Baranzini SE, Zhao C, Yuk DI, Irvine KA, Kaing S, Sanai N, Franklin RJ, Rowitch DH (2009) Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. Genes Dev 23(13):1571–1585

Ferrara G, Errede M, Girolamo F, Morando S, Ivaldi F, Panini N, Bendotti C, Perris R, Furlan R, Virgintino D, Kerlero de Rosbo N, Uccelli A (2016) NG2, a common denominator for neuroinflammation, blood-brain barrier alteration, and oligodendrocyte precursor response in EAE, plays a role in dendritic cell activation. Acta Neuropathol 132(1):23–42

Franke H, Parravicini C, Lecca D, Zanier ER, Heine C, Bremicker K, Fumagalli M, Rosa P, Longhi L, Stocchetti N, De Simoni MG, Weber M, Abbracchio MP (2013) Changes of the GPR17 receptor, a new target for neurorepair, in neurons and glial cells in patients with traumatic brain injury. Purinergic Signal 9(3):451–462

Franklin RJ, Ffrench-Constant C (2008) Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 9 (11):839–855

Fratangeli A, Parmigiani E, Fumagalli M, Lecca D, Benfante R, Passafaro M, Buffo A, Abbracchio MP, Rosa P (2013) The regulated expression, intracellular trafficking and membrane recycling of the P2Y-like receptor GPR17 in Oli-neu oligodendroglial cells. J Biolog Chem 288(7):5241–5256

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63(6):1256–1272

Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio MP (2004) Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. Biochem Pharmacol 68 (1):113–124

Fumagalli M, Daniele S, Lecca D, Lee PR, Parravicini C, Fields RD, Rosa P, Antonucci F, Verderio C, Trincavelli ML, Bramanti P, Martini C, Abbracchio MP (2011a) Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation. J Biol Chem 286(12):10593–10604

Fumagalli M, Lecca D, Abbracchio MP (2011b) Role of purinergic signalling in neuro-immune cells and adult neural progenitors. Front Biosci 16:2326–2341

Fumagalli M, Bonfanti E, Daniele S, Lecca D, Martini C, Trincavelli ML, Abbracchio MP (2015) The ubiquitin ligase Mdm2 controls oligodendrocyte maturation by intertwining mTOR with G protein-coupled receptor kinase 2 in the regulation of GPR17 receptor desensitization. Glia in press. doi:10.1002/glia.22896

Fumagalli M, Lecca D, Abbracchio MP (2016) CNS remyelination as a novel reparative approach to neurodegenerative diseases: The roles of purinergic signaling and the P2Y-like receptor GPR17. Neuropharmacology 104:82–93

Garenc C, Julien P, Levy E (2010) Oxysterols in biological systems: the gastrointestinal tract, liver, vascular wall and central nervous system. Free Radic Res 44(1):47–73

Hannedouche S, Zhang J, Yi T, Shen W, Nguyen D, Pereira JP, Guerini D, Baumgarten BU, Roggo S, Wen B, Knochenmuss R, Noel S, Gessier F, Kelly LM, Vanek M, Laurent S, Preuss I, Miault C, Christen I, Karuna R, Li W, Koo DI, Suply T, Schmedt C, Peters EC, Falchetto R, Katopodis A, Spanka C, Roy MO, Detheux M, Chen YA, Schultz PG, Cho CY, Seuwen K, Cyster JG, Sailer AW (2011) Oxysterols direct immune cell migration via EBI2. Nature 475(7357):524–527

Haupt VJ, Daminelli S, Schroeder M (2013) Drug promiscuity in PDB: protein binding site similarity is key. PLoS One 8(6):e65894

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9(12):1512–1519

Hennen S, Wang H, Peters L, Merten N, Simon K, Spinrath A, Blattermann S, Akkari R, Schrage R, Schroder R, Schulz D, Vermeiren C, Zimmermann K, Kehraus S, Drewke C, Pfeifer A, Konig GM, Mohr K, Gillard M, Muller CE, Lu QR, Gomeza J, Kostenis E (2013) Decoding signaling and function of the orphan G protein-coupled receptor GPR17 with a small-molecule agonist. Sci Signal 6 (298):ra93

Huber C, Marschallinger J, Tempfer H, Furtner T, Couillard-Despres S, Bauer HC, Rivera FJ, Aigner L (2011) Inhibition of leukotriene receptors boosts neural progenitor proliferation. Cell Physiol Biochem 28(5):793–804

Im DS (2004) Discovery of new G protein-coupled receptors for lipid mediators. J Lipid Res 45 (3):410–418

Im DS (2013) Intercellular lipid mediators and GPCR drug discovery. Biomol Ther 21(6):411–422

Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, Schachter JB, Harden TK, Jacobson KA (1997) A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol Pharmacol 52(3):499–507

Kanaoka Y, Maekawa A, Austen KF (2013) Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. J Biol Chem 288(16):10967–10972

Kenakin T (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. FASEB J 15(3):598–611

Kotani M, Mollereau C, Detheux M, Le Poul E, Brezillon S, Vakili J, Mazarguil H, Vassart G, Zajac JM, Parmentier M (2001) Functional characterization of a human receptor for neuropeptide FF and related peptides. Br J Pharmacol 133(1):138–144

Lecca D, Abbracchio MP (2008) Deorphanisation of G protein-coupled receptors: a tool to provide new insights in nervous system pathophysiology and new targets for psycho-active drugs. Neurochem Int 52 (3):339–351

Lecca D, Ceruti S (2008) Uracil nucleotides: from metabolic intermediates to neuroprotection and neuroinflammation. Biochem Pharmacol 75 (10):1869–1881

Lecca D, Trincavelli ML, Gelosa P, Sironi L, Ciana P, Fumagalli M, Villa G, Verderio C, Grumelli C, Guerrini U, Tremoli E, Rosa P, Cuboni S, Martini C, Buffo A, Cimino M, Abbracchio MP (2008) The recently identified P2Y-like receptor GPR17 is a sensor of brain damage and a new target for brain repair. PLoS One 3(10):e3579

Lecca D, Marangon D, Coppolino GT, Mendez AM, Finardi A, Costa GD, Martinelli V, Furlan R, Abbracchio MP (2016) MiR-125a-3p timely inhibits oligodendroglial maturation and is pathologically up-regulated in human multiple sclerosis. Sci Rep 6:34503

Levit A, Beuming T, Krilov G, Sherman W, Niv MY (2014) Predicting GPCR promiscuity using binding site features. J Chem Inf Model 54(1):184–194

Li M, Hale JS, Rich JN, Ransohoff RM, Lathia JD (2012) Chemokine CXCL12 in neurodegenerative diseases: an SOS signal for stem cell-based repair. Trends Neurosci 35(10):619–628

Li C, Xiao L, Liu X, Yang W, Shen W, Hu C, Yang G, He C (2013) A functional role of NMDA receptor in regulating the differentiation of oligodendrocyte precursor cells and remyelination. Glia 61(5):732–749

Liu C, Yang XV, Wu J, Kuei C, Mani NS, Zhang L, Yu J, Sutton SW, Qin N, Banie H, Karlsson L, Sun S,

Lovenberg TW (2011a) Oxysterols direct B-cell migration through EBI2. Nature 475(7357):519–523

Liu C, Sage JC, Miller MR, Verhaak RG, Hippenmeyer S, Vogel H, Foreman O, Bronson RT, Nishiyama A, Luo L, Zong H (2011b) Mosaic analysis with double markers reveals tumor cell of origin in glioma. Cell 146(2):209–221

Maekawa A, Balestrieri B, Austen KF, Kanaoka Y (2009) GPR17 is a negative regulator of the cysteinyl leukotriene 1 receptor response to leukotriene D4. Proc Natl Acad Sci U S A 106(28):11685–11690

Mamedova L, Capra V, Accomazzo MR, Gao ZG, Ferrario S, Fumagalli M, Abbracchio MP, Rovati GE, Jacobson KA (2005) CysLT1 leukotriene receptor antagonists inhibit the effects of nucleotides acting at P2Y receptors. Biochem Pharmacol 71(1–2):115–125

Mao FX, Li WJ, Chen HJ, Qian LH, Buzby JS (2012) Periventricular leukomalacia long-term prognosis may be improved by treatment with UDP-glucose, GDNF, and memantine in neonatal rats. Brain Res 1486:112–120

Marschallinger J, Schaffner I, Klein B, Gelfert R, Rivera FJ, Illes S, Grassner L, Janssen M, Rotheneichner P, Schmuckermair C, Coras R, Boccazzi M, Chishty M, Lagler FB, Renic M, Bauer HC, Singewald N, Blumcke I, Bogdahn U, Couillard-Despres S, Lie DC, Abbracchio MP, Aigner L (2015) Structural and functional rejuvenation of the aged brain by an approved anti-asthmatic drug. Nat Commun 6:8466

Marteau F, Le Poul E, Communi D, Labouret C, Savi P, Boeynaems JM, Gonzalez NS (2003) Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 64(1):104–112

Marucci G, Dal Ben D, Lambertucci C, Santinelli C, Spinaci A, Thomas A, Volpini R, Buccioni M (2016) The G protein-coupled receptor GPR17: overview and update. ChemMedChem 11(23):2567–2574

Mei F, Wang H, Liu S, Niu J, Wang L, He Y, Etxeberria A, Chan JR, Xiao L (2013) Stage-specific deletion of Olig2 conveys opposing functions on differentiation and maturation of oligodendrocytes. J Neurosci 33(19):8454–8462

Meraviglia V, Ulivi AF, Boccazzi M, Valenza F, Fratangeli A, Passafaro M, Lecca D, Stagni F, Giacomini A, Bartesaghi R, Abbracchio MP, Ceruti S, Rosa P (2016) SNX27, a protein involved in down syndrome, regulates GPR17 trafficking and oligodendrocyte differentiation. Glia 64 (8):1437–1460

Mitew S, Hay CM, Peckham H, Xiao J, Koenning M, Emery B (2013) Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. Neuroscience 276:29–47

Nakatani H, Martin E, Hassani H, Clavairoly A, Maire CL, Viadieu A, Kerninon C, Delmasure A, Frah M, Weber M, Nakafuku M, Zalc B, Thomas JL, Guillemot F, Nait-Oumesmar B, Parras C (2013) Ascl1/Mash1 promotes brain oligodendrogenesis during myelination and remyelination. J Neurosci 33 (23):9752–9768

Ou Z, Sun Y, Lin L, You N, Liu X, Li H, Ma Y, Cao L, Han Y, Liu M, Deng Y, Yao L, Lu QR, Chen Y (2016) Olig2-targeted G-protein-coupled receptor Gpr17 regulates oligodendrocyte survival in response to lysolecithin-induced demyelination. J Neurosci 36 (41):10560–10573

Parravicini C, Ranghino G, Abbracchio MP, Fantucci P (2008) GPR17: molecular modeling and dynamics studies of the 3-D structure and purinergic ligand binding features in comparison with P2Y receptors. BMC Bioinf 9:263

Parravicini C, Abbracchio MP, Fantucci P, Ranghino G (2010) Forced unbinding of GPR17 ligands from wild type and R255I mutant receptor models through a computational approach. BMC Struct Biol 10:8

Parravicini C, Daniele S, Palazzolo L, Trincavelli ML, Martini C, Zaratin P, Primi R, Coppolino G, Gianazza E, Abbracchio MP, Eberini I (2016) A promiscuous recognition mechanism between GPR17 and SDF-1: molecular insights. Cell Signal 28(6):631–642

Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, Kanaoka Y, Conley P, Boyce JA (2009) Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. J Exp Med 206 (11):2543–2555

Patel JR, McCandless EE, Dorsey D, Klein RS (2010) CXCR4 promotes differentiation of oligodendrocyte progenitors and remyelination. Proc Natl Acad Sci U S A 107(24):11062–11067

Pugliese AM, Trincavelli ML, Lecca D, Coppi E, Fumagalli M, Ferrario S, Failli P, Daniele S, Martini C, Pedata F, Abbracchio MP (2009) Functional characterization of two isoforms of the P2Y-like receptor GPR17: [35S]GTPgammaS binding and electrophysiological studies in 1321N1 cells. Am J Physiol Cell Physiol 297(4):C1028–C1040

Qi AD, Harden TK, Nicholas RA (2013) Is GPR17 a P2Y/leukotriene receptor? examination of uracil nucleotides, nucleotide sugars, and cysteinyl leukotrienes as agonists of GPR17. J Pharmacol Exp Ther 347(1):38–46

Qin Y, Verdegaal EM, Siderius M, Bebelman JP, Smit MJ, Leurs R, Willemze R, Tensen CP, Osanto S (2011) Quantitative expression profiling of G-protein-coupled receptors (GPCRs) in metastatic melanoma: the constitutively active orphan GPCR GPR18 as novel drug target. Pigment Cell Melanoma Res 24 (1):207–218

Raccosta L, Fontana R, Maggioni D, Lanterna C, Villablanca EJ, Paniccia A, Musumeci A, Chiricozzi E, Trincavelli ML, Daniele S, Martini C, Gustafsson JA, Doglioni C, Feo SG, Leiva A, Ciampa MG, Mauri L, Sensi C, Prinetti A, Eberini I, Mora JR, Bordignon C, Steffensen KR, Sonnino S, Sozzani S, Traversari C, Russo V (2013) The oxysterol-CXCR2

axis plays a key role in the recruitment of tumor-promoting neutrophils. J Exp Med 210(9):1711–1728

Rajagopalan L, Rajarathnam K (2004) Ligand selectivity and affinity of chemokine receptor CXCR1. Role of N-terminal domain. J Biol Chem 279 (29):30000–30008

Rosenkilde MM, Benned-Jensen T, Andersen H, Holst PJ, Kledal TN, Luttichau HR, Larsen JK, Christensen JP, Schwartz TW (2006) Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity. J Biol Chem 281(19):13199–13208

Salituro FG, Harrison BL, Baron BM, Nyce PL, Stewart KT, Kehne JH, White HS, McDonald IA (1992) 3-(2-Carboxyindol-3-yl)propionic acid-based antagonists of the N-methyl-D-aspartic acid receptor associated glycine binding site. J Med Chem 35 (10):1791–1799

Sensi C, Daniele S, Parravicini C, Zappelli E, Russo V, Trincavelli ML, Martini C, Abbracchio MP, Eberini I (2014) Oxysterols act as promiscuous ligands of class-A GPCRs: in silico molecular modeling and in vitro validation. Cell Signal 26(12):2614–2620

Temporini C, Ceruti S, Calleri E, Ferrario S, Moaddel R, Abbracchio MP, Massolini G (2009) Development of an immobilized GPR17 receptor stationary phase for binding determination using frontal affinity chromatography coupled to mass spectrometry. Anal Biochem 384(1):123–129

Tyler WA, Jain MR, Cifelli SE, Li Q, Ku L, Feng Y, Li H, Wood TL (2011) Proteomic identification of novel targets regulated by the mammalian target of rapamycin pathway during oligodendrocyte differentiation. Glia 59(11):1754–1769

Uhlenbrock K, Gassenhuber H, Kostenis E (2002) Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. Cell Signal 14(11):941–953

Vigano F, Schneider S, Cimino M, Bonfanti E, Gelosa P, Sironi L, Abbracchio MP, Dimou L (2016) GPR17 expressing NG2-Glia: oligodendrocyte progenitors serving as a reserve pool after injury. Glia 64 (2):287–299

Wang L, Du C, Lv J, Wei W, Cui Y, Xie X (2011) Antiasthmatic drugs targeting the cysteinyl leukotriene receptor 1 alleviate central nervous system inflammatory cell infiltration and pathogenesis of experimental autoimmune encephalomyelitis. J Immunol 187(5):2336–2345

Wheatley M, Wootten D, Conner MT, Simms J, Kendrick R, Logan RT, Poyner DR, Barwell J (2012) Lifting the lid on GPCRs: the role of extracellular loops. Br J Pharmacol 165(6):1688–1703

Whitney LW, Ludwin SK, McFarland HF, Biddison WE (2001) Microarray analysis of gene expression in multiple sclerosis and EAE identifies 5-lipoxygenase as a component of inflammatory lesions. J Neuroimmunol 121(1–2):40–48

Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science 330(6007):1066–1071

Ye F, Chen Y, Hoang T, Montgomery RL, Zhao XH, Bu H, Hu T, Taketo MM, van Es JH, Clevers H, Hsieh J, Bassel-Duby R, Olson EN, Lu QR (2009) HDAC1 and HDAC2 regulate oligodendrocyte differentiation by disrupting the beta-catenin-TCF interaction. Nat Neurosci 12(7):829–838

Yu GL, Wei EQ, Wang ML, Zhang WP, Zhang SH, Weng JQ, Chu LS, Fang SH, Zhou Y, Chen Z, Zhang Q, Zhang LH (2005a) Pranlukast, a cysteinyl leukotriene receptor-1 antagonist, protects against chronic ischemic brain injury and inhibits the glial scar formation in mice. Brain Res 1053(1–2):116–125

Yu GL, Wei EQ, Zhang SH, Xu HM, Chu LS, Zhang WP, Zhang Q, Chen Z, Mei RH, Zhao MH (2005b) Montelukast, a cysteinyl leukotriene receptor-1 antagonist, dose- and time-dependently protects against focal cerebral ischemia in mice. Pharmacology 73 (1):31–40

Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, Moss SM, Paoletta S, Kiselev E, Lu W, Fenalti G, Zhang W, Muller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q (2014) Structure of the human P2Y12 receptor in complex with an antithrombotic drug. Nature 509 (7498):115–118

Zhang D, Gao ZG, Zhang K, Kiselev E, Crane S, Wang J, Paoletta S, Yi C, Ma L, Zhang W, Han GW, Liu H, Cherezov V, Katritch V, Jiang H, Stevens RC, Jacobson KA, Zhao Q, Wu B (2015) Two disparate ligand-binding sites in the human P2Y1 receptor. Nature 520(7547):317–321

Zhao B, Zhao CZ, Zhang XY, Huang XQ, Shi WZ, Fang SH, Lu YB, Zhang WP, Xia Q, Wei EQ (2012) The new P2Y-like receptor G protein-coupled receptor 17 mediates acute neuronal injury and late microgliosis after focal cerebral ischemia in rats. Neuroscience 202:42–57

Adv Exp Med Biol - Protein Reviews (2017) 19:193–232
DOI 10.1007/5584_2017_61

Published online: 5 July 2017

Biochemical and Pharmacological Role of A_1 Adenosine Receptors and Their Modulation as Novel Therapeutic Strategy

Katia Varani, Fabrizio Vincenzi, Stefania Merighi, Stefania Gessi, and Pier Andrea Borea

Abstract

Adenosine, the purine nucleoside, mediates its effects through activation of four G-protein coupled adenosine receptors (ARs) named as A_1, A_{2A}, A_{2B} and A_3. In particular, A_1ARs are distributed through the body, primarily inhibitory in the regulation of adenylyl cyclase activity and able to reduce the cyclic AMP levels. Considerable advances have been made in the pharmacological and molecular characterization of A_1ARs, which had been proposed as targets for the discovery and drug design of antagonists, agonists and allosteric enhancers. Several lines of evidence indicate that adenosine interacting with A_1ARs may be an endogenous protective agent in the human body since it prevents the damage caused by various pathological conditions, such as in ischemia/hypoxia, epileptic seizures, excitotoxic neuronal injury and cardiac arrhythmias in cardiovascular system. It has also been reported that one of the most promising targets for the development of new anxiolytic drugs could be A_1ARs, and that their activation may reduce pain signaling in the spinal cord. A_1AR antagonists induce diuresis and natriuresis in various experimental models, mediating the inhibition of A_1ARs in the proximal tubule which is primarily responsible for reabsorption and fluid uptake. In addition, the results of various studies indicate that adenosine is present within pancreatic islets and is implicated through A_1ARs in the regulation of insulin secretion and in glucose concentrations. In the present paper it will become apparent that A_1ARs could be implicated in the pharmacological treatment of several pathologies with an important influence on human health.

K. Varani (✉), F. Vincenzi, S. Merighi, S. Gessi, and P.A. Borea
Department of Medical Science, Pharmacology Section, University of Ferrara, Via Fossato di Mortara 17-19, 44121 Ferrara, Italy
e-mail: vrk@unife.it; vncfrz@unife.it; mhs@unife.it; gss@unife.it; bpa@unife.it

Keywords
A_1 adenosine receptors • Tissue distribution • Biochemical pathways • A_1AR agonists, antagonists and allosteric modulators • Pharmacological role • Therapeutic applications

Abbreviations

AMP	adenosine monophosphate
AR	Adenosine receptors
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB-1	cAMP responsive element binding protein 1
HD	Huntington's disease
TM	transmembrane

1 Structure, Distribution and Biochemical Pathways of A_1ARs

1.1 Molecular Structure of A_1ARs

To date adenosine receptors (ARs) are grouped into four subtypes based on structural similarity named as A_1, A_{2A}, A_{2B} and A_3 ARs (Borea et al. 2015). In particular, A_1ARs represent the first identified subtype widely distributed in the central nervous system (CNS) and in peripheral tissues (Fredholm et al. 2011a). From the molecular point of view, A_1ARs, as well as the other ARs, belong to the G-protein coupled receptor superfamily sharing the characteristic seven transmembrane (TM1–7) domain structure. The A_1AR is a sequence of 326 amino acids organized in TM domains constituted by α helices connected by three extracellular (EL1, EL2 and EL3) and three intracellular (IL1, IL2 and IL3) hydrophobic loops. The N-terminal tail is exposed to the extracellular environment and the C-terminal tail is located in the cytoplasm to interact with G proteins (Klinger et al. 2002). Each of these domains provides specific properties to various receptor proteins even if a significant homology is found for ARs. By progressively truncating the A_1AR, two inherent functions of the C-terminal tail have been identified. First of all, deletion of the last 22 and 26 amino acids reduced and completely abolished surface expression of the receptor, respectively. Furthermore, the removal of the distal portion of the C-tail resulted in a functional receptor that transferred the agonist-mediated signal more efficiently than the full-length receptor (Pankevych et al. 2003). It is well known that agonist binding causes conformational changes to the receptors and activates the G protein transferring extracellular signals to intracellular targets. Fluorescence Resonance Energy Transfer analyses revealed that the active conformation of the A_1AR is unstable without G protein binding and that the stabilizing effects of G protein on the active conformation of the A_1AR differ depending on the types of Gα (Tateyama and Kubo 2016). Much work has been done to elucidate that TM3 and TMs 5–7 are highly conserved regions responsible for the binding of receptor ligands. Although it is well established that A_1AR orthosteric site is located within the receptor TM bundle, prior studies have implicated EL2 as having a significant role in contributing to orthosteric ligand affinity and signaling (Peeters et al. 2012; Nguyen et al. 2016). Moreover, molecular modeling studies of A_1ARs have suggested the presence in proximity to the orthosteric site of the allosteric site within the boundaries of the second extracellular loop of the receptor (Narlawar et al. 2010). Like most other transmembrane G-protein coupled receptors, the A_1AR crystal structure has not been obtained even if several studies have tried to predict the binding site by using 3D–models based on the available site-directed mutagenesis data (Giordanetto et al. 2003). These models

have been used for molecular docking of the native agonist, adenosine and of its derivatives (Ivanov et al. 2007). Site-directed mutagenesis of the A_1AR has resulted in the identification of amino acid residues in TM domains 6 and 7, that are critical in both agonist and antagonist binding. These include the distal region of the second extracellular loop of ARs which has a role in the binding of both agonist and antagonist ligands. A segment of the exofacial portion of TM domain 5 of ARs appears to be involved in the selective recognition of agonists containing a substitution at the 5′-position of the ribose moiety. Isolation of the genomic sequence of human A_1AR, in combination with analysis of the transcript distribution in several tissues, indicates that alternative splicing of human A_1AR occurs in the 5′-untranslated region of the gene. The exon 4, 5 and 6 transcript has been detected in all tissues that express the A_1AR, while the exon 3, 5 and 6 mRNA is found in tissues that display a relatively high A_1AR expression (Olah et al. 1995). A recent study using molecular docking and molecular dynamics simulation confirmed that Thr270, His278 and Asn70, Phe171, Glu172, Tyr271 and Ile274 were crucial for the interaction between A_1AR agonist and antagonist and the receptor (Mansourian et al. 2015). Cysteine residues, with their ability to form covalent bonds with other cysteine residues through a disulfide bond, have been shown to be important for receptor structure. Mutagenesis data revealed that Cys80 and Cys169 are the only two cysteines in the human A_1ARs that are absolutely required for delivery of the receptor to the plasma membrane (Scholl and Wells 2000). In addition, site-directed mutagenesis studies have also been performed in order to identify the amino acids involved in the binding site of A_1AR allosteric modulators (Canals et al. 2012). Several papers have reported that A_1ARs could be present as homomers or heteromers with various membrane receptors such as A_{2A}ARs, $P2Y_1$, $P2Y_2$ and D_1 subtypes (Fredholm et al. 2011b). Western blotting analysis and bioluminescence resonance energy transfer experiments based on bioluminescence signals were indicative for A_1-A_1AR association among overexpressed recombinant receptors (Yoshioka et al. 2002). A_1-A_{2A}AR heterodimers have been found in striatal glutamatergic nerve terminals, both pre- and postsynaptically, suggesting a fine modulation of glutamatergic release by adenosine (Ciruela et al. 2006). It has been also found that A_1-A_{2A}AR heterodimers act as a concentration-dependent switch controlling striatal glutamatergic neurotransmission (Ferré et al. 2007). It has been speculated that A_1- $P2Y_1$ heteromerization might be one of the mechanisms for the adenine nucleotide-mediated inhibition of neurotransmitter release (Nakata et al. 2005). The simultaneous activation of A_1-$P2Y_2$ heteromers induces a differential effect on $A_1/G_{i/o}$ and $P_{2Y}/G_{q/11}$ signaling while the stimulation of the monomeric receptors have additive effects (Lazarowski et al. 2000). In cotransfected Ltk-fibroblast cells a specific coimmunoprecipitation has been detected between A_1ARs and D_2 receptors in the absence of the respective agonists (Ginés et al. 2000). Moreover, heteromers of A_1AR and α2-adrenoceptor within the nucleus tractus solitarii could be very important for the sympathetic regulation of physiological cardiovascular homeostasis (Santiago et al. 2015). Deregulation of these systems may result in an elevated sympathetic tone which could cause neurogenic hypertension (Carrettiero et al. 2008). The physiological crosstalk between these receptors is deregulated in animal models of hypertension, which suggests their involvement in the development of this pathology (Carrettiero et al. 2009). In addition, A_1ARs heterodimerize with β_1- and/or β_2-adrenergic receptors generating constitutive heterodimers which significantly influence orthosteric ligand binding affinity of both adrenergic receptors without altering the ligand binding properties of A_1AR. The formation of these heteromers leads to altered receptor pharmacology, functional coupling, and intracellular signaling pathways (Chandrasekera et al. 2013). Interestingly, the receptor cross-talk between these two important receptor families may offer the opportunity to fine-tune crucial signaling responses and to develop more specific therapeutic interventions. It is well reported that the desensitization of the

receptor could be homologous if directly stimulated by the drug, or heterologous if involves other receptors. On the other hand, antagonist treatment may lead to sensitization and to an increase in receptor density (Kiesman et al. 2009). The activation of A_1ARs may lead to various degrees of desensitization and down-regulation of receptor expression (Schulte and Fredholm 2002a). It is also noteworthy that A_1ARs may interact with proteins other than receptors, which may affect receptor signaling and may even play a key role in receptor trafficking, internalization and desensitization (Ruiz et al. 2011).

1.2 Tissue and Cellular Distribution of A_1ARs

A_1ARs are highly expressed throughout the CNS in different regions, such as cortex, hippocampus, cerebellum and spinal cord. In particular, the highest densities of A_1ARs in rat brain occur in the molecular layer of the cerebellum, the molecular and polymorphic layers of the hippocampus and dentate gyrus, the medial geniculate body, certain thalamic nuclei, and the lateral septum. High densities also are observed in certain layers of the cerebral cortex, the piriform cortex, the caudate-putamen, the nucleus accumbens, and the granule cell layer of the cerebellum (Goodman and Synder 1982). A_1ARs have been cloned from several species including mouse, rat, guinea-pig, rabbit, dog, bovine and human, and have been found to have a high overall sequence homology of 87% (Nell and Albrecht-Küpper 2009). Differences in binding characteristics between species have been reported, which might be due to small variations in the A_1AR aminoacid sequences (Nell and Albrecht-Küpper 2009). Autoradiographic localization of A_1ARs in human brain tissues has revealed a heterogeneous anatomical distribution with high levels, particularly in the hippocampal formation, striatum, neocortex and some thalamic nuclei (Fastbom et al. 1986). The distribution of receptors in human brain is similar to that seen in rat brain even if some regional differences have been observed in the cerebral cortex, the striatum and the cerebellar cortex that may prove to be functionally relevant (Fastbom et al. 1987). In a quantitative autoradiographic study performed in human postmortem brain, A_1ARs were widely distributed with the highest densities in the stratum radiatum/pyramidale of the hippocampal region CA1. In particular, A_1ARs were non homogeneously distributed in nucleus caudatus, globus pallidus, and cortical areas: in the cingulate and frontal cortex, the deep layers showed the highest labeling, while in the occipital, parietal, temporal, and insular cortex it was highest in the superficial layers (Svenningsson et al. 1997). As for the cellular localization A_1ARs were found in both neurons and glial cells (Acton and Miles 2015; Gessi et al. 2013; Merighi et al. 2015). Different techniques in humans have been used to quantify the A_1ARs such as positron emission tomography and autoradiography which have revealed similar results in the distribution of this receptor subtype (Meyer et al. 2004). In the cardiovascular system, regional expression of A_1ARs, have been found in left and right atria and ventricular myocardium, with higher levels in the atria (Böhm et al. 1989; Musser et al. 1993; Kapicka et al. 2003). A_1ARs have also been reported on coronary arterial smooth muscle in pigs and mice and on coronary endothelial cells in guinea-pig (Zahler et al. 1994; Hussain and Mustafa 1995; Shen et al. 2005; Headrick et al. 2013). It is well reported that A_1ARs represent the main AR type in cardiac muscle and that a fraction named A_1 receptor reserve exists in the atrium, that has been quantified by using the Furchgott method (Srinivas et al. 1997). This method makes it possible to calculate the agonist equilibrium dissociation constant and the fractional receptor occupancy through analysis of agonist concentration-response curves obtained before and after irreversible inactivation of a fraction of the receptor population (Zhang et al. 1997). Intermediate expression of A_1ARs has been found in the sarcolemma and cytosol of skeletal muscle cells and in vascular cells of skeletal muscle tissue (Lynge and Hellsten 2000). A_1ARs have also been identified in airway

smooth muscle, in coronary smooth muscle cells and in intestinal muscle cells (Ethier and Madison 2006; Nayeem and Mustafa 2002; Murthy et al. 1995). A_1AR mRNA is also present in peripheral tissues including the stomach, spleen, lung, pancreas, kidney, small intestine and liver at varying levels (Spicuzza et al. 2006; Kolachala et al. 2008). In particular, A_1ARs are expressed at low levels in lung endothelial cells, airway epithelial cells and alveolar epithelial cells and are most pronounced on alveolar macrophages (Sun et al. 2005). In kidney, A_1ARs are most abundant in collecting ducts of the papilla, inner medulla and cells of the juxtaglomerular apparatus (Weaver and Reppert 1992). Moreover, A_1AR mRNA has been detected at low levels in the large intestine where it mediates secretory refluxes (Christofi et al. 2001). The identification of A_1ARs has been found in human adipocyte membranes from different anatomical localizations, such as abdominal, femoral and omental fat deposits (Larrouy et al. 1991). A_1ARs are expressed in pancreas tissues where they have been associated with the regulation of glucose homeostasis by reduction of insulin and stimulation of glucagone (Salazar-Martinez et al. 2004). In addition, A_1ARs are differentially present on a variety of immune cells, including neutrophils, eosinophils, macrophages and monocytes where they are able to mediate a pro-inflammatory immune response (Sachdeva and Gupta 2013; Boros et al. 2016). A_1ARs have also been autoradiographically localized in rat, guinea pig, monkey, and human retina. The highest levels of A_1AR binding sites occur in the nerve fiber, ganglion cell, and inner plexiform layers of the retina in the various species examined (Braas et al. 1987).

1.3 Biochemical Pathways of A_1ARs

The transduction of some external signals into internal signals involves sequential activation of three membrane proteins: a membrane receptor, a guanosine triphosphate-binding protein or named G protein that couples the receptor to specific effector enzymes generating second messengers able to activate various protein kinases (Murthy et al. 2000; Gessi et al. 2011). It is well known that G protein coupled receptors play crucial roles in mediating cellular responses to external stimuli, and increasing evidence suggests that they function as multiple units (Schulte and Fredholm 2002b). The G protein coupled receptor activation causes the exchange of guanosine diphosphate for the guanosine triphosphate bound to G protein α subunit and the dissociation of βγ heterodimer (Olah and Stiles 2000). In particular, A_1ARs are primarily coupled to members of G_i/G_o family of G proteins inducing inhibition of adenylyl cyclase activity therefore reducing the intracellular levels of cyclic adenosine monophosphate (cAMP) (Fredholm et al. 2000) (Fig. 1). Recently, in human brains, the activation of A_1AR-mediated G-protein involves preferentially Gαi-3 which belongs to the α subunits of Gi/o class (Odagaki et al. 2015). Interestingly, it has been found that the activation of Gαi-coupled receptors often causes enhancement of the inositol phosphate signal triggered by Gαq-coupled receptors. This mechanism of the synergistic receptor crosstalk has been investigated between A_1ARs, α2C adrenergic receptors and the αq-coupled bradykinin receptors suggesting that Gβγ exchange between Gαi- and Gq-coupled receptors mediates this type of receptor crosstalk (Quitterer and Lohse 1999). The decrease in cAMP levels could lead to the attenuation of cAMP responsive element binding protein 1 (CREB-1) phosphorylation by cAMP-dependent protein kinase A because, for full transcriptional activation of CREB, cofactors such as CREB binding protein are necessary (Ellis et al. 1995). In particular, CREB phosphorylation requires protein kinase A, phosphorylation and activation of CREB binding protein which could be involved in cross signaling with other kinases such as the mitogen-activated protein kinases (Grewal et al. 2000). In addition, A_1ARs might activate phospholipase C, which is known to increase inositol 1,4,5-triphosphate mediating a rapid and transient increase in the cytoplasmatic Ca^{2+} concentrations that stimulate various calcium-dependent protein kinases or calmodulin

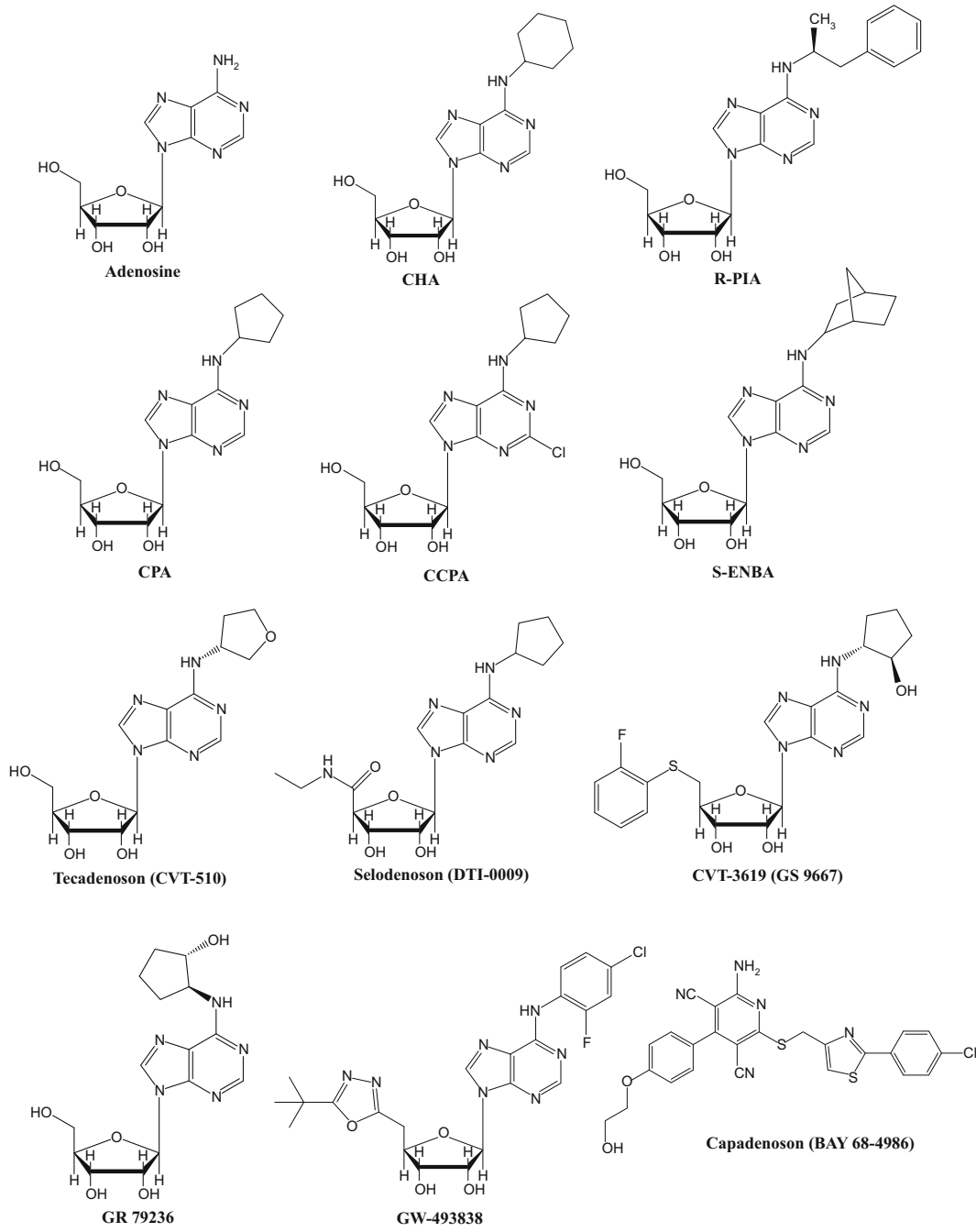

Fig. 1 Chemical structures of some of the most interesting A_1AR agonists

(Basheer et al. 2002; Nalli et al. 2014). Moreover, G protein βγ subunits seem to be the relevant signal transducers for A_1AR-mediated phospholipase C activation (Biber et al. 1997). In cardiac tissue and neurons, it has been reported that A_1AR activation is linked to the

opening of K+ channels (Kunduri et al. 2013). A_1AR could also inhibit Q, P and N-type Ca^{2+} channels and modulate extracellular signal-regulated protein kinases via the release of βγ subunits (Schulte and Fredholm 2002a).

2 A_1AR Agonists, Antagonists and Allosteric Modulators

Intense efforts of many pharmaceutical companies and academicians in the AR field have led to the discovery of clinical candidates that are agonists, antagonists and allosteric enhancers (Preti et al. 2015). In particular, the majority of A_1AR agonists are structurally related to the endogenous ligand adenosine possessing a purine and a ribose moiety (Fig. 2). Changes in either structural properties, including stereochemistry, of the ribose moiety in most cases lead to a loss of receptor-binding potency and intrinsic activity. Most of the adenosine-related compounds are modified in the N^6- or C^2- position of the adenine moiety. In addition, modifications in the 3′, 4′ or 5′ position of the ribose moiety lead to a number of highly selective agonists with improved metabolic stability compared to adenosine, that has a short-life in the body (Fig. 2).

N^6-substitution with large and hydrophobic groups is generally well tolerated leading to potent and selective A_1AR agonists. In the past several active molecules have been found including N^6-cyclopentyl-adenosine (CPA), N^6-cyclohexyladenosine (CHA), 2-chloro-N^6-cyclopentyladenosine (CCPA), N^6-(endo-norborn-2-yl)-adenosine (S-ENBA), N^6-(endo-5,6-epoxynorborn-2-yl)-adenosine (S-epoxy-ENBA) (Gao et al. 2003; Gao and Jacobson 2007) (Fig. 2). Interestingly, a hydrogen at N^6 position is required for the activity, suggesting that it may be involved in a hydrogen bond interaction with the receptor or in an unfavorable steric hindrance between the N^6-disubstituted derivatives and the receptor itself. The substitution of the 5′-hydroxyl group of the adenosine ribose moiety is also well tolerated and leads to very potent compounds, while that of the 2′ or 3′-hydroxyl groups is less favorable for the activity (Schenone et al. 2010). The 5′-chloro-5′-deoxy derivative of S-ENBA, 5′-chloro-5′-deoxy-N^6-(endo-norborn-2-yl)-adenosine (Cl-ENBA), showed high affinity at A_1ARs, with a relevant selectivity versus the other AR subtypes (Franchetti et al. 2009). The most interesting N^6-substituted derivative is CVT-510 (tecadenoson) by CV Therapeutics and represents one of the few full agonists with favorable solubility to reach clinical trials (Snowdy et al. 1999) (Fig. 2). The 5′-position of tecadenoson was chosen as the site of substitution to potentially provide partial agonism by removing the hydrogen bond donor capabilities in this specific position (van Tilburg et al. 2001). A number of similar derivatives of tecadenoson have been synthesized by various research groups as potent and selective A_1AR agonists (Ashton et al. 2007; Cappellacci et al. 2008).

Different substitutions at N^6-position have been investigated, such as selodenoson (DTI 0009), by Aderis Pharmaceuticals, that represents the most relevant N^6-cyclopentyl substituted compound (Gao and Jacobson 2007) (Fig. 2). A series of 5′-substituted CPA have been synthesized where the 5′-hydroxyl group has been converted to a methoxy group resulting in partial agonists (van Tilburg et al. 2001). A family of N^6-cyclopentyl adenosine derivatives substituted on C2 or on the 2′-position of the ribose mojety have been synthesized as potent and selective A_1AR agonists (Elzein et al. 2007; Maione et al. 2007). Moreover, CVT 3619 by CV Therapeutics Inc. substituted on the 5′-position of the ribose mojety with a 2-fluorophenylthiomethyl group resulted in a partial agonist for A_1AR with a good oral bioavailability (Dhalla et al. 2007a; Shearer et al. 2009) (Fig. 2). Various *in vitro* and *in vivo* studies have been performed by Glaxo Smith Kline on GW-493838, an A_1AR partial agonist substituted in 5′ position with a bulky moiety (Giorgi and Nieri 2013) (Fig. 2). Another compound synthesized by Glaxo Smith Kline, named as GR 79236 and defined as A_1AR agonist with high affinity, showed a reduction in lipid and glucose activity in an animal model of

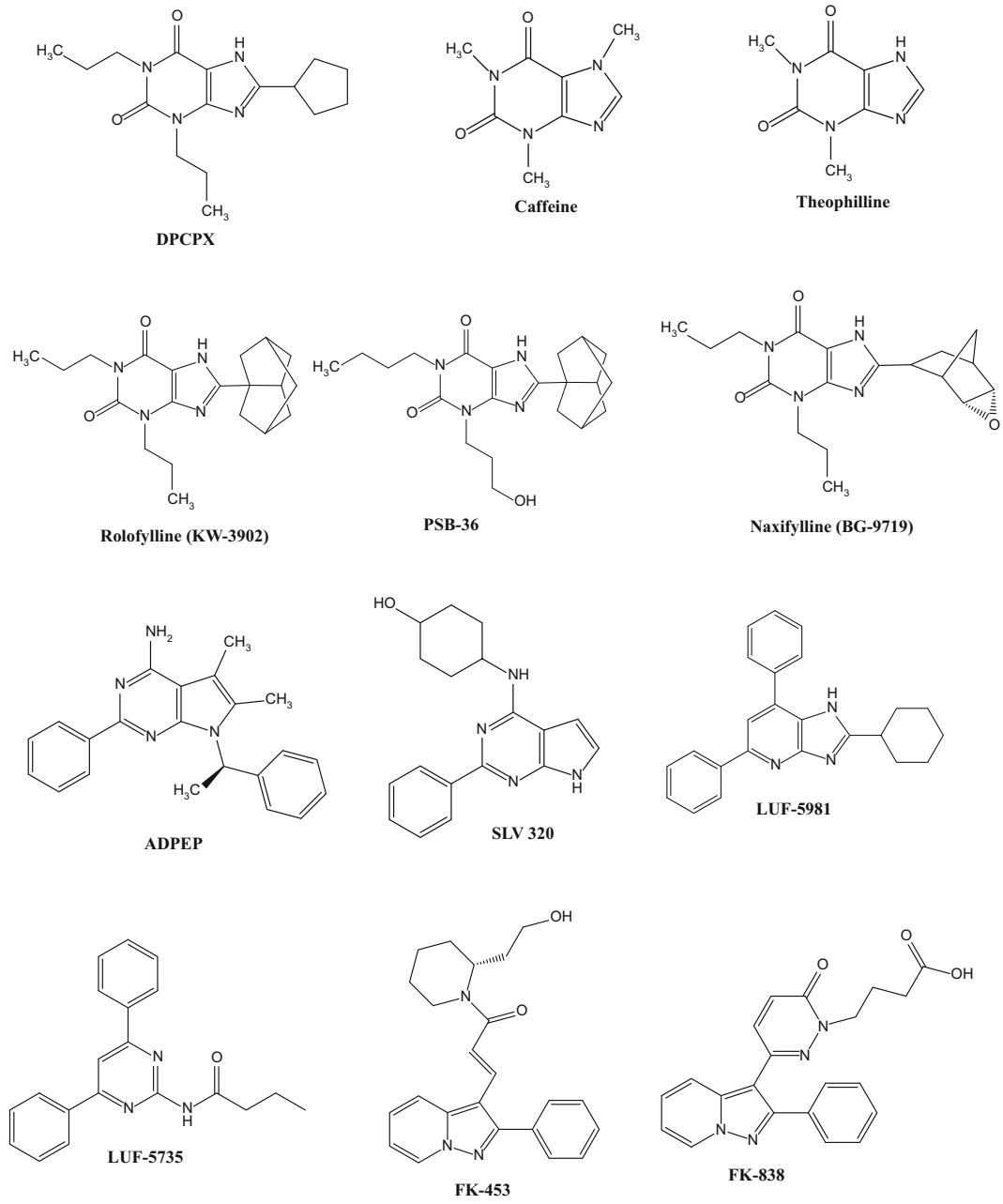

Fig. 2 Chemical structures of some of the most well-known A_1AR antagonists

diabetes (Fig. 2). The only non nucleosidic agonist family being aminopyridine derivatives by Bayer Schering Pharma was capadenoson, BAY 68–4986 which possess neither the purine ring structure nor containing the ribose (Schenone et al. 2010) (Fig. 2). Table 1 reports the affinity values expressed as Ki (nM) of selected A_1AR agonists to the A_1, A_{2A}, A_{2B} and A_3ARs in human or rat substrates.

It is well reported that the prototypic A_1AR antagonists are the xanthines, theophylline and caffeine, and a multitude of xanthine derivatives

Table 1 Affinity values of selected AR agonists to A_1, A_{2A}, A_{2B} and A_3ARs

Compounds	A_1ARs Ki (nM)	A_{2A}ARs Ki (nM)	A_{2B}ARs Ki (nM)	A_3ARs Ki (nM)	References
Adenosine	100 (h)	310 (h)	15,000 (h)	290 (h)	Fredholm et al. (2011a)
	73 (r)	150 (r)	5100 (r)	6500 (r)	
CHA	1.31 (h)	310 (h)	160,000 (r)	1025 (h)	Daly et al. (1993) and van Galen et al. (1994)
	0.85 (r)	460 (r)		176 (r)	
R-PIA	2.04 (h)	859 (h)	3500 (h)	33 (h)	Salvatore et al. (1993) and Klotz et al. (1998)
	1.2 (r)	220 (r)	730 (r)	158 (r)	
CPA	2.3 (h)	794 (h)	18,600 (h)	72 (h)	Jacobson and Gao (2006)
	0.12 (r)	845 (r)		213 (r)	
CCPA	0.83 (h)	2270 (h)	18,800 (h)	38 (h)	Jacobson and Gao (2006)
	0.09 (r)	950 (r)		237 (r)	
S-ENBA	0.34 (r)	477 (r)	–	282 (h)	Müller and Jacobson (2011)
				915 (r)	
Cl-ENBA	0.51 (h)	1340 (h)	2740 (h)	1290 (h)	Franchetti et al. (2009)
Tecadenoson (CVT-510)	3.1(b)	7300 (b)	–	–	Cappellacci et al. (2008) and Müller and Jacobson (2011)
	6.5 (p)	2315 (h)			
		9756 (p)			
Selodenoson (DTI-0009)	1.11 (r)	306 (r)	–	–	(Merkel et al. 1993)
CVT-3619 (GS 9667)	55 (h)	>10,000 (h)	>50,000 (h)	>1000 (h)	Müller and Jacobson (2011)
GR 79236	3.1 (r)	1300 (h)	–	–	Müller and Jacobson (2011)

The data are expressed as Ki (nM) reported in human (h), rat (r), pig (p) and bovine (b) substrates

has been synthesized and studied for this activity (Fig. 3). However, a high number of non-xanthine derivatives has been reported as potent and selective A_1AR antagonists. Both xanthine and non-xanthine derivatives are devoid of the sugar moiety that characterizes the majority of A_1AR agonists. From the chemical point of view the A_1AR antagonists are bicyclic or tricyclic compounds where the hydrophobic substituents may enhance affinity, whereas hydrophilic substituents render many of the high affinity antagonists quite insoluble in water (Müller and Jacobson 2011). Among A_1AR antagonists, xanthine derivatives represent a class of interesting compounds, for example, the 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) due to its high affinity and selectivity. The most important derivatives of this family are 1,3 dipropyl-8-(3-noradamantyl)xanthine (KW-3902, rolofylline, NovaCardia, Inc.) and 1,3 dipropyl 8-[2-(5,6-epoxynorbornyl)xanthine (BG 9719, naxifylline, CV Therapeutics & Biogen) (Fig. 3). Different compounds have been synthesized as derivatives of KW-3902 or BG 9719 compounds with the aim to identify novel and selective A_1AR antagonists with greater aqueous solubility (Kiesman et al. 2006). The most potent compound of this series is 1-butyl-3-3-hydroxypropyl)-8-(3-noradamntyl)xanthine (PSB 36) (Müller and

Jacobson 2011) (Fig. 3). Considerable effort, due to the nonspecific effects of xanthines, has been made to identify non xanthine A_1AR antagonists with increased potency and selectivity, such as a variety of heterocycles compounds including pyrazolopyridines, thiazolopyrimidines, imidazolopyridines and benzimidazoles (Baraldi et al. 2008). A_1AR antagonists are usually bi- and tricyclic heteroaromatic compounds even if, some examples of monocyclic derivatives,

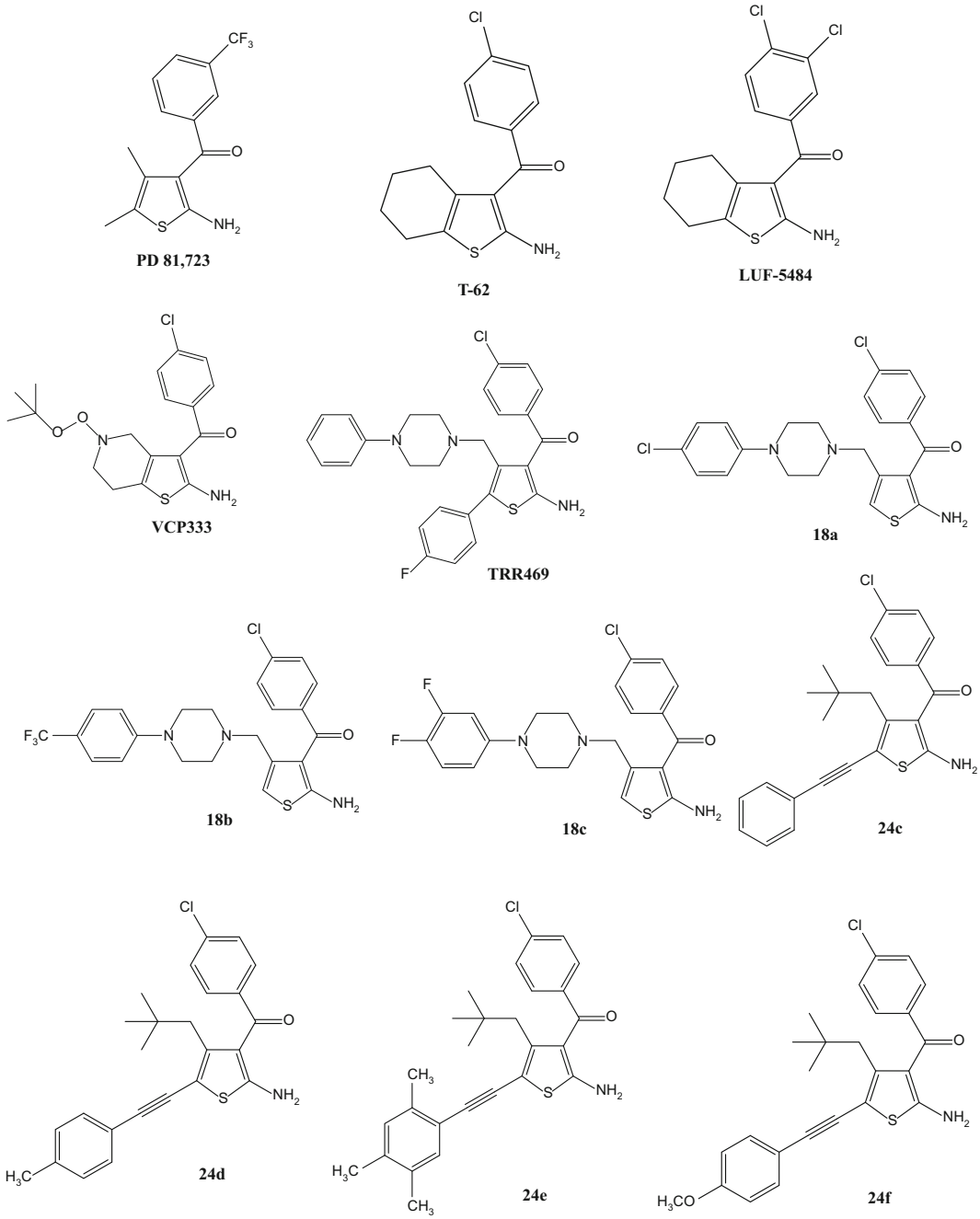

Fig. 3 Chemical structures of selected A_1AR allosteric enhancers

including thiazoles, thiadiazoles and pyrimidines, have been reported as A_1AR antagonists (Chang et al. 2004a). A series of 7-deazadenine derivatives bearing polar substituents has been synthesized as analogues of the potent A_1AR antagonist named as ADPEP (Müller et al. 1996) or substituted with a side chain and identified as SLV320 by Solvay Pharmaceuticals (Kalk et al. 2007) (Fig. 3).

A number of 1-deazapurine derivatives has been identified and characterized by a high affinity and selectivity versus A_1ARs, such as LUF-5981, which in functional assays behaves like an inverse agonist (Chang et al. 2007) (Fig. 3). Different compounds, like LUF-5740 and LUF-5735, have been synthesized, showing good potency at the A_1AR and an optimal polar surface area value suggesting a good brain permeation (Chang et al. 2004b) (Fig. 3). It is interesting to note the role of cyano-substitution at the pyrimidine core structure and the introduction of the methylene bridge give high affinity to the compounds and selectivity to A_1ARs and behave only as antagonists (van Veldhoven et al. 2008).

Another class of analogs, structurally related to the xanthine core consists of pyrazolo pyridines derivatives such as FK-453 and FK-838 which show potent and selective A_1AR antagonist activity (Akahane et al. 1999) (Fig. 3). Further structure-activity work has identified various adenine derivatives like N-0861 as a lead compound and its derivative, WRC-0571, which is a highly potent and selective A_1AR antagonist with a good potency and aqueous solubility showing specific competitive antagonism at low concentrations but nonspecific inhibitory activity at higher concentrations (Shryock et al. 1992) (Fig. 3). Table 2 summarizes various examples of A_1AR antagonists with their affinity values versus A_1, A_{2A}, A_{2B} and A_3ARs in human or rat substrates.

It is well reported that allosteric modulation may provide an alternative approach to highly selective agonists for A_1ARs with the specific advantage of their ability to induce responses

Table 2 Affinity values of selected AR antagonists to A_1, A_{2A}, A_{2B} and A_3ARs

Compounds	A_1ARs Ki (nM)	A_{2A}ARs Ki (nM)	A_{2B}ARs Ki (nM)	A_3ARs Ki (nM)	References
DPCPX	3.0 (h)	129 (h)	51 (h)	795 (h)	Müller and Jacobson (2011)
	1.0 (r)	500 (r)	186 (r)	> 10,000 (r)	
Caffeine	44,900 (h)	23,400 (h)	33,800 (h)	13,300 (h)	Müller and Jacobson (2011)
	41,000 (r)	32,500 (r)	30,000 (r)	> 100,000 (r)	
Theophylline	6770 (h)	1710 (h)	9070 (h)	22,300 (h)	Müller and Jacobson (2011)
	14,000 (r)	22,000 (r)	15,100 (r)	> 100,000 (r)	
Rolofylline (KW-3902)	0.72 (h)	108 (h)	296 (h)	4390 (h)	Müller and Jacobson (2011)
	12.6 (r)	510 (r)			
PSB-36	0.7 (h)	980 (h)	187 (h)	2300 (h)	Müller and Jacobson (2011)
	0.124 (r)	552 (r)		6500 (r)	
Naxifylline (BG-9719)	0.45 (h)	1100 (h)	611 (h)	4810 (h)	Müller and Jacobson (2011)
	0.67 (r)	1250 (r)			
ADPEP	4.7 (r)	3700 (r)	–	–	Müller (2001)
SLV 320	1.0 (h)	398 (h)	3981 (h)	200 (h)	Müller and Jacobson (2011)
	2.51 (r)		501 (r)		
LUF-5981	0.90 (h)	194 (h)	–	637 (h)	Chang et al. (2007)
LUF-5735	3.70 (h)	> 10,000 (h)	> 10,000 (h)	> 10,000 (h)	Chang et al. (2004a)
FK-453	18 (h)	1300 (h)	980 (h)	> 10,000 (h)	Müller and Jacobson (2011)
FK-838	12 (h)	1202 (r)	–	–	Scheiff et al. (2010)
	6.61 (r)				

The data are expressed as Ki (nM) reported in the references in human (h) and rat (r) substrates

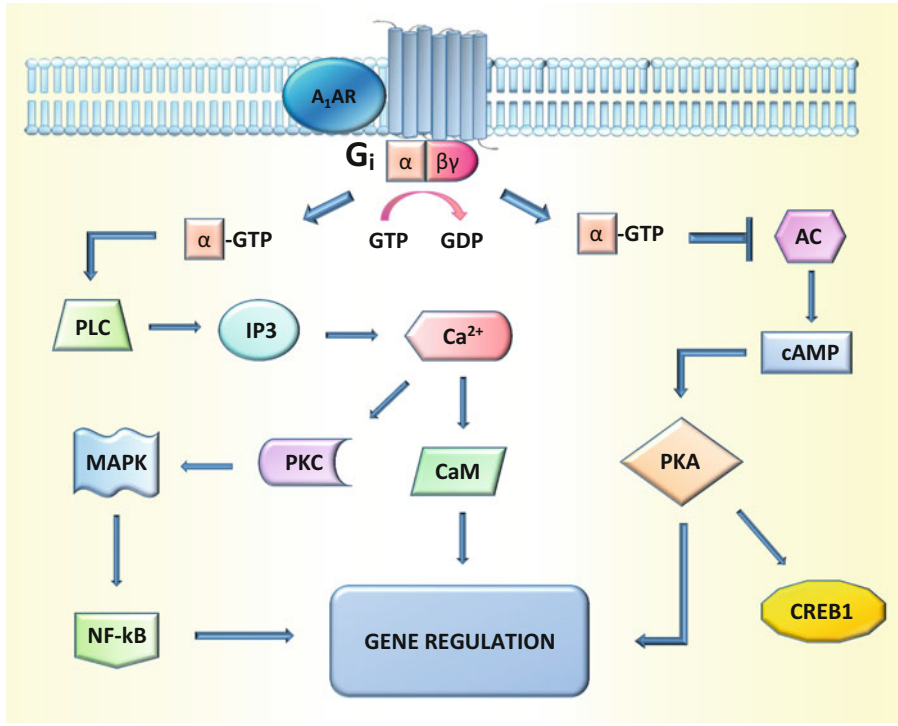

Fig. 4 Schematic representation of intracellular signaling pathways mediated by A_1AR stimulation. Upon activation of A_1ARs by adenosine: (**a**) the Gα subunit is dissociated from the receptor and Gβγ, and decreases adenylate cyclase activity and cAMP production; (**b**) PLC is activated by Gα-GTP and mediates the increase in IP3 and $Ca2^+$ levels

only under conditions in which the endogenous agonist is able to exert its physiological effects (Romagnoli et al. 2015). Allosteric modulators can be divided as negative or positive allosteric modulators inducing either a negative or a positive effect on cellular signaling and receptor coupling, respectively (Christopoulos 2002). The first allosteric modulator of A_1ARs is represented by [(2-amino-4,5-dimethylthiophen-3-yl) (3-trifluoromethyl) methanone] (PD 81,723) which has been found to stabilize the high affinity conformational state of A_1AR-G protein complex (Bruns and Fergus 1990) (Fig. 4). Structure-activity relationship studies have indicated that the benzoyl group is essential for activity, and lipophilic substituents on the phenyl of the benzoyl moiety favors good allosteric activity. As a consequence, novel 2-amino-3-benzoylthiophene derivatives named as T-62 and LUF-5484 have been characterized (van der Klein et al. 1999) (Fig. 4). A further series of 2-amino-3-benzoyl thiophene derivatives has been developed and characterized (Baraldi et al. 2000). By systematic modification of PD 81,723 new thiophene derivatives have been synthesized and investigated such as, for example, an analog of T-62 with a 7-membered heterocyclic ring fused to the thiophene named as VCP333 (Butcher et al. 2013). Several A_1AR allosteric compounds have been studied by using different chemical approaches on the thiophene nucleus, and pharmacologically characterized as showing a strong ability to increase the A_1AR density and cause a shift of more than seven fold (Baraldi et al. 2003; Baraldi et al. 2004; Romagnoli et al. 2008, 2012a, 2013, 2014a) (Fig. 4). From these studies it appears evident that the amino and carbonyl group of the benzoyl moiety are fundamental for allosteric activity and that any modification of the amino function has led to a reduction in activity (Fig. 4). Table 3 shows the A_1AR density expressed as Bmax values

Table 3 Effect of A_1AR allosteric enhancers on the A_1AR density (A) and their modulation on CCPA affinity (B)

Compounds	(A) [^{3}H]CCPA		(B) [^{3}H]DPCPX		References
	Bmax (fmol/ mg protein)	Bmax shift (fold of increase)	CCPA Ki (nM)	CCPA Ki shift (fold of decrease)	
PD 81,723	680	1.3	9.8	1.6	Romagnoli et al. (2015)
T-62	887	1.7	8.4	1.8	Vincenzi et al. (2014)
TRR469	7308	14.0	1.2	12.8	Vincenzi et al. (2014)
18a	3626	7.0	2.7	5.6	Romagnoli et al. (2008, 2012a)
18b	3989	7.7	2.4	6.3	Romagnoli et al. (2008, 2012a)
18c	3708	7.2	2.8	5.5	Romagnoli et al. (2008, 2012a)
24c	4925	9.5	1.5	9.9	Romagnoli et al. (2014a)
24d	3723	7.2	2.0	7.5	Romagnoli et al. (2014a)
24e	3633	7.0	2.0	7.5	Romagnoli et al. (2014a)
24f	3826	7.4	1.8	8.2	Romagnoli et al. (2014a)

Bmax (fmol/mg protein) and Bmax shift obtained in [^{3}H]CCPA saturation binding experiments on hA_1CHO membranes performed in the absence (K_D = 1.1 nM; Bmax = 522 fmol/mg protein) or in the presence of 10 μM enhancers (A). Ki values of CCPA obtained in [^{3}H]DPCPX competition binding experiments on hA_1CHO membranes in the presence of 10 μM tested compounds and CCPA shift = Ki(CCPA)/Ki(CCPA + 10 μM enhancers) (B) where the Ki of CCPA was 15.1 nM

All the values indicated are expressed as the mean

obtained by [^{3}H]CCPA saturation binding assays in hA_1CHO membranes in the presence of PD 81,723 and allosteric enhancers at the 10 μM concentration, as well as the relative calculated Bmax shift (A). The modulation by the same compounds on CCPA affinity (CCPA Ki shift) in [^{3}H]DPCPX competition binding experiments is also reported (B). These data highlight the capability of the novel enhancers to mediate a significant increase in A_1AR density. The enhancers are able to mediate a shift of the A_1ARs toward the high-affinity state as suggested by the increase in CCPA affinity in the presence of the allosteric enhancers as compared with the control conditions. As a consequence, the novel enhancers may have a therapeutic potential due to their ability to increase the efficacy of the endogenous agonist, adenosine. Moreover, A_1AR enhancers offer the prospect of increasing receptor selectivity where it has not been possible to design selective orthosteric drugs without side effects.

3 Pharmacological Role and Preclinical Studies of A_1ARs

Several lines of evidence indicate that adenosine may be an endogenous protective agent in the human body since it prevents the damage caused by various pathological conditions such as in CNS ischemia, excitotoxicity or epileptic seizures and, in the cardiovascular system, cardiac arrhythmias (Burnstock 2011; Borea et al. 2016). It is a well-established fact that a low concentration of adenosine is normally present in the extracellular fluid, but this rises dramatically in relation to different pathological disorders, and the activation of A_1AR pathway can affect a number of organs or systems, like the CNS, gastrointestinal or cardiovascular systems, kidney, lung and eye diseases, as well as the pancreas and metabolism regulation (Borea et al. 2016; Burnstock 2013) (Fig. 5; Table 4).

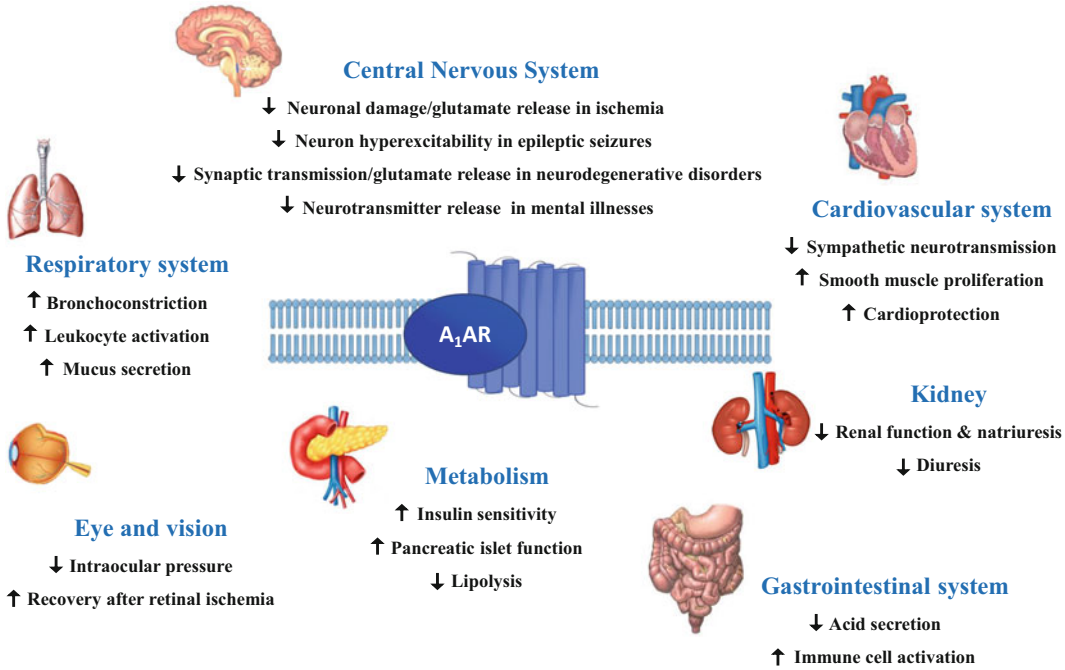

Fig. 5 Schematic diagram illustrating the functional role of A_1ARs. Activation of A_1AR pathway by adenosine can affect central nervous, cardiovascular and respiratory systems, as well as metabolism, kidney and gastrointestinal system thus regulating various biologic functions

3.1 Ischemia/Hypoxia

It is well known that ischemia-induced cell death may be partly mediated by excitotoxic actions of glutamate or aspartate which result in membrane depolarization, elevated intracellular calcium levels and cell death (Wardas 2002). Great interest has been devoted to the potential role of A_1ARs in the control of calcium influx and glutamate release, membrane potential and cellular metabolism, events which are associated with ischemia damage. It has been reported that A_1AR agonists tend to decrease the extent of ischemic brain damage, whilst A_1AR antagonists exacerbate cerebral damage (de Mendonça et al. 2000). In particular, the selective A_1AR agonist, CHA, reduces the morphologically evaluated neuronal damage by alteration of the lactate dehydrogenase release or deoxyglucose transport, whereas A_1AR antagonists enhance hypoxia-induced cell damage (Lynch et al. 1998). A similar neuroprotective effect has been observed in a model of histotoxic anoxia where the selective A_1AR agonist, CPA, attenuated neuronal cell death in hippocampal cell cultures, and A_1AR antagonists blocked this effect (Wardas 2002). In cortical or hippocampal brain slices the effect of A_1AR agonists has been seen to attenuate the neuronal cell loss and degeneration evoked by superfusion with a hypoxic or hypoglycemic medium (Newman et al. 1998). The reduced function of A_1ARs after ischemia is accompanied by a decreased density of these receptors and their desensitization, which is most likely due to oxygen deprivation (Coelho et al. 2006). It has been observed that in hippocampal slices, CHA reduces the cell death induced by a glucose-deprived medium (Barth et al. 2007). Moreover, acute systemic or intracerebroventricular injection of CHA attenuates the neuronal loss in the hippocampus and improves neurological deficits in rats subjected to global forebrain ischemia (Rudolphi and Schubert 1997). Well-known A_1AR antagonists, such as DPCPX, potentiate the mortality or degeneration of hippocampal neurons in rats subjected to focal

Table 4 Potential involvement of A_1ARs and their pharmacological role in various diseases

System/ Disease	Proposed Role A_1AR activation	Potential use in therapy	References
Ischemia/Hypoxia	Reduction of neuronal damage; – Decrease of glutamate	Ischemic stroke	de Mendonça et al. (2000)
Epilepsy	Reduction of neuron hyperexcitability; – Control of glutamatergic transmission	Epileptic seizures	Berman et al. (2000), Gomes et al. 2011)
Neurodegenerative disorders	Inhibitory effect on D_1 receptors; – Reduction of synaptic transmission; – Inhibition of glutamate release; – Regulation of inflammation and neuronal death	Parkinson's disease; Huntington's disease; Spinal cord injury	Jaberi et al. (2016), Palacios et al. (2012) and Ferrante et al. (2014)
Mental illness	Blocking of neurotransmitter release; - Control of dopaminergic and glutamatergic systems	Anxiety-related disorders; Schizophrenia	Gomes and Shinnar (2011) and Serchov et al. (2015)
Chronic & Neuropathic Pain	Reduce pain signaling and hypersensitivity	Chronic and neuropathic pain associated to different disease state	Gao and Jacobson (2007) and Li et al. (2002)
Cardiovascular diseases	Regulation of cytoprotection and cell survival; – Repression of sympathetic neurotransmission	Heart failure; Ischemia and reperfusion injury	Vyas et al. (2016) and Burgdorf et al. (2001)
Renal disorders	Antidiuresis; – Vasocontriction; – Reduction of renal function and natriuresis	Hypertension; Renal diseases; Post-ischemic acute renal failure	Welch 2015; Vallon and Osswald (2009)
Pulmonary pathologies	Bronchoconstriction; – Increase of leucocyte activation and mucus secretion	Asthma; Chronic airway inflammation	Björck et al. (1992), Cronstein et al. (1992) and McNamara et al. (2004)
Eye & vision problems	Modulation of inflammation, neuroprotection and angiogenesis; – Control of intraocular pressure; – Recovery after retinal ischemia	Retinopathy; Glaucoma; Retinal ischemia	Zhang et al. (2015) and Zhong et al. (2013)
Pancreas diseases	Regulation of insulin secretion – Control of pancreatic islet function	Type 1 diabetes; Type 2 diabetes	Andersson (2014) and Johansson et al. (2007a)
Gastrointestinal function modulation	Regulation of enteric nervous and immune systems – Decrease of acid secretion	Intestinal inflammation	Antonioli et al. (2008) , Lee et al. (2001) and Bours et al. (2006)
Metabolism & adipose tissue	Reduction of lipolysis	Metabolic syndrome; Obesity	Dong et al. (2001) and Dhalla et al. (2007b)

ischemia (Von Lubitz et al. 1994). Interestingly, a reverse effect appears after chronic administration of A_1AR antagonists, such as DPCPX or caffeine, which, if administered for 2–3 weeks before an ischemic insult, lower the neuronal injury assessed by histopathological examination (Von Lubitz et al. 1995). It has been suggested that the beneficial effect seen after chronic administration of A_1AR antagonists may be due to an up-regulation of these receptor subtypes (Jacobson and Gao 2006). As a consequence, a sustained A_1AR activation could be crucial to define the threshold of tissue sensitivity to ischemic damage and very helpful in decreasing brain damage. Moreover, A_1ARs also prevent ischemia-induced cytotoxicity in non-neuronal tissues and control the neuronal viability involving general mechanisms common to the various cell types (Arrigoni et al. 2005; Peart and Headrick 2007). Adenosine via presynaptic A_1ARs may attenuate the influx of calcium through voltage-dependent calcium channels

and may decrease the glutamate release that represents the major mechanism underlying the neuroprotection (de Mendonça et al. 2000). On the other hand, adenosine interacting with postsynaptic A_1ARs counteracts excessive membrane depolarization by the activation of potassium channels and increases the efflux of potassium which leads to hyperpolarization of postsynaptic neurons (de Mendonça et al. 2000). Therefore, by stabilizing membrane potentials and maintaining intracellular calcium homeostasis in postsynaptic neurons adenosine may act as a neuroprotector controlling metabolic recovery after hypoxia (Duarte et al. 2009).

Several studies have reported dose-dependent effects of caffeine which are positively or negatively associated with ischemic stroke (Fredholm et al. 2016). In particular, one study showed that elevated caffeine intake is associated with a transiently increased risk of ischemic stroke (Mostofsky et al. 2010). Another study evaluated the long-term moderate consumption of caffeine which provides significantly protective effects in reducing the risk of stroke (Bøhn et al. 2012). Recently, it has been found that a hypothermic and hypometabolic state can be triggered by combining activation of A_1ARs with a reduced ambient temperature. As a consequence, the pharmacological manipulation of central autonomic thermoregulatory circuits could represent a potential target for the induction of a hypothermic state that is able to reduce the cellular metabolism, chemical reaction kinetics and oxygen demand of ischemic tissue (Tupone et al. 2016). A study performed in mice showed that Cl-ENBA, one of the most selective A_1AR agonist, caused dose-dependent hypothermia and hypoactivity through the activation of central A_1ARs (Carlin et al. 2017).

3.2 Epilepsy

The potential of adenosine as an antiepileptic mediator has emerged from the observation that A_1ARs are enriched in the synapses where they inhibit glutamate release, decrease glutamatergic responsiveness and hyperpolarize neurons that decrease the hyperexcitability associated with epilepsy (Cieślak et al. 2016). In addition, the levels of endogenous extracellular adenosine rise upon seizure activity, suggesting that adenosine could represent an endogenous anti-epileptic compound (Berman et al. 2000). Different studies have confirmed that the acute administration of A_1AR agonists attenuates seizure and/or convulsive activity in a wide variety of animal models of epilepsy (Świąder et al. 2014). Moreover, acute administration of non-selective A_1AR antagonists, such as caffeine or theophylline or selective A_1AR antagonists, enhance the duration and severity of seizures and/or convulsions (Boison 2008). Several studies have identified a decreased density and efficiency of synaptic A_1ARs in various models of epilepsy which act as an inhibitory system to avoid initiating seizure-like events (Rebola et al. 2005). The long-term consumption of caffeine has been found to prevent neuronal damage in different models of epilepsy, most likely due to a significant A_1AR upregulation which reveals a beneficial effect on seizure-induced neuronal damage (Duarte et al. 2009). A clear association between the ability of A_1ARs to control glutamatergic transmission and/or other features present in epilepsy has been reported (Gomes and Shinnar 2011). Another suggestion could be that the decreased function of the endogenous A_1AR-mediated inhibitory system is associated to the lack of adequate adenosinergic tonus as a result of the modified purinergic metabolism and of long term reduction in the extracellular levels of adenosine (Khan et al. 2001). A decrease in the density of presynaptic A_1ARs and their activation may still be an attractive and effective manner to restrain seizure activity in chronic epilepsy and in drug-resistant forms of epilepsy (Gouder et al. 2003). In epilepsy the presence of a robust increase has been observed in the expression and activity of adenosine kinase that mediates an ATP dependent phosphorylation of adenosine (Tomé et al. 2010). As a consequence, the adenosine kinase inhibitors could represent novel candidates for anti-epileptic drugs aimed at increasing the levels of adenosine (Li et al. 2007). Among the ATP hydrolysis product, it

has been proposed that adenosine monophosphate (AMP) act as a A_1AR full agonist (Rittiner et al. 2012). It has been also found that AMP directly activates A_1ARs in the rat and mouse brain by reducing the neuronal firing rate of spontaneously active thermoregulatory neurons in hypothalamus and in the excitatory postsynaptic potential in hippocampus (Muzzi et al. 2013). The alteration of AMP metabolism, including those occurring in patients affected by status epilectus or devastating neurological disorders due to purine nucleotides, may concur to decrease the epileptic threshold (Camici et al. 2010). Some of the actions of AMP are not related to A_1AR activation and its mechanism of action is not still elucidated (Carlin et al. 2017).

3.3 Neurodegenerative Disorders

Several data have been generated as showing the crucial role of adenosine as a modulator of neurotransmission and neuroprotective agent against excitotoxic neuronal injury (Burnstock 2013; Stone et al. 2009). The known biological activities of A_1ARs in brain functions, including its physical interaction with and inhibitory effect on dopamine receptor $D_{1,}$ provide supportive evidence that disruptions of A_1ARs may result in neurological dysfunction (Jaberi et al. 2016; Cunha 2016). The gene expressing A_1ARs may be the susceptibility gene within in Parkinson's disease with a quantitative effect on the interaction with dopamine receptors (Jaberi et al. 2016). The presence of an epidemiologic inverse correlation between the consumption of caffeine, a well-known adenosine antagonist, and the risk of developing Parkinson's disease has also been found (Ross et al. 2000; Palacios et al. 2012).

Huntington's disease (HD) is a neurodegenerative disorder caused by a mutation of the gene encoding for the huntingtin that in healthy subjects presents different functions associated with vesicular membranes, microtubules and synaptic function (Zuccato and Cattaneo 2014). In HD patients the mutated huntingtin alters synaptic transmission and induces glutamatergic dysfunctions with an increase in glutamate release (Zeron et al. 2002). The deregulation of glutamate transmission involves morphological and functional changes in synapses in the medium-sized spiny neurons co-expressing A_1ARs and dopamine D_1 receptors (Gomes et al. 2011). A_1AR stimulation exerts beneficial effects in a lesional transgenic mice model of HD, as suggested by its greater efficacy in reducing synaptic transmission in corticostriatal slices due to a stronger inhibition of glutamate release (Ferrante et al. 2014). These data suggest that, in HD mutation, the A_1ARs undergo profound changes in terms of reduced expression even if with an increased functional activity (Ferrante et al. 2014).

In spinal cord injury, ARs have been shown to play a major role in regulating inflammatory responses and neuronal death. Moreover, the blockade of A_1ARs by caffeine has been involved in mediating neuroprotective effects against spinal cord injury (Rivera-Oliver and Díaz-Ríos 2014). Daily caffeine intake has been shown to inhibit the process of increased tolerance to pain by modulation of A_1ARs, as demonstrated by using DPCPX which mimics the effects of caffeine (Sawynok and Reid 2012).

3.4 Mental Illness

Several studies have suggested that one of the most promising targets for the development of new anxiolytic drugs could be A_1ARs, the activation of which modulates neuronal activity by blocking neurotransmitter release and reducing the firing rate (Chen et al. 2013). It has also been shown that upregulating A_1ARs in forebrain neurons evokes both resilience to depressive-like behavior and antidepressant effects in a chronic depression model (Serchov et al. 2015). Different studies have highlighted that mice lacking adenosine A_1ARs display enhanced anxiety (Giménez-Llort et al. 2002; Lang et al. 2003). Moreover, the anxiogenic actions of adenosine antagonists, such as caffeine, have generally been attributed to the blockade of these

receptors, as suggested by the caffeine effects on mood changes (Millan 2003; Cunha 2008). High doses of caffeine seem to increase anxiety, whereas lower doses reduce anxiety levels in humans (Lopes et al. 2011). As a consequence, drugs that facilitate A_1AR-mediated actions could be effective in anxiety as observed by A_1AR agonists which have anxiolytic actions (Johansson et al. 2001). Patients suffering from panic disorder, a serious form of anxiety disorder, appear to be sensitive to small amounts of caffeine which promote an anxious behavior with the possibility of precipitating panic attacks (El Yacoubi et al. 2000). In addition, it has been reported that activation of A_1ARs in mice mediates the anxiolytic-like effect induced by ethanol (Prediger et al. 2004, 2006). Despite their promising therapeutic potential, the use of A_1AR agonists has been hampered by poor receptor subtype selectivity and various side effects such as negative chronotropic and inotropic effects in the heart, catalepsy and a depressant effect on locomotor activity (Chen et al. 2013; Romagnoli et al. 2010). As a consequence, a positive allosteric modulation has proven to represent a valuable alternative to orthosteric agonists by acting on a distinct site and potentiating the effect of the endogenous agonist (Childers et al. 2005; Gao et al. 2005). Different series of A_1AR positive allosteric modulators, some of which represent most potent and effective compounds, have been synthesized and pharmacologically characterized (Romagnoli et al. 2008, 2014b). In particular, the A_1AR positive allosteric modulator 2-amino-4-[(4-(phenyl) piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl)-(4-chlorophenyl) methanone (TRR469) has been shown to display an anxiolytic profile similar to diazepam in various anxiety tests in mice even if, in contrast to diazepam, it does not show interaction with ethanol to induce sedation and motor impairment. These data provide evidence to support the positive allosteric modulation of A_1ARs as a new and interesting pharmacological strategy for the treatment of anxiety-related disorders (Vincenzi et al. 2016).

3.5 Chronic and Neuropathic Pain

It has been found that A_1AR agonists reduce pain signaling in the spinal cord where the receptors are highly expressed, suggesting that A_1AR activation produces inhibitory effects on pain in a number of preclinical models (Sawynok 2016). Intravenous infusions of adenosine in humans reduce some aspects of neuropathic pain and can decrease postoperative pain mainly through the A_1AR (Gao and Jacobson 2007). Increased nociceptive response in mice lacking A_1ARs has been reported, suggesting that the A_1AR agonists may be tested clinically as analgesics in neuropathic pain (Johansson et al. 2001). Systemic administration of CCPA decreases the nociceptive response in plantar and formalin tests abrogated by the use of A_1AR antagonists involving different pathways in pain modulation (Maione et al. 2007). The local application of CCPA inhibits mechanical allodynia and thermal hyperalgesia, suggesting that the activation of A_1ARs could play a role in the regulation of nerve injury linked to the enhanced excitability of spinal dorsal horn neurons (Yamaguchi et al. 2014). It has also been reported that high doses of an agonist might result in a level of receptor activation that causes dangerous side effects (Kiesman et al. 2009; Romagnoli et al. 2010). On the other hand, mice lacking A_1ARs or the potential use of A_1AR antagonists have exhibited moderate hyperalgesia to heat stimulation (Wu et al. 2005). In addition, A_1AR agonists and allosteric enhancers could be of potential interest for the treatment of neuropathic pain. It has been shown that allosteric enhancers could increase the number of G proteins activated by the agonist, but have no effect on the affinity of activated G protein, providing the neuroanatomical basis for understanding potential therapeutic effects of such drugs (Childers et al. 2005). Moreover, A_1AR allosteric modulators acting in a site distinct from the agonist binding site could have potential therapeutic advantage over orthosteric ligands (Gao et al. 2005). The antinociceptive effect mediated by allosteric enhancers is blocked by using a selective A_1AR

antagonist, thus supporting that the enhanced activation of the A_1ARs in the brain reduces pain (Childers et al. 2005). Positive allosteric modulation of the A_1AR by novel enhancers could reduce hypersensitivity, suggesting the use of such modulators in the treatment of chronic pain associated with hyperalgesia and allodynia (Li et al. 2002). A wide range of allosteric enhancers on the structure of PD 81,723 has been synthesized by various research groups (Baraldi et al. 2000, 2003, 2004; Tranberg et al. 2002; Lütjens et al. 2003). Different series have been synthesized by introducing some substitutions on the phenyl ring exerting a fundamental influence on the allosteric activity (Romagnoli et al. 2008, 2012a, b, 2006,). In particular, the antinociceptive properties of TRR469 have been reported in two models of acute pain, such as writhing and formalin tests, and in chronic streptozotocin-induced diabetic neuropathy (Vincenzi et al. 2014). These interesting data suggest that A_1AR allosteric enhancers are promising candidates to treat acute and chronic pain, with the potential advantages of their unique mechanism of action. TRR469 dramatically increases adenosine affinity in mouse spinal cord membranes, suggesting the possibility of exploiting the anti-nociceptive effect of endogenous adenosine in a more physiological way than using an exogenous direct receptor agonist (Vincenzi et al. 2014).

3.6 Cardiovascular Diseases

In the heart, adenosine-induced protective and regenerative effects are mediated predominantly by A_1ARs that are expressed in cardiomyocytes, smooth muscle cells, in atria and ventricular tissues (Mustafa et al. 2009; Burnstock and Pelleg 2015). Myocardial A_1AR activation inhibits a variety of myocardial pathologies associated with ischemia and reperfusion injury, including stunning, arrhythmogenesis, coronary and ventricular dysfunction, acute myocardial infarction, apoptosis and chronic heart failure (Peart and Headrick 2000; Headrick et al. 2003). These results have suggested that A_1AR is implicated in different cardiovascular therapies for heart diseases like angina pectoris, control of cardiac rhythm, ischemic injury during an acute coronary syndrome or heart failure (Thompson et al. 2002; Albrecht-Küpper et al. 2012).

In particular, ARs and adenosine are important determinants of myocardial resistance to ischemia with the relevance of AR function in ischemic heart disease and cardiomyopathy (Morrison et al. 2006; Tang et al. 2007). Recently, it has been found that A_1ARs mediate the regulation of tissue transglutaminase activity in cytoprotection and in cardiomyocyte-like cell survival that have been investigated by monitoring hypoxia-induced cell death (Vyas et al. 2016). The ability of ARs to limit myocardial damage with ischemia-reperfusion may be negatively influenced by associated diseases including diabetes, obesity, hypertension and hyperthrophy (Peart and Headrick 2009). Some studies support a role for endogenous adenosine in electrophysiological changes and arrhythmogenesis during cardiac hypoxia and ischemia (Kaplan et al. 2003).

It is well reported that several acute functional consequences of myocardial ischemia are AR-dependent, as can be seen in post-ischemic repression of sympathetic neurotransmission which appears to involve endogenous adenosine and A_1AR activity (Burgdorf et al. 2001). In ischemic heart, an unexpected positive A_1AR-mediated inotropic response to adenosine in atria from coronary heart disease patients has been observed, whilst for some time adenosine via A_1ARs has been associated to a negative inotropic effect in human atrial preparations (Gergs et al. 2009). Pregnant mice treated during embryogenesis with caffeine, developed into adult mice which had abnormal heart function because adenosine acting via A_1ARs plays an essential role in protecting the embryo against intrauterine stress (Rivkees and Wendler 2012).

It is well reported that adenosine plays a role in vascular remodeling and is implicated in key processes of atherosclerosis where the A_1ARs stimulate smooth muscle proliferation and are involved in promoting stenosis with their

expression being increased in proximity to the vascular stents (Edwards et al. 2008). In pressure-overload hypertrophy, elevated interstitial adenosine levels are accompanied by an increased expression of A_1ARs mediating the major cardioprotective effects of this autacoid, which could be of relevance to hypertensive heart disease (Perlini et al. 2007). The hypertrophic response could be mediated by α-adrenoceptor stimulation which is accompanied by a compensatory A_1AR up-regulation able to antagonize Gαq-coupled receptors or block their signaling (Puhl et al. 2016). A_1AR expression may be relevant to cardiac disease progression since A_1AR overexpression can induce cardiomyopathy and cardiac dilatation. Thus, the inducible A_1AR transgenic mouse model provides novel insights into the role of adenosine signaling in heart failure and illustrates the potentially deleterious consequences of selective versus nonselective activation of adenosine-signaling pathways in the heart (Funakoshi et al. 2006).

It has been reported that full A_1AR agonists show an important role in the broad physiologic spectrum of cardiac actions from bradycardia to atrioventricular block, negative inotropy and dromotropy (Mustafa et al. 2009). In particular, A_1AR may mediate an atrio-ventricular block related to infarct and atrial fibrillation with infarction and ischemia (Yavuz et al. 2004). In a placebo-controlled study, enhanced levels of adenosine were shown to play a role in ischemia pain as suggested by patients with silent myocardial ischemia who had a decreased sensitivity to adenosine-provoked chest pain if compared with angina pectoris patients (Sadigh-Lindell et al. 2003). The protective and regenerative cardiovascular effects of A_1ARs might be counter-regulated by several side effects and by receptor desensitization which could represent a potential problem due to chronic use of full agonists (Roman et al. 2008).

Moreover, the potential use of partial A_1AR agonists, which are low efficacy ligands and elicit only a submaximal response, can be used to trigger some of the physiological responses of receptor activation depending on the organ/tissue involved. These ligands could induce less A_1AR desensitization than full agonists and be ideal for therapeutic chronic treatment with broader dose ranges (Albrecht-Küpper et al. 2012). Different reports indicate that ischemic preconditioning activates ARs to induce cardioprotective effects in various animal models and intracoronary infusion by combination of adenosine and dipyridamole significantly reduced infarct size (Montero et al. 2014). Interestingly, the postconditioning-dependent reduction in infarct size is modulated via A_1AR activation and targeted deletion of these receptors results in loss of cardioprotective effects, indicating their involvement in cardioprotection (Xi et al. 2008). The ischemic post-conditioning and adenosine significantly reduces myocardial infarct size and improves cardiac function (Randhawa and Jaggi 2016). Furthermore, adenosine post-conditioning significantly decreases some of the most important inflammatory cytokines in the myocardium indicating that adenosine post-conditioning dependent cardioprotection could involve the reduced formation of inflammatory cytokines (Ke et al. 2011).

3.7 Renal Disorders

It is well reported that A_1AR antagonists induce diuresis and natriuresis on various experimental models in animals and humans (Burnstock et al. 2014). Much of these effects could be due to inhibition of A_1ARs in the proximal tubule, which is primarily responsible for the reabsorption of filtered Na+ and fluid uptake (Welch 2015). Caffeine and theophylline, components of coffee and tea, respectively, are methylxanthines and act as non-selective AR antagonists mediating diuresis and natriuresis (Rieg et al. 2005). However, caffeine and theophylline were used traditionally to increase urine output until more potent diuretics became available in the middle of the last century. The dose of caffeine that elicits a significant acute diuresis has been reported to be in the order of 300 mg, the equivalent of about 4–5 cups of coffee (Riesenhuber et al. 2006). These effects have

recently been attributed to their action on A_1ARs present with high density in the proximal tubule and in the kidney nephron. Caffeine in high concentration supplied in a Ca^{2+}-free medium has been shown to prevent the Ca^{2+} increase caused by vasopressin in rat renal papillary collecting duct cells, and this may blunt the increase of cAMP and the effect of vasopressin on water permeability (Osswald and Schnermann 2011). Caffeine appears to exert this effect by depletion of endoplasmatic Ca^{2+} stores, suggesting that its action may be caused by interaction with ryanodine sensitive Ca^{2+} release channels. In some studies methylxanthines have been found to increase the tubular Na^+ load. Since significant natriuresis can occur without changes in glomerular filtration rate or renal blood flow, the natriuresis caused by methylxanthines is predominantly the result of inhibition of tubular salt transport (Shirley et al. 2002). There is considerable evidence to indicate that methylxanthine-induced natriuresis is mainly a consequence of inhibition of salt transport along the proximal convoluted tubule. As a consequence, potential novel drugs targeting A_1ARs induce diuresis and natriuresis in several experimental animal models. Proximal reabsorption is reduced in A_1AR knock-out mice and salt-sensitive hypertension is less effective in mice deficient of A_1ARs because the absence of these receptors in distal nephron segments might have prevented the excess Na + and fluid retention (Lee et al. 2012). Experimentally, an acute saline volume load has been observed to excrete more rapidly in A_1AR knock-out mice, suggesting that A_1ARs participate in reabsorption of excess fluid and electrolytes in the proximal tubules under normal conditions (Bell et al. 2010). It is well reported that cAMP production plays a role in the secretion of fluid that is thought to be partly responsible for the accumulation of fluid in renal cystic disease (Belibi et al. 2002). Thus, by augmenting cAMP levels the methylxanthines could contribute to the progression of cyst formation. In primary cultures of renal cysts from patients with autosomal dominant polycystic kidney disease, caffeine increases levels of cellular cAMP and this is associated with an increase in trans-epithelial chloride secretion (Tanner and Tanner 2001). In several experimental disease models, chronic caffeine administration has been found to exacerbate the development of hypertension and renal disease, perhaps through the effect of caffeine on renin secretion. In association with the increase in renin secretion, prolonged caffeine ingestion causes a faster decline in renal function and a significant enhancement of urinary protein excretion (Tofovic et al. 2002, 2007). Moreover, theophylline partially prevents the reduction in medullary blood flow induced by iodixanol by antagonizing vasoconstriction mediated A_1ARs (Lancelot et al. 2002). This hypothesis is supported by studies in which the A_1ARs were blocked by selective antagonists that were more effective than theophylline in attenuating iohexol-induced renal functional impairment in animal models with pre-existing renal insufficiency (Osswald and Schnermann 2011). The potential of methylxanthines to improve renal function following postischemic acute renal failure has arisen from the fact that ischemia is associated with the increase in adenosine levels in the kidney and in other organs (Vallon and Osswald 2009). Adenosine can produce vasoconstriction, a response that has been suggested to be an organ-specific version of metabolic control designed to restrict organ perfusion when transport work increases. However, the vasoconstriction elicited by an intravenous infusion of adenosine is only short lasting, being replaced within 1–2 min by vasodilatation. A_1AR in afferent arterioles are selectively activated from the interstitial aspect of the vessel. This property can dissociate A_1AR activation from changes in vascular adenosine concentration, a characteristic that is ideally suited for the role of renal adenosine as a paracrine factor in the control of glomerular function (Hansen and Schnermann 2003).

3.8 Pulmonary Pathologies

A_1AR activation reduces alveolar fluid clearance in mice and may lead to alveolar fluid

accumulation and reduced lipopolysaccharide-induced pulmonary edema formation (Heller et al. 2007). On the other hand, A_1AR antagonists cause dose-dependent pulmonary infiltration of neutrophils, lymphocytes and alveolar edema (Burnstock et al. 2012). Pretreatment with A_1AR antagonist blocks the increase in pulmonary capillary filtration in an animal model of acute lung injury (Schepp and Reutershan 2008). Application of the A_1AR antagonist before and after the maximum dose of endotoxin completely abolishes the alveolar injury (Schepp and Reutershan 2008). The predominant pro-inflammatory role of A_1ARs, as shown in different models of acute lung injury, is counter to its protective function in chronically inflamed airways. The deletion of A_1ARs in mice has resulted in markedly enhanced pulmonary inflammation, suggesting an anti-inflammatory role of A_1ARs in chronically inflamed airways (Sun et al. 2005). Interestingly, lipopolysaccharide has been shown to be a ligand for A_1ARs on human pulmonary endothelial cells and is able to displace a selective A_1AR antagonist from the receptor in a dose-dependent and competitive manner. The binding of lipopolysaccharide to A_1ARs leads to increased secretion of interleukin-6 and thromboxane A2, which can be significantly reduced by administration of a selective A_1AR antagonist (Wilson and Batra 2002).

Convincing evidence suggests that A_1ARs could well play a significant role in the pathophysiology of asthma and that airway obstruction in response to aerosol administration of adenosine and allergen is inhibited in a rabbit model of allergic airway inflammation following treatment with antisense oligonucleotides as well as A_1AR antagonist (Nyce and Metzger 1997). These data suggest that the A_1ARs not only directly mediate bronchoconstriction following administration of exogenous adenosine, but that endogenous adenosine is an important component of the allergic response. It has been demonstrated that adenosine-induced contraction of isolated bronchial tissue *in vitro* is greater in tissues obtained from asthmatic subjects than healthy subjects, and that this contraction could be significantly inhibited following preincubation with a selective A_1AR antagonist (Björck et al. 1992). Compared with healthy subjects, A_1AR expression is increased in bronchial biopsies obtained from mild asthmatic subjects in the airway epithelium and smooth muscle regions of the tissue, as confirmed with the preclinical findings in the rabbit model of allergic asthma (Brown et al. 2008). It has been demonstrated that A_1AR activation on human airway smooth muscle cells *in vitro* results in an increase in intracellular calcium mobilization, which could potentially mediate the airway smooth muscle contraction (Ethier and Madison 2006). The finding of A_1AR up-regulation and increased sensitivity of the airways to adenosine could well be of clinical significance. In asthmatics, the level of adenosine in plasma and exhaled breath condensate is increased following allergen or exercise challenge and could, therefore, lead to A_1AR activation contributing to airway obstruction during an acute asthma exacerbation (Vizi et al. 2002). Other studies using experimental animals have implicated a role for A_1AR activation in mediating airway obstruction in sensitized guinea pigs and allergic rabbits (Keir et al. 2006). A_1AR expression has also been identified on a number of inflammatory cells where it appears to be proinflammatory and on human eosinophils where it promotes anion superoxide release (Ezeamuzie and Philips 1999). It is well reported that adenosine induces monocyte phagocytosis and chemotaxis of immature dendritic cells and increases the release of cytotoxic substances from endothelial cells and the endothelial cell permeability (Wilson and Batra 2002; Panther et al. 2001). A_1ARs mediate the respiratory burst in neutrophils, in addition to chemotaxis and their adherence to endothelial cells (Cronstein et al. 1992). Many studies have demonstrated the pro-inflammatory action attributed to A_1AR activation even if a preclinical study has documented an anti-inflammatory effect of A_1AR signaling (Sun et al. 2005). The mechanism by which adenosine mediates airway obstruction may

constitute indirect components by activating pulmonary C fibers and cholinergic neural pathways in various animal models (Lee et al. 2004; Hua et al. 2007). In addition to its effects on bronchoconstriction, leukocyte activation and inflammation, A_1ARs may play an important role in mucus secretion and airway remodeling of human asthma. It has been shown that adenosine is able to induce mucus secretion and increase the expression of the mucin gene (McNamara et al. 2004). Thus, it could be speculated that the reported A_1AR up-regulation on asthmatic bronchial epithelium promotes adenosine-induced mucin secretion (Brown et al. 2008).

3.9 Eye and Vision Problems

Adenosine is an established neuromodulator in the mammalian retina with A_1ARs being especially prevalent in the nerve fiber layer, ganglion cell layer, photoreceptor cell layer and in retinal pigment epithelium (Zhang et al. 2006; Wan et al. 2011). It is well known that several diseases are related to retinal ganglion cell death, such as glaucoma, diabetes and other retinopathies (Jacobson and Civan 2016). Many studies have attempted to identify factors that could increase neuroprotection of these cells. Interleukin-6 is able to increase the survival and regeneration of retinal ganglion cells in *in vitro* as well as *in vivo,* and its concentration is linked to AR modulation (Perígolo-Vicente et al. 2014). The potential of AR-based therapy for retinopathy is supported by the ability of ARs to modulate inflammation, neuroprotection and angiogenesis in retina where A_1AR expression and density are well detected (Zhang et al. 2015). In A_1AR knockout mice in oxygen-induced retinopathy model, the A_1AR activation does not affect normal retinal vascular development, but distinctly controls hyperoxia-induced vaso-obliteration and hypoxia-induced revascularization (Brito et al. 2012). The modification of the responses of photosensitive retinal ganglion cells through a cAMP/PKA pathway is a general feature of rat ganglion cell photoreceptors, and this pathway can be inhibited by A_1AR activation (Sodhi and Hartwick 2014). The adenosine levels in the retina rise at night, and adenosinergic modulation of these photoreceptors may serve as an internal regulatory mechanism to limit the transmission of nocturnal photic signals to the brain which are significantly reduced through the suppression of cAMP-related pathway mediated by A_1ARs (Sun et al. 2002). Stimulation of A_1ARs on non-photosensitive rat ganglion cells results in the inhibition of voltage-gated calcium channels and glutamate-induced calcium influx (Hartwick et al. 2004). It is well reported that adenosine and several AR agonists modulate the intraocular pressure that is maintained in equilibrium when the rate of humor aqueous production is equal to the rate of aqueous outflow, suggesting that the adenosine system could be one potential target system for therapeutic approaches in glaucoma (Zhong et al. 2013). Selective A_1AR activation has been shown to be able to reduce intraocular pressure by an early reduction in aqueous flow followed by a subsequent increase in outflow in different animal models (Crosson 2001). The reduction in aqueous flow may be mediated by post-junctional A_1ARs in the ciliary body which mediate the suppression of cAMP accumulation (Avila et al. 2001). A_1AR agonists increase the outflow of the humor aqueous flow through sequential activation of Gi/o, phospholipase C, protein kinase C and mitogen-activated protein kinase leading to secretion of metalloproteinases-2 (Husain et al. 2007). Adenosine, by the interaction with A_1ARs of the trabecular meshwork, is able to reduce outflow resistance and lower intraocular pressure (Li et al. 2012). It has also been observed that, in the retina ischemic damage, the protective role of A_1AR activation appears after brief periods even if the protection is not effective in prolonged ischemia. The data are consistent with the use of A_1AR antagonists that can diminish recovery after retinal ischemia (Zhong et al. 2013). It is well known that ARs could also play a key role in the function of the lacrimal gland where different signaling pathways modulate the protein secretion at the basis of normal and pathological responses (Hodges and Dartt 2016).

3.10 Pancreas Diseases

Under physiological conditions, insulin secretion from pancreatic β-cells is tightly regulated by various factors, including nutrients, the nervous system, and other hormones. Pancreatic β-cells are also influenced by paracrine and autocrine interactions (Chandra and Liddle 2014). The results of various studies indicate that adenosine is present within pancreatic islets and is implicated in the regulation of insulin secretion and in glucose concentrations. In rat islets, low adenosine has been shown to decrease glucose-induced insulin secretion via A_1ARs whilst high adenosine concentrations exert an opposite effect (Andersson 2014). It is also well established that suppression of adenosine action increases insulin-secretory response of β-cells to glucose, whilst low adenosine concentrations do not affect insulin secretion (Szkudelski and Szkudelska 2015). Type 1 diabetes is reported to be a complex polygenic disease triggered by various environmental factors in genetically susceptible individuals. A_1ARs are differentially expressed and alternatively spliced in the pancreatic lymph nodes or islets of type 1 diabetes patients to form dominant-negative non-functional isoforms (Yip and Fathman 2014). In particular, diminished A_1AR expression in pancreatic α-cells may contribute to the pathology of type 1 diabetes (Yip et al. 2013). The selective A_1AR agonists are less efficacious in ameliorating the course of diabetes compared to the nonselective AR agonists which prevent diabetes development in two different animal models (Németh et al. 2007). The removal of adenosine or the use of A_1AR antagonists, such as caffeine, decrease insulin-stimulated glucose transport and improve glucose tolerance suggesting that long term coffee consumption is associated with a decreased risk for type 2 diabetes (Salazar-Martinez et al. 2004; van Dam and Hu 2005). Briefly, A_1ARs have a minimal effect on muscle glucose uptake, but are important in regulating pancreatic islet function (Johansson et al. 2007a). Glucose injection induces sustained increases in plasma insulin and glucagon levels in mice without A_1ARs, whereas control mice react with a transient increase in insulin and decrease in glucagon levels (Yang et al. 2012). Pancreas perfusion experiments demonstrate that A_1AR mice have a higher basal insulin secretion than control mice and that insulin-mediated glucose uptake in skeletal muscle shows no significant difference between the two groups of mice (Yang et al. 2012).

3.11 Gastrointestinal Function Modulation

Intestinal functions result from an integrated regulatory interplay between the enteric nervous system, smooth muscle and the mucosal/immune system aimed at maintaining a homeostatic status and ensuring adaptative responses in the presence of pathological conditions (Wood 2004). The complex network is regulated by various mediators among which adenosine is one of the most important modulating agents (Antonioli et al. 2008). There is evidence for a prominent role of adenosine in the interactions between enteric neurons and smooth muscle with fine tuning operated by this molecule on motility peristaltic reflex and transit in the small and large intestine of various animal models (Burnstock 2014). In particular, A_1AR activation has been shown to mediate the inhibitory action of adenosine on excitatory cholinergic transmission of motor neurons innervating circular and longitudinal smooth muscle (Lee et al. 2001). This inhibitory control is ascribed to the ability of A_1ARs to reduce the availability of Ca^{2+} levels via inhibition of N type calcium channels (Lee and Parsons 2000).

In large intestine an inhibitory effect mediated by A_1ARs has been observed in colonic preparations from various animal models (Zizzo et al. 2006). These results are supported by *in vivo* experiments demonstrating a significant increase in the colonic propulsion of animals treated with A_1AR antagonists (Kadowaki et al. 2000). The intestinal secretory reflexes may involve A_1ARs because in A_1AR knock-out

mice a significant reduction in adenosine-induced Cl- secretion has been observed, confirming an important role of this receptor subtype in the regulation of gut functions (Christofi et al. 2001). It is well known that the concentration of extracellular adenosine is a critical determinant for the activation of ARs on enterochromaffin cells and that the release of serotonin is under the control of ARs (Christofi et al. 2001). In particular, the basal release of serotonin is under tonic control of A_1ARs predominantly by using a cAMP-dependent inhibitory pathway (Christofi et al. 2004). A large body of experimental evidence suggests that the modulation of intrinsic primary afferent neurons is mainly ascribable to the activation of pre-junctional A_1ARs which mediate a membrane hyperpolarization with a decrease in the sensitivity of these neurons to excitatory stimuli (Christofi et al. 2001).

An inhibitory effect of adenosine has also been observed in colonic submucosal nerves of various animal models which is probably due to a significant expression of A_1ARs, suggesting a relevant role of endogenous adenosine as a physiological brake in the modulation of reflex-evoked chloride secretion (Cooke et al. 1999). A_1ARs are also expressed in mesenteric nerves innervating in rat jejunum where they facilitate the excitation of peripheral sensory terminals (Kirkup et al. 2001). Increasing evidence highlights the existence of close interactions between the enteric nervous system and enteric immune cells deputed to establish a first line of defence against foreign invasion. In particular, the stimulation of A_1ARs induces up-regulation of the neutrophil adhesion receptor and increased expression of complement receptors responsible for enhanced adhesion of neutrophils to vascular endothelium (Bours et al. 2006). In addition, the activation of A_1ARs enhances the phagocytosis process and promotes the production of reactive oxygen species (Antonioli et al. 2008). Immature human dendritic cells express A_1ARs which mediate chemotaxis via an increase in intracellular calcium, thus allowing the recruitment of dendritic cells into inflammation sites (Schnurr et al. 2004). Under inflammatory conditions, changes in AR expression appear to contribute to the modulation of gut inflammatory responses like, for example, the significant up-regulation of A_1ARs in the presence of intestinal inflammation (Sundarama et al. 2003).

3.12 Metabolism and Adipose Tissue Regulation

Adenosine is an important regulator of metabolism and its interaction with A_1ARs modulates metabolism, obesity and islet endocrine and vascular functions during ageing through the involvement of oxidative stress and inflammatory responses (Yang et al. 2015). Since adenosine can modify myocardial substrate, handling and circulating levels of glucose, insulin and fatty acids, ARs have the ability to modulate the detrimental cardiovascular effects of metabolic disorders such as obesity, diabetes, metabolic syndrome and may also participate in disease pathogenesis (Grden et al. 2006). Moreover, the abrogation of A_1AR signaling improves the metabolic profile during ageing by using multifactorial mechanisms at the base of lipolysis and lipogenesis (Fredholm et al. 2011c). A direct effect of A_1ARs has been reported on peripheral insulin signaling, presumably via attenuation of nicotinamide adenine dinucleotide phosphate oxidase function, as well as on the modulation of inflammatory pathways in visceral adipose tissues and on the islet microvasculature (Dhalla et al. 2009). Today, adenosine is considered to be a major endogenous anti-lipolytic factor and it is surprising that A_1AR antagonists do not cause any compensatory changes in other agents, like insulin, involved in lipolysis (Johansson et al. 2007b). On the other hand, it has been found that long-term activation of A_1ARs leads to a desensitization of both adenosine and insulin effects, and some aspects of insulin signaling are enhanced by A_1AR activation (Cheng et al. 2000). As a consequence, the possibility that adenosine and insulin interact with each other in adipose tissue has potential clinical relevance in type 2 diabetes secondary to obesity (Cheng et al. 2000). *In vitro* and *in vivo*

assays have demonstrated the presence of an A_1AR upregulation in adipose tissue of transgenic mice, and that the activation of these receptors improves insulin sensitivity (Dong et al. 2001; Schoelch et al. 2004). It is well known that high levels of free fatty acids are associated with obesity and can cause insulin resistance in skeletal muscle (Boden 2003). Drugs that lower free fatty acids may be used for treating obesity, type 2 diabetes or dyslipidemia, even if these drugs have sometimes shown rebound effects causing increased levels of free fatty acids. This rebound phenomenon does not occur when A_1AR partial agonists are used for decreasing the free fatty acid levels in rats (Dhalla et al. 2007b). From the pharmacological point of view, the A_1AR reserve in the adipose tissue makes it possible for a partial agonist to elicit a maximum antilypolitic response with only minor effects on organs where spare receptors are not present (Johansson et al. 2008).

4 Therapeutic Applications of A_1AR Compounds in Clinical Trials

Over the past few years, a number of new agents from the purinergic field has reached clinical applications after having passed the preclinical phase where novel mechanisms of action have been adequately characterized. Adenosine represents the drug of choice, used under the name of Adenocard, for controlling paroxysmal supraventricular tachycardia by activating A_1ARs. Adenosine's mechanism of action can be thought of as a temporary paralyzing of supraventricular tissue. Pharmacologically, adenosine hyperpolarizes the cells by stimulating an inward potassium current and temporarily inhibiting calcium migration. The pacemaker activity of the sinoatrial node, spontaneous atrial activity and conduction through the atrioventricular node are slowed or temporarily stopped. Adenosine does not show selective actions and has moderate side effects due to its extremely short-life within seconds (Chen et al. 2013). As a consequence, the development of novel selective AR agonists with a long-life but reduced side effects is highly demanded. In the A_1AR agonist area, clinical candidates have been discovered for the following conditions: atrial arrhythmias (e.g. tecadenoson, selodenoson and PJ-875); type II diabetes and insulin sensitizing agents (e.g. GR79236, ARA, RPR-749, and CVT-3619); and angina (e.g. BAY 68–4986). Tecadenoson (CVT-510), a full agonist for A_1ARs, has been investigated in Phase III trial for patients with paroxysmal supraventricular tachycardia, atrial fibrillation and atrial flutter. It appears that low doses of tecadenoson have minimal effects on atrium-ventricular, nodal conduction and blood pressure compared with adenosine (Cheung and Lerman 2003). However, at high doses, like other full agonists for the A_1ARs, it may also display atrium-ventricular block. A Phase II clinical trial of another full A_1AR agonist, selodenoson, for atrial fibrillation has also been completed. Both agonists he shown promising therapeutic efficacy and further development will probably depend on potential side effects (Aurora et al. 2005). A Phase II trial of BAY 68–4986 (capadenoson), a non-nucleoside A_1AR agonist, in patients with persistent or permanent atrial fibrillation, has been completed and represents a novel chemical structure among AR agonists for clinical development (Tendera et al. 2012). A full A_1AR agonist, GR79236, has been studied in a clinical trial showing several cardiovascular side effects. The challenges associated with the development of any A_1AR agonists are to obtain tissue-specific effects while avoiding off-target tissue side effects and A_1AR desensitization leading to tachyphylaxis. For the antiarrhythmic agents that act as ventricular rate control agents, a selective response can be accomplished by careful dosing paradigms. It is well known that type 2 diabetes patients have increased levels of non-esterified fatty acids, in part due to β-adrenergic agonism, which decrease insulin sensitivity. Thus, lowering the fatty acid levels could lead to an insulin-sensitizing effect. Treatment of type II diabetes using A_1AR agonists in human clinical trials was blocked by cardiovascular side effects and by a

desensitization due to the full agonists such as GR79236, ARA, and RPR 749 (Kiesman et al. 2009). In the literature, several papers have reported that the A_1ARs are abundantly expressed in spinal cord and other neuronal tissues, and their activation has produced pain-relieving effects in a number of preclinical animal models. As a consequence, adenosine has been studied in Phase II trials for patients with neuropathic pain, and selective A_1AR agonists have been developed as analgesics. Unfortunately, some A_1AR agonists, such as SDZ WAG 994, GR79236 and GW 493838, have been withdrawn in phase II clinical trials, possibly due to their inability to penetrate CNS sufficiently to cause a substantial effect (Gao and Jacobson 2011).

Moreover, new partial A_1AR agonists including CVT-3619 (GS 9667) with a good selectivity versus A_1ARs, have the potential to provide enhanced insulin sensitivity without cardiovascular side effects and tachyphylaxis (Shearer et al. 2009). CVT-3619 was investigated in phase I, single-blind, placebo-controlled evaluating the safety, tolerability and pharmacokinetics of the oral administration, in healthy and obese volunteers. No clinically meaningful changes in heart rate or blood pressure were observed in the study, suggesting that CVT-3619 may increase insulin sensitivity and subsequently decrease blood glucose without causing severe cardiovascular effects (Staehr et al. 2013). The non nucleosidic A_1AR agonist BAY 68–4986 (capadenoson) represents a novel approach to angina wherein both animal studies and early human studies are promising. These studies showed that long-term therapy with capadenoson improves phosphorylation and total protein level of PGC-1α in the myocardium of animal models of heart failure (Sabbah et al. 2013). A clinical trial found that in patients with stable angina capadenoson lowers exercise at comparable maximum workload, which is associated with improved total exercise time and prolongation of ischaemia time (Tendera et al. 2012). In the literature it has been reported that the challenges associated with developing allosteric enhancers for therapeutic intervention are also well defined in humans; for example, T-62, that is an A_1AR allosteric enhancer, was evaluated in phase I clinical trials as a potential treatment for neuropathic pain (Gao and Jacobson 2011). Moreover, T62 has produced beneficial effects in several *in vivo* pain models, such as carrageenan-inflamed rats, and reduces hypersensitivity following peripheral inflammation by using a central mechanism (Li et al. 2003).

Significant progress has been made in identifying several A_1AR antagonists that have been investigated in clinical trials for a number of different medical indications, and they appear to be safe and well tolerated in humans (Dittrich et al. 2007; Doggrell 2005; Givertz et al. 2007; Greenberg et al. 2007). The A_1AR antagonists which have been in clinical development are KW3902 (Kyowa Hakko), BG9928, (CV therapeutics & Biogen Idec) and SLV320 (Solvay). These compounds have high affinity for human A_1ARs showing more than 200-fold selectivity over the A_1AR, and demonstrate renal protective effects in various animal models of disease and pharmacologic effects in human subjects. A_1AR antagonists have reduced the risk of persistent worsening renal failure in a clinical study in patients with chronic heart failure. A placebo-controlled randomized trial involving patients with acute heart failure was carried out with A_1AR antagonist rolofylline, although the results did not prevent worsening renal functions. Pharmacological studies have clearly demonstrated that A_1ARs mediate protective effects against ischaemic kidney injury, which is consistent with the increased frequency of stroke and seizure activity in clinical trials of A_1AR antagonists (Müller and Jacobson 2011). NovaCardia has developed both intravenous and oral formulations of KW 3902 in phase III studies with the aim to evaluate the effect of this compound in the presence of furosemide on heart failure signs and symptoms, renal functions and safety in subjects with acute heart failure syndrome. These studies suggested that the combination of these drugs was effective in maintaining renal function in the patients examined. KW 3902 has been patented for heart

failure and hypertension, renal disorders and renal failure (Wider et al. 2004). CV therapeutics and Biogen Idec have studied BG 9719 in phase II for the treatment of congestive heart failure. By blocking the A_1ARs, BG 9719 may reduce the amount of fluid waste that the kidneys retain without the associated decline in kidney function. It has also been found that BG 9719 increases both urine output and glomerular filtration rate. In particular, if this compound is given in addition with furosemide, urine volume increases and the deterioration in glomerular filtration rate is not present. Interestingly, A_1AR antagonism might preserve renal function and promote natriuresis during heart failure. As a consequence, BG 9719 has been patented for renal and cardiovascular disorders (Otsuki et al. 2005). An A_1AR antagonist, SLV 320, has been studied in phase II clinical trials at Solvay for the treatment of chronic heart failure and renal failure. A randomized placebo controlled and multicenter study has also been carried out to evaluate the cardiac hemodynamics and safety of this compound in patients with chronic heart failure (Pang et al. 2011).

Another A_1AR antagonist, N-0861, developed at Aderis together with other structurally similar compounds, have been patented for heart failure therapy, prevention and treatment of ischemia-reperfusion and endotoxin-related injury (Neely 1995). FK-453 belongs to a series of pyrazolopyridines, synthesized by Astellas Pharma, that were shown to have antihypertensive and diuretic effects. The renal effects and diuretic and natriuretic properties of the A_1AR antagonists are similar to the thiazide diuretics, and the most common side effects are headache and skin rash (Vallon et al. 2008). Thus, FK-453 has been studied in phase II trials for acute renal failure (Li et al. 2016). FK-838 is a diuretic and antihypertensive A_1AR antagonist developed at Astellas Pharma and under phase II studies for hypertension and as a diuretic agent. Its diuretic and natriuretic effects appear to be due to both its renal hemodynamic effects and direct inhibition of proximal tubular reabsorption (Kieć-Kononowicz et al. 2001). In combination with furosemide, FK-838 enhances the diuretic and natriuretic actions of furosemide to the same extent as hydrochlorothiazide and does not increase potassium loss. In addition, FK-838 has also been studied in phase II trials for the treatment of anemia (Kohno et al. 1993). It is well known that the activation of A_1ARs on different cell types produces bronchoconstriction, inflammation, mucous gland hyperplasia, angiogenesis, and fibrosis. An A_1AR antagonist, L 97-1 (Endacea, Inc.), has been investigated in the pathophysiology of human asthma, as a once-daily and oral treatment. L 97-1 is a water-soluble, small-molecule A_1AR antagonist with high affinity and selectivity for the human A_1ARs (Obiefuna et al. 2005). In an animal model of allergic asthma, L 97-1 blocks allergic airway responses, bronchial hyperresponsiveness to histamine, and airway inflammation (Obiefuna et al. 2005; Nadeem et al. 2006). As a consequence, L 97-1 has been patented for asthma and respiratory disorders (Wilson and Partridge 2007). In addition, administration of therapeutically effective amounts of A_1AR antagonist in combination with an antibiotic agent has been hypothesized, suggesting their potential use for treating and preventing sepsis (Wilson 2006). Moreover, several clinical studies have been performed regarding the effect of L 97-1 on coronary artery disease, heart failure and hypertension, dementia, Alzheimer's disease and depression (Partridge and Wilson 2005).

In conclusion, the investigation of A_1ARs and their ligands is rapidly growing with an increasing impact in the drug discovery process. There is now extensive evidence for the involvement of A_1ARs in the physiological regulation of several diseases (Table 4). The chemical development of several classes of agonists, antagonists and allosteric modulators versus A_1ARs has led to the identification of clinical candidates with an important impact on the drug discovery process. The results reported clearly suggest that A_1ARs could represent a possible target for pharmacological intervention to prevent different pathologies in the CNS, cardiovascular system, renal and lung disorders, regulation of gastrointestinal functions and metabolism. The important preclinical and clinical advances reported in the

present paper suggest that A_1ARs could be implicated in the pharmacological treatment of several diseases with the opportunity to improve the human health.

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Acton D, Miles GB (2015) Stimulation of glia reveals modulation of mammalian spinal motor networks by adenosine. PLoS One 10:e0134488

Akahane A, Katayama H, Mitsunaga T, Kato T, Kinoshita T, Kita Y, Kusunoki T, Terai T, Yoshida K, Shiokawa Y (1999) Discovery of 6-oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1 (6H)- pyridazinebutanoic acid (FK 838): a novel non-xanthine adenosine A_1 receptor antagonist with potent diuretic activity. J Med Chem 42:779–783

Albrecht-Küpper BE, Leineweber K, Nell PG (2012) Partial adenosine A_1 receptor agonists for cardiovascular therapies. Purinergic Signal 8:91–99

Andersson O (2014) Role of adenosine signalling and metabolism in β-cell regeneration. Exp Cell Res 321:3–10

Antonioli L, Fornai M, Colucci R, Ghisu N, Tuccori M, Del Tacca M, Blandizzi C (2008) Regulation of enteric functions by adenosine: pathophysiological and pharmacological implications. Pharmacol Ther 120:233–253

Arrigoni E, Crocker AJ, Saper CB, Greene RW, Scammell TE (2005) Deletion of presynaptic adenosine A1 receptors impairs the recovery of synaptic transmission after hypoxia. Neuroscience 132:575–558

Ashton TD, Aumann KM, Baker SP, Schiesser CH, Scammells PJ (2007) Structure-activity relationships of adenosines with heterocyclic N6-substituents. Bioorg Med Chem Lett 1724:6779–6784

Aurora J, Manmohan S, Kenneth KH (2005) Pharmaceutical formulation for controlled release of selodenoson. WO patent 200525545, 24 mar 2005

Avila MY, Stone RA, Civan MM (2001) A_1, A_{2A} and A_3 subtype adenosine receptors modulate intraocular pressure in the mouse. Br J Pharmacol 13:241–245

Baraldi PG, Zaid AN, Lampronti I, Fruttarolo F, Pavani MG, Tabrizi MA, Shryock JC, Leung E, Romagnoli R (2000) Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A1 adenosine receptor. Bioorg Med Chem Lett 10:1953–1957

Baraldi PG, Romagnoli R, Pavani MG, Nuñez Mdel C, Tabrizi MA, Shryock JC, Leung E, Moorman AR, Uluoglu C, Iannotta V, Merighi S, Borea PA (2003) Synthesis and biological effects of novel 2-amino-3-naphthoylthiophenes as allosteric enhancers of the A1 adenosine receptor. J Med Chem 46:794–809

Baraldi PG, Pavani MG, Shryock JC, Moorman AR, Iannotta V, Borea PA, Romagnoli R (2004) Synthesis of 2-amino-3-heteroaroylthiophenes and evaluation of their activity as potential allosteric enhancers at the human A1 receptor. Eur J Med Chem 39:855–865

Baraldi PG, Tabrizi MA, Gessi S, Borea PA (2008) Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. Chem Rev 108:238–263

Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, Edlich F, Katschinski DM, Stiehl DP, Wenger RH, Camenisch G (2007) The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol 27:3758–3768

Basheer R, Arrigoni E, Thatte HS, Greene RW, Ambudkar IS, McCarley RW (2002) Adenosine induces inositol 1,4,5-trisphosphate receptor-mediated mobilization of intracellular calcium stores in basal forebrain cholinergic neurons. J Neurosci 22:7680–7686

Belibi FA, Wallace DP, Yamaguchi T, Christensen M, Reif G, Grantham JJ (2002) The effect of caffeine on renal epithelial cells from patients with autosomal dominant polycystic kidney disease. J Am Soc Nephrol 13:2723–2729

Bell TD, Luo Z, Welch WJ (2010) Glomerular tubular balance is suppressed in adenosine type 1 receptor-deficient mice. Am J Physiol Renal Physiol 299: F1158–F1163

Berman RF, Fredholm BB, Aden U, O'Connor WT (2000) Evidence for increased dorsal hippocampal adenosine release and metabolism during pharmacologically induced seizures in rats. Brain Res 872:44–53

Biber K, Klotz KN, Berger M, Gebicke-Härter PJ, van Calker D (1997) Adenosine A_1 receptor-mediated activation of phospholipase C in cultured astrocytes depends on the level of receptor expression. J Neurosci 17:4956–4964

Björck T, Gustafsson LE, Dahlén SE (1992) Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. Am Rev Respir Dis 145:1087–1091

Boden G (2003) Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. Exp Clin Endocrinol Diabetes 111:121–124

Böhm M, Pieske B, Ungerer M, Erdmann E (1989) Characterization of A_1 adenosine receptors in atrial and ventricular myocardium from diseased human hearts. Circ Res 65:1201–1211

Bøhn SK, Ward NC, Hodgson JM, Croft KD (2012) Effects of tea and coffee on cardiovascular disease risk. Food Funct 3:575–591

Boison D (2008) The adenosine kinase hypothesis of epileptogenesis. Prog Neurobiol 84:249–262

Borea PA, Varani K, Vincenzi F, Baraldi PG, Tabrizi MA, Merighi S, Gessi S (2015) The A_3 adenosine receptor: history and perspectives. Pharmacol Rev 67:74–102

Borea PA, Gessi S, Merighi S, Varani K (2016) Adenosine as a multi-signalling guardian angel in human diseases: when, where and how does it exert its protective effects? Trends Pharmacol Sci 37:419–434

Boros D, Thompson J, Larson DF (2016) Adenosine regulation of the immune response initiated by ischemia reperfusion injury. Perfusion 31:103–110

Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112:358–404

Braas KM, Zarbin MA, Snyder SH (1987) Endogenous adenosine and adenosine receptors localized to ganglion cells of the retina. Proc Natl Acad Sci U S A 84:3906–3910

Brito R, Pereira MR, Paes-de-Carvalho R, Calaza Kda C (2012) Expression of A_1 adenosine receptors in the developing avian retina: in vivo modulation by A_{2A} receptors and endogenous adenosine. J Neurochem 123:239–249

Brown RA, Clarke GW, Ledbetter CL, Hurle MJ, Denyer JC, Simcock DE, Coote JE, Savage TJ, Murdoch RD, Page CP, Spina D, O'Connor BJ (2008) Elevated expression of adenosine A_1 receptor in bronchial biopsy specimens from asthmatic subjects. Eur Respir J 31:311–319

Bruns RF, Fergus JH (1990) Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. Mol Pharmacol 38:939–949

Burgdorf C, Richardt D, Kurz T, Seyfarth M, Jain D, Katus HA, Richardt G (2001) Adenosine inhibits norepinephrine release in the postischemic rat heart: the mechanism of neuronal stunning. Cardiovasc Res 49:713–720

Burnstock G (2011) Introductory overview of purinergic signalling. Front Biosci 3:896–900

Burnstock G (2013) Introduction to purinergic signalling in the brain. Adv Exp Med Biol 986:1–12

Burnstock G (2014) Purinergic signalling in the gastrointestinal tract and related organs in health and disease. Purinergic Signal 10:3–50

Burnstock G, Pelleg A (2015) Cardiac purinergic signalling in health and disease. Purinergic Signal 11:1–46

Burnstock G, Brouns I, Adriaensen D, Timmermans JP (2012) Purinergic signaling in the airways. Pharmacol Rev 64:834–868

Burnstock G, Evans LC, Bailey MA (2014) Purinergic signalling in the kidney in health and disease. Purinergic Signal 10:71–101

Butcher A, Scammells PJ, White PJ, Devine SM, Rose'meyer RB (2013) An allosteric modulator of the adenosine A_1 receptor improves cardiac function following ischaemia in murine isolated hearts. Pharmaceuticals (Basel) 6:546–556

Camici M, Micheli V, Ipata PL, Tozzi MG (2010) Pediatric neurological syndromes and inborn errors of purine metabolism. Neurochem Int 56:367–378

Canals M, Lane JR, Wen A, Scammells PJ, Sexton PM, Christopoulos A (2012) A Monod-Wyman-Changeux mechanism can explain G protein-coupled receptor (GPCR) allosteric modulation. J Biol Chem 287:650–659

Cappellacci L, Franchetti P, Vita P, Petrelli R, Lavecchia A, Costa B, Spinetti F, Martini C, Klotz KN, Grifantini M (2008) 5′-Carbamoyl derivatives of 2′-C-methyl-purine nucleosides as selective A1 adenosine receptor agonists: affinity, efficacy, and selectivity for A1 receptor from different species. Bioorg Med Chem 16:336–353

Carlin JL, Jain S, Gizewski E, Wan TC, Tosh DK, Xiao C, Auchampach JA, Jacobson KA, Gavrilova O, Reitman ML (2017) Hypothermia in mouse is caused by adenosine A_1 and A_3 receptor agonists and AMP via three distinct mechanisms. Neuropharmacology 114:101–113

Carrettiero DC, Almeida RS, Fior-Chadi DR (2008) Adenosine modulates alpha2-adrenergic receptors within specific subnuclei of the nucleus tractus solitarius in normotensive and spontaneously hypertensive rats. Hypertens Res 31:2177–2186

Carrettiero DC, da Silva SM, Fior-Chadi DR (2009) Adenosine modulates alpha2-adrenergic receptors through a phospholipase C pathway in brainstem cell culture of rats. Auton Neurosci 151:174–177

Chandra R, Liddle RA (2014) Recent advances in the regulation of pancreatic secretion. Curr Opin Gastroenterol 30:490–494

Chandrasekera PC, Wan TC, Gizewski ET, Auchampach JA, Lasley RD (2013) Adenosine A_1 receptors heterodimerize with β1- and β2-adrenergic receptors creating novel receptor complexes with altered G protein coupling and signaling. Cell Signal 25:736–742

Chang LC, Brussee J, Ijzerman AP (2004a) Non-xanthine antagonists for the adenosine A_1 receptor. Chem Biodivers 1:1591–1626

Chang LC, Spanjersberg RF, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, van den Hout G, Beukers MW, Brussee J, Ijzerman AP (2004b) 2,4,6-trisubstituted pyrimidines as a new class of selective adenosine A_1 receptor antagonists. J Med Chem 47:6529–6540

Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Westerhout J, Spangenberg T, Brussee J, Ijzerman AP (2007) 2,6,8-trisubstituted 1-deazapurines as adenosine receptor antagonists. J Med Chem 50:828–834

Chen JF, Eltzschig HK, Fredholm BB (2013) Adenosine receptors as drug targets--what are the challenges? Nat Rev Drug Discov 12:265–286

Cheng JT, Chi TC, Liu IM (2000) Activation of adenosine A_1 receptors by drugs to lower plasma glucose in streptozotocin-induced diabetic rats. Auton Neurosci 83:127–133

Cheung JW, Lerman BB (2003) CVT-510: a selective A_1 adenosine receptor agonist. Cardiovasc Drug Rev 21:277–292

Childers SR, Li X, Xiao R, Eisenach JC (2005) Allosteric modulation of adenosine A_1 receptor coupling to G-proteins in brain. J Neurochem 93:715–723

Christofi FL, Zhang H, Yu JG, Guzman J, Xue J, Kim M, Wang YZ, Cooke HJ (2001) Differential gene expression of adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors in the human enteric nervous system. J Comp Neurol 439:46–64

Christofi FL, Kim M, Wunderlich JE, Xue J, Suntres Z, Cardounel A, Javed NH, Yu JG, Grants I, Cooke HJ (2004) Endogenous adenosine differentially modulates 5-hydroxytryptamine release from a human enterochromaffin cell model. Gastroenterology 127:188–202

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. Nat Rev Drug Discov 1:198–210

Cieślak M, Wojtczak A, Komoszyński M (2016) Role of the purinergic signaling in epilepsy. Pharmacol Rep 69:130–138

Ciruela F, Casadó V, Rodrigues RJ, Luján R, Burgueño J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J, Cortés A, Canela EI, López-Giménez JF, Milligan G, Lluis C, Cunha RA, Ferré S, Franco R (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 26(7):2080–2087

Coelho JE, Rebola N, Fragata I, Ribeiro JA, de Mendonça A, Cunha RA (2006) Hypoxia-induced desensitization and internalization of adenosine A_1 receptors in the rat hippocampus. Neuroscience 138:1195–1203

Cooke HJ, Wang Y, Liu CY, Zhang H, Christofi FL (1999) Activation of neuronal adenosine A_1 receptors suppresses secretory reflexes in the guinea pig colon. Am J Phys 276:G451–G462

Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G (1992) Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. J Immunol 148:2201–2206

Crosson CE (2001) Intraocular pressure responses to the adenosine agonist cyclohexyladenosine: evidence for a dual mechanism of action. Invest Ophthalmol Vis Sci 42:1837–1840

Cunha RA (2008) Caffeine, adenosine receptors, memory and Alzheimer disease. Med Clin (Barc) 131:790–795

Cunha RA (2016) How does adenosine control neuronal dysfunction and neurodegeneration? J Neurochem 139:1019–1055

Daly JW, Padgett WL, Secunda SI, Thompson RD, Olsson RA (1993) Structure-activity relationships for 2-substituted adenosines at A_1 and A_2 adenosine receptors. Pharmacology 46:91–100

de Mendonça A, Sebastião AM, Ribeiro JA (2000) Adenosine: does it have a neuroprotective role after all? Brain Res Brain Res Rev 33:258–274

Dhalla AK, Wong MY, Voshol PJ, Belardinelli L, Reaven GM (2007a) A_1 adenosine receptor partial agonist lowers plasma FFA and improves insulin resistance induced by high-fat diet in rodents. Am J Physiol Endocrinol Metab 292:E1358–E1363

Dhalla AK, Santikul M, Smith M, Wong MY, Shryock JC, Belardinelli L (2007b) Antilipolytic activity of a novel partial A_1 adenosine receptor agonist devoid of cardiovascular effects: comparison with nicotinic acid. J Pharmacol Exp Ther 321:327–333

Dhalla AK, Chisholm JW, Reaven GM, Belardinelli L (2009) A_1 adenosine receptor: role in diabetes and obesity. Handb Exp Pharmacol 193:271–295

Dittrich HC, Gupta DK, Hack TC, Dowling T, Callahan J, Thomson S (2007) The effect of KW-3902, an adenosine A_1 receptor antagonist, on renal function and renal plasma flow in ambulatory patients with heart failure and renal impairment. J Card Fail 13:609–617

Doggrell SA (2005) BG-9928 (Biogen Idec). Curr Opin Investig Drugs 6:962–968

Dong Q, Ginsberg HN, Erlanger BF (2001) Overexpression of the A_1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. Diabetes Obes Metab 3:360–366

Duarte JM, Carvalho RA, Cunha RA, Gruetter R (2009) Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. J Neurochem 111:368–379

Edwards JM, Alloosh MA, Long XL, Dick GM, Lloyd PG, Mokelke EA, Sturek M (2008) Adenosine A_1 receptors in neointimal hyperplasia and in-stent stenosis in Ossabaw miniature swine. Coron Artery Dis 19:27–31

El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM (2000) The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A_{2A} adenosine receptor antagonists. Psychopharmacol (Berl) 148:153–163

Ellis MJ, Lindon AC, Flint KJ, Jones NC, Goodbourn S (1995) Activating transcription factor-1 is a specific antagonist of the cyclic adenosine 3′.5-′-monophosphate (cAMP) response element-binding protein-1-mediated response to cAMP. Mol Endocrinol 9:255–265

Elzein E, Kalla R, Li X, Perry T, Marquart T, Micklatcher M, Li Y, Wu Y, Zeng D, Zablocki J (2007) N6-Cycloalkyl-2-substituted adenosine derivatives as selective, high affinity adenosine A_1 receptor agonists. Bioorg Med Chem Lett 17:161–166

Ethier MF, Madison JM (2006) Adenosine A_1 receptors mediate mobilization of calcium in human bronchial smooth muscle cells. Am J Respir Cell Mol Biol 35:496–502

Ezeamuzie CI, Philips E (1999) Adenosine A_3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. Br J Pharmacol 127:188–194

Fastbom J, Pazos A, Probst A, Palacios JM (1986) Adenosine A_1 receptors in human brain: characterization and autoradiographic visualization. Neurosci Lett 65:127–132

Fastbom J, Pazos A, Palacios JM (1987) The distribution of adenosine A_1 receptors and 5′-nucleotidase in the brain of some commonly used experimental animals. Neurosci 22:813–826

Ferrante A, Martire A, Pepponi R, Varani K, Vincenzi F, Ferraro L, Beggiato S, Tebano MT, Popoli P (2014) Expression, pharmacology and functional activity of adenosine A_1 receptors in genetic models of Huntington's disease. Neurobiol Dis 71:193–204

Ferré S, Ciruela F, Quiroz C, Luján R, Popoli P, Cunha RA, Agnati LF, Fuxe K, Woods AS, Lluis C, Franco R (2007) Adenosine receptor heteromers and their integrative role in striatal function. Sci World J 7:74–85

Franchetti P, Cappellacci L, Vita P, Petrelli R, Lavecchia A, Kachler S, Klotz KN, Marabese I, Luongo L, Maione S, Grifantini M (2009) N6-Cycloalkyl- and N6-bicycloalkyl-C5′(C2′)-modified adenosine derivatives as high-affinity and selective agonists at the human A_1 adenosine receptor with antinociceptive effects in mice. J Med Chem 52:2393–2406

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W (2000) Structure and function of adenosine receptors and their genes. Naunyn Schmiedeberg's Arch Pharmacol 362:364–374

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE (2011a) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors an update. Pharmacol Rev 63:1–34

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE (2011b) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors-an update. Pharmacol Rev 63:1–34

Fredholm BB, Johansson S, Wang YQ (2011c) Adenosine and the regulation of metabolism and body temperature. Adv Pharmacol 61:77–94

Fredholm BB, Yang J, Wang Y (2016) Low, but not high, dose caffeine is a readily available probe for adenosine actions. Mol Asp Med S0098-2997:30080–30082

Funakoshi H, Chan TO, Good JC, Libonati JR, Piuhola J, Chen X, MacDonnell SM, Lee LL, Herrmann DE, Zhang J, Martini J, Palmer TM, Sanbe A, Robbins J, Houser SR, Koch WJ, Feldman AM (2006) Regulated overexpression of the A_1 adenosine receptor in mice results in adverse but reversible changes in cardiac morphology and function. Circulation 114:2240–2250

Gao ZG, Jacobson KA (2007) Emerging adenosine receptor agonists. Expert Opin Emerg Drugs 12:479–492

Gao ZG, Jacobson KA (2011) Emerging adenosine receptor agonists: an update. Expert Opin Emerg Drugs 16:597–602

Gao ZG, Melman N, Erdmann A, Kim SG, Müller CE, IJzerman AP, Jacobson KA (2003) Differential allosteric modulation by amiloride analogues of agonist and antagonist binding at A_1 and A_3 adenosine receptors. Biochem Pharmacol 65:525–534

Gao ZG, Kim SK, Ijzerman AP, Jacobson KA (2005) Allosteric modulation of the adenosine family of receptors. Mini Rev Med Chem 5:545–553

Gergs U, Boknik P, Schmitz W, Simm A, Silber RE, Neumann J (2009) A positive inotropic effect of adenosine in cardiac preparations of right atria from diseased human hearts. Naunyn Schmiedeberg's Arch Pharmacol 379:533–540

Gessi S, Merighi S, Fazzi D, Stefanelli A, Varani K, Borea PA (2011) Adenosine receptor targeting in health and disease. Expert Opin Investig Drugs 20:1591–1609

Gessi S, Merighi S, Stefanelli A, Fazzi D, Varani K, Borea PA (2013) A_1 and A_3 adenosine receptors inhibit LPS-induced hypoxia-inducible factor-1 accumulation in murine astrocytes. Pharmacol Res 76:157–170

Giménez-Llort L, Fernández-Teruel A, Escorihuela RM, Fredholm BB, Tobeña A, Pekny M, Johansson B (2002) Mice lacking the adenosine A_1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci 16:547–550

Ginés S, Hillion J, Torvinen M, Le Crom S, Casadó V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Verniera P, Lluis C, Ferré S, Fuxe K, Franco R (2000) Dopamine D_1 and adenosine A_1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci U S A 97:8606–8611

Giordanetto F, Fossa P, Menozzi G, Schenone S, Bondavalli F, Ranise A, Mosti L (2003) Exploring the molecular basis of selectivity in A1 adenosine receptors agonists: a case study. J Comput Aided Mol Des 17:39–51

Giorgi I, Nieri P (2013) Adenosine A_1 modulators: a patent update (2008 to present). Expert Opin Ther Pat 23:1109–1121

Givertz MM, Massie BM, Fields TK, Pearson LL, Dittrich HC (2007) The effects of KW-3902, an adenosine A_1 receptor antagonist, on diuresis and renal function in patients with acute decompensated heart failure and renal impairment or diuretic resistance. J Am Coll Cardiol 50:1551–1560

Gomes WA, Shinnar S (2011) Prospects for imaging-related biomarkers of human epileptogenesis: a critical review. Biomark Med 5:599–606

Gomes CV, Kaster MP, Tomé AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta 1808:1380–1399

Goodman RR, Synder SH (1982) Autoradiographic localization of adenosine receptors in rat brain using [^{3}H] cyclohexyladenosine. J Neurosci 2:1230–1241

Gouder N, Fritschy JM, Boison D (2003) Seizure suppression by adenosine A_1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia 44:877–885

Grden M, Podgorska M, Kocbuch K, Szutowicz A, Pawelczyk T (2006) Expression of adenosine receptors in cardiac fibroblasts as a function of insulin and glucose level. Arch Biochem Biophys 455:10–17

Greenberg B, Thomas I, Banish D, Goldman S, Havranek E, Massie BM, Zhu Y, Ticho B, Abraham WT (2007) Effects of multiple oral doses of an A_1 adenosine antagonist, BG9928, in patients with heart failure: results of a placebo-controlled, dose-escalation study. J Am Coll Cardiol 50:600–606

Grewal SS, Fass DM, Yao H, Ellig CL, Goodman RH, Stork PJ (2000) Calcium and cAMP signals differentially regulate cAMP-responsive element-binding protein function via a Rap1-extracellular signal-regulated kinase pathway. J Biol Chem 275:34433–34441

Hansen PB, Schnermann J (2003) Vasoconstrictor and vasodilator effects of adenosine in the kidney. Am J Physiol Renal Physiol 285:F590–F599

Hartwick AT, Lalonde MR, Barnes S, Baldridge WH (2004) Adenosine A_1 receptor modulation of glutamate-induced calcium influx in rat retinal ganglion cells. Invest Ophthalmol Vis Sci 45:3740–3748

Headrick JP, Hack B, Ashton KJ (2003) Acute adenosinergic cardioprotection in ischemic-reperfused hearts. Am J Physiol Heart Circ Physiol 285:H1797–H1818

Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN (2013) Cardiovascular adenosine receptors: expression, actions and interactions. Pharmacol Ther 140:92–111

Heller AR, Rothermel J, Weigand MA, Plaschke K, Schmeck J, Wendel M, Bardenheuer HJ, Koch T (2007) Adenosine A_1 and A_2 receptor agonists reduce endotoxin-induced cellular energy depletion and oedema formation in the lung. Eur J Anaesthesiol 24:258–266

Hodges RR, Dartt DA (2016) Signaling pathways of Purinergic receptors and their interactions with cholinergic and adrenergic pathways in the lacrimal gland. J Ocul Pharmacol Ther 32:490–497

Hua X, Erikson CJ, Chason KD, Rosebrock CN, Deshpande DA, Penn RB, Tilley SL (2007) Involvement of A_1 adenosine receptors and neural pathways in adenosine-induced bronchoconstriction in mice. Am J Physiol Lung Cell Mol Physiol 29:L25–L32

Husain S, Shearer TW, Crosson CE (2007) Mechanisms linking adenosine A_1 receptors and extracellular signal-regulated kinase 1/2 activation in human trabecular meshwork cells. J Pharmacol Exp Ther 320:258–265

Hussain T, Mustafa SJ (1995) Binding of A_1 adenosine receptor ligand [3H] 8-cyclopentyl-1,3-dipropylxanthine in coronary smooth muscle. Circ Res 77:194–198

Ivanov AA, Palyulin VA, Zefirov NS (2007) Computer aided comparative analysis of the binding modes of the adenosine receptor agonists for all known subtypes of adenosine receptors. J Mol Graph Model 25:740–754

Jaberi E, Rohani M, Shahidi GA, Nafissi S, Arefian E, Soleimani M, Moghadam A, Arzenani MK, Keramatian F, Klotzle B, Fan JB, Turk C, Steemers F, Elahi E (2016) Mutation in ADORA1 identified as likely cause of early-onset parkinsonism and cognitive dysfunction. Mov Disord 31:1004–1011

Jacobson KA, Civan MM (2016) Ocular purine receptors as drug targets in the eye. J Ocul Pharmacol Ther 32:534–547

Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 5:247–264

Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Giménez-Llort L, Escorihuela RM, Fernández-Teruel A, Wiesenfeld-Hallin Z, Xu XJ, Hårdemark A, Betsholtz C, Herlenius E, Fredholm BB (2001) Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A_1 receptor. Proc Natl Acad Sci U S A 98:9407–9412

Johansson SM, Salehi A, Sandström ME, Westerblad H, Lundquist I, Carlsson PO, Fredholm BB, Katz A (2007a) A_1 receptor deficiency causes increased insulin and glucagon secretion in mice. Biochem Pharmacol 74:1628–1635

Johansson SM, Yang JN, Lindgren E, Fredholm BB (2007b) Eliminating the antilipolytic adenosine A_1 receptor does not lead to compensatory changes in the antilipolytic actions of PGE2 and nicotinic acid. Acta Physiol (Oxf) 190:87–96

Johansson SM, Lindgren E, Yang JN, Herling AW, Fredholm BB (2008) Adenosine A_1 receptors regulate lipolysis and lipogenesis in mouse adipose tissue-interactions with insulin. Eur J Pharmacol 597:92–101

Kadowaki M, Tokita K, Nagakura Y, Takeda M, Hanaoka K, Tomoi M (2000) Adenosine A_1 receptor blockade reverses dysmotility induced by ischemia-reperfusion in rat colon. Eur J Pharmacol 409:319–323

Kalk P, Eggert B, Relle K, Godes M, Heiden S, Sharkovska Y, Fischer Y, Ziegler D, Bielenberg GW, Hocher B (2007) The adenosine A_1 receptor antagonist SLV320 reduces myocardial fibrosis in rats with 5/6 nephrectomy without affecting blood pressure. Br J Pharmacol 151:1025–1032

Kapicka CL, Montamat SC, Olson RD, Musser B, Mudumbi RV, Vestal RE (2003) Species comparison of adenosine A_1 receptors in isolated mammalian atrial and ventricular myocardium. Life Sci 72:2825–2838

Kaplan JL, Gao E, De Garavilla L, Victain M, Minczak B, Dalsey WC (2003) Adenosine A_1 antagonism attenuates atropine-resistant hypoxic bradycardia in rats. Acad Emerg Med 10:923–930

Ke JJ, Yu FX, Rao Y, Wang YL (2011) Adenosine postconditioning protects against myocardial ischemia-reperfusion injury though modulate

production of TNF-α and prevents activation of transcription factor NF-kappaB. Mol Biol Rep 38:531–538

Keir S, Boswell-Smith V, Spina D, Page C (2006) Mechanism of adenosine-induced airways obstruction in allergic guinea pigs. Br J Pharmacol 147:720–728

Khan RB, Yerremsetty PK, Lindstrom D, McGill LJ (2001) Emergency EEG and factors associated with nonconvulsive status epilepticus. J Natl Med Assoc 93:359–362

Kieć-Kononowicz K, Drabczyńska A, Pękala E, Michalak B, Müller CE, Schumacher B, Karolak-Wojciechowska J, Duddeck H, Rockitt S, Wartchow R (2001) New developments in A_1 and A_2 adenosine receptor antagonists. Pure App Chem 73:1411–1420

Kiesman WF, Zhao J, Conlon PR, Petter RC, Jin X, Smits G, Lutterodt F, Sullivan GW, Linden J (2006) Norbornyllactone-substituted xanthines as adenosine A_1 receptor antagonists. Bioorg Med Chem 14:3654–3661

Kiesman WF, Elzein E, Zablocki J (2009) A_1 adenosine receptor antagonists, agonists, and allosteric enhancers. Handb Exp Pharmacol 193:25–58

Kirkup AJ, Brunsden AM, Grundy D (2001) Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol 280:G787–G794

Klinger M, Freissmuth M, Nanoff C (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. Cell Signal 14:99–108

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. Naunyn Schmiedeberg's Arch Pharmacol 357:1–9

Kohno Y, Nagatomi I, Hanaoka K (1993) Pyrazolopyridine compounds for the treatment of anemia. WO patent 1993025205, 23 dec 1993

Kolachala VL, Bajaj R, Chalasani M, Sitaraman SV (2008) Purinergic receptors in gastrointestinal inflammation. Am J Physiol Gastrointest Liver Physiol 294: G401–G410

Kunduri S, Dick G, Nayeem M, Mustafa S (2013) Adenosine A_1 receptor signaling inhibits BK channels through a PKCα-dependent mechanism in mouse aortic smooth muscle. Physiol Rep 1:e00037

Lancelot E, Idée JM, Laclédère C, Santus R, Corot C (2002) Effects of two dimeric iodinated contrast media on renal medullary blood perfusion and oxygenation in dogs. Investig Radiol 37:368–375

Lang UE, Lang F, Richter K, Vallon V, Lipp HP, Schnermann J, Wolfer DP (2003) Emotional instability but intact spatial cognition in adenosine receptor 1 knock out mice. Behav Brain Res 145:179–188

Larrouy D, Galitzky J, Lafontan M (1991) A_1 adenosine receptors in the human fat cell: tissue distribution and regulation of radioligand binding. Eur J Pharmacol 206:139–147

Lazarowski ER, Boucher RC, Harden TK (2000) Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J Biol Chem 275:31061–31068

Lee JJ, Parsons ME (2000) Signaling mechanisms coupled to presynaptic A_1 and H3 receptors in the inhibition of cholinergic contractile responses of the guinea pig ileum. J Pharmacol Exp Ther 295:607–613

Lee JJ, Talubmook C, Parsons ME (2001) Activation of presynaptic A_1 receptors by endogenous adenosine inhibits acetylcholine release in the guinea-pig ileum. J Auton Pharmacol 21:29–38

Lee HK, Choi SS, Han KJ, Han EJ, Suh HW (2004) Roles of adenosine receptors in the regulation of kainic acid-induced neurotoxic responses in mice. Brain Res Mol Brain Res 125:76–85

Lee DL, Bell TD, Bhupatkar J, Solis G, Welch WJ (2012) Adenosine A_1 receptor knockout mice have a decreased blood pressure response to low-dose ANG II infusion. Am J Physiol Regul Integr Comp Physiol 303:R683–R688

Li X, Conklin D, Ma W, Zhu X, Eisenach JC (2002) Spinal noradrenergic activation mediates allodynia reduction from an allosteric adenosine modulator in a rat model of neuropathic pain. Pain 97:117–125

Li X, Conklin D, Pan HL, Eisenach JC (2003) Allosteric adenosine receptor modulation reduces hypersensitivity following peripheral inflammation by a central mechanism. J Pharmacol Exp Ther 305:950–955

Li T, Quan Lan J, Fredholm BB, Simon RP, Boison D (2007) Adenosine dysfunction in astrogliosis: cause for seizure generation? Neuron Glia Biol 3:353–366

Li A, Leung CT, Peterson-Yantorno K, Stamer WD, Mitchell CH, Civan MM (2012) Mechanisms of ATP release by human trabecular meshwork cells, the enabling step in purinergic regulation of aqueous humor outflow. J Cell Physiol 227:172–182

Li RH, Ding CK, Jiang YN, Ding ZC, An XM, Tang HT, Jing QW, Zhan ZP (2016) Synthesis of 5,6-Dihydropyrazolo[5,1-a]isoquinolines through indium(III)-promoted Halocyclizations of N-Propargylic Sulfonylhydrazones. Org Lett 18:1666–1669

Lopes LV, Sebastião AM, Ribeiro JA (2011) Adenosine and related drugs in brain diseases: present and future in clinical trials. Curr Top Med Chem 11:1087–1101

Lütjens H, Zickgraf A, Figler H, Linden J, Olsson RA, Scammells PJ (2003) 2-amino-3-benzoylthiophene allosteric enhancers of A_1 adenosine agonist binding: new 3, 4-, and 5-modifications. J Med Chem 46:1870–1877

Lynch JJ, Alexander KM, Jarvis MF, Kowaluk EA (1998) Inhibition of adenosine kinase during oxygen-glucose deprivation in rat cortical neuronal cultures. Neurosci Lett 252:207–210

Lynge J, Hellsten Y (2000) Distribution of adenosine A_1, A_{2A} and A_{2B} receptors in human skeletal muscle. Acta Physiol Scand 169:283–290

Maione S, de Novellis V, Cappellacci L, Palazzo E, Vita D, Luongo L, Stella L, Franchetti P, Marabese I, Rossi F, Grifantini M (2007) The antinociceptive effect of 2-chloro-2′-C-methyl-N6-cyclopentyladenosine (2′-me-CCPA), a highly selective adenosine A_1 receptor agonist, in the rat. Pain 131:281–292

Mansourian S, Bina P, Fehri A, Karimi AA, Boroumand MA, Abbasi K (2015) Preoperative oral pentoxifylline in case of coronary artery bypass grafting with left ventricular dysfunction (ejection fraction equal to/less than 30%). Anatol J Cardiol 15:1014–1019

McNamara N, Gallup M, Khong A, Sucher A, Maltseva I, Fahy J, Basbaum C (2004) Adenosine up-regulation of the mucin gene, MUC2, in asthma. FASEB J 18:1770–1772

Merighi S, Borea PA, Stefanelli A, Bencivenni S, Castillo CA, Varani K, Gessi S (2015) A_{2A} and A_{2B} adenosine receptors affect HIF-1α signaling in activated primary microglial cells. Glia 63:1933–1952

Merkel LA, Rivera LM, Colussi DJ, Perrone MH, Smits GJ, Cox BF (1993) In vitro and in vivo characterization of an A_1 selective adenosine agonist, RG14202. J Pharmacol Exp Ther 265:699–706

Meyer PT, Bier D, Holschbach MH, Boy C, Olsson RA, Coenen HH, Zilles K, Bauer A (2004) Quantification of cerebral A_1 adenosine receptors in humans using [18F]CPFPX and PET. J Cereb Blood Flow Metab 24:323–333

Millan MJ (2003) The neurobiology and control of anxious states. Prog Neurobiol 70:83–244

Montero MF, Saurim R, Bonservizi WG, Koike MK, Taha MO (2014) Heart injury following intestinal ischemia reperfusion in rats is attenuated by association of ischemic preconditioning and adenosine. Acta Cir Bras 29:67–71

Morrison RR, Teng B, Oldenburg PJ, Katwa LC, Schnermann JB, Mustafa SJ (2006) Effects of targeted deletion of A_1 adenosine receptors on postischemic cardiac function and expression of adenosine receptor subtypes. Am J Physiol Heart Circ Physiol 291: H1875–H1882

Mostofsky E, Schlaug G, Mukamal KJ, Rosamond WD, Mittleman MA (2010) Coffee and acute ischemic stroke onset: the stroke onset study. Neurology 75:1583–1588

Müller CE (2001) A_1 adenosine receptors and their ligands: overview and recent developments. Farmaco 56:77–80

Müller CE, Jacobson KA (2011) Recent developments in adenosine receptor ligands and their potential as novel drugs. Biochim Biophys Acta 1808:1290–1308

Müller CE, Geis U, Grahner B, Lanzner W, Eger K (1996) Chiral pyrrolo[2,3-d]pyrimidine and pyrimido[4,5-b] indole derivatives: structure-activity relationships of potent, highly stereoselective A_1 adenosine receptor antagonists. J Med Chem 39:2482–2491

Murthy KS, McHenry L, Grider JR, Makhlouf GM (1995) Adenosine A_1 and A_{2B} receptors coupled to distinct interactive signaling pathways in intestinal muscle cells. J Pharmacol Exp Ther 274:300–306

Murthy KS, Grider JR, Makhlouf GM (2000) Heterologous desensitization of response mediated by selective PKC-dependent phosphorylation of G(i-1) and G(i-2). Am J Cell Physiol 279:C925–C934

Musser B, Morgan ME, Leid M, Murray TF, Linden J, Vestal RE (1993) Species comparison of adenosine and β-adrenoceptors in mammalian atrial and ventricular myocardium. Eur J Pharmacol 246:105–111

Mustafa SJ, Morrison RR, Teng B, Pelleg A (2009) Adenosine receptors and the heart: role in regulation of coronary blood flow and cardiac electrophysiology. Handb Exp Pharmacol 193:161–188

Muzzi M, Coppi E, Pugliese AM, Chiarugi A (2013) Anticonvulsant effect of AMP by direct activation of adenosine A_1 receptor. Exp Neurol 250:189–193

Nadeem A, Obiefuna PC, Wilson CN, Mustafa SJ (2006) Adenosine A_1 receptor antagonist versus montelukast on airway reactivity and inflammation. Eur J Pharmacol 551:116–124

Nakata H, Yoshioka K, Kamiya T, Tsuga H, Oyanagi K (2005) Functions of heteromeric association between adenosine and P2Y receptors. J Mol Neurosci 26:233–238

Nalli AD, Kumar DP, Al-Shboul O, Mahavadi S, Kuemmerle JF, Grider JR, Murthy KS (2014) Regulation of Gβγi-dependent PLC-β3 activity in smooth muscle: inhibitory phosphorylation of PLC-β3 by PKA and PKG and stimulatory phosphorylation of Gαi-GTPase-activating protein RGS2 by PKG. Cell Biochem Biophys 70:867–880

Narlawar R, Lane JR, Doddareddy M, Lin J, Brussee J, Ijzerman AP (2010) Hybrid ortho/allosteric ligands for the adenosine A1 receptor. J Med Chem 53:3028–3037

Nayeem MA, Mustafa SJ (2002) Protein kinase C isoforms and A_1 adenosine receptors in porcine coronary smooth muscle cells. Vasc Pharmacol 39:47–54

Neely C (1995) Prevention and treatment of ischemia-reperfusion and endotoxin-related injury using adenosine and purino receptor antagonists. WO patent 1995026728, 12 oct 1995

Nell PG, Albrecht-Küpper B (2009) The adenosine A_1 receptor and its ligands. Prog Med Chem 47:163–195

Németh ZH, Bleich D, Csóka B, Pacher P, Mabley JG, Himer L, Vizi ES, Deitch EA, Szabó C, Cronstein BN, Haskó G (2007) Adenosine receptor activation ameliorates type 1 diabetes. FASEB J 21:2379–2388

Newman GC, Hospod FE, Trowbridge SD, Motwani S, Liu Y (1998) Restoring adenine nucleotides in a brain slice model of cerebral reperfusion. J Cereb Blood Flow Metab 18:675–685

Nguyen AT, Baltos JA, Thomas T, Nguyen TD, Muñoz LL, Gregory KJ, White PJ, Sexton PM, Christopoulos A, May LT (2016) Extracellular loop 2 of the adenosine A1 receptor has a key role in orthosteric ligand affinity and agonist efficacy. Mol Pharmacol 90:703–714

Nyce JW, Metzger WJ (1997) DNA antisense therapy for asthma in an animal model. Nature 385:721–725

Obiefuna PC, Batra VK, Nadeem A, Borron P, Wilson CN, Mustafa SJ (2005) A novel A_1 adenosine receptor antagonist, L-97-1 [3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione], reduces allergic responses to house dust mite in an allergic rabbit model of asthma. J Pharmacol Exp Ther 315:329–336

Odagaki Y, Kinoshita M, Ota T, Meana JJ, Callado LF, García-Sevilla JA (2015) Adenosine A1 receptors are selectively coupled to Gα(i-3) in postmortem human brain cortex: Guanosine-5′-O-(3-[(35)S]thio)triphosphate ([(35)S]GTPγS) binding/immunoprecipitation study. Eur J Pharmacol 764:592–598

Olah ME, Stiles GL (2000) The role of receptor structure in determining adenosine receptor activity. Pharmacol Ther 85:55–75

Olah ME, Ren H, Stiles GL (1995) Adenosine receptors: protein and gene structure. Arch Int Pharmacodyn Ther 329(1):135–150

Osswald H, Schnermann J (2011) Methylxanthines and the kidney. Handb Exp Pharmacol 200:391–412

Otsuki L, Dittrich HC, Widder KJ, Blantz R, Thomson S (2005) Method of treatment of disease using an adenosine A_1 receptor antagonist. WO patent 200515083, 10 nov 2005

Palacios N, Gao X, McCullough ML, Schwarzschild MA, Shah R, Gapstur S, Ascherio A (2012) Caffeine and risk of Parkinson's disease in a large cohort of men and women. Mov Disord 27:1276–1282

Pang PS, Mehra M, Maggioni AP, Filippatos G, Middlebrooks J, Turlapaty P, Kazei D, Gheorghiade M (2011) Rationale, design, and results from RENO-DEFEND 1: a randomized, dose-finding study of the selective A_1 adenosine antagonist SLV320 in patients hospitalized with acute heart failure. Am Heart J 161:1012–1023

Pankevych H, Korkhov V, Freissmuth M, Nanoff C (2003) Truncation of the A_1 adenosine receptor reveals distinct roles of the membrane-proximal carboxyl terminus in receptor folding and G protein coupling. J Biol Chem 278:30283–30293

Panther E, Idzko M, Herouy Y, Rheinen H, Gebicke-Haerter PJ, Mrowietz U, Dichmann S, Norgauer J (2001) Expression and function of adenosine receptors in human dendritic cells. FASEB J 15:1963–1970

Partridge JJ, Wilson CN (2005) A_1 adenosine receptor antagonists. WO patent 2005009343, 3 feb 2005

Peart J, Headrick JP (2000) Intrinsic A_1 adenosine receptor activation during ischemia or reperfusion improves recovery in mouse hearts. Am J Physiol Heart Circ Physiol 279:H2166–H2175

Peart JN, Headrick JP (2007) Adenosinergic cardioprotection: multiple receptors, multiple pathways. Pharmacol Ther 114:208–221

Peart JN, Headrick JP (2009) Clinical cardioprotection and the value of conditioning responses. Am J Physiol Heart Circ Physiol 296:H1705–H1720

Peeters MC, Wisse LE, Dinaj A, Vroling B, Vriend G, Ijzerman AP (2012) The role of the second and third extracellular loops of the adenosine A_1 receptor in activation and allosteric modulation. Biochem Pharmacol 84:76–87

Perígolo-Vicente R, Ritt K, Gonçalves-de-Albuquerque CF, Castro-Faria-Neto HC, Paes-de-Carvalho R, Giestal-de-Araujo E (2014) IL-6, A_1 and A_{2A}ARs: a crosstalk that modulates BDNF and induces neuroprotection. Biochem Biophys Res Commun 449:477–482

Perlini S, Arosio B, Parmeggiani L, Santambrogio D, Palladini G, Tozzi R, Gatti C, Annoni G, Meyer TE, Ferrari AU (2007) Adenosine A_1 receptor expression during the transition from compensated pressure overload hypertrophy to heart failure. J Hypertens 25:449–454

Prediger RD, Batista LC, Takahashi RN (2004) Adenosine A_1 receptors modulate the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. Eur J Pharmacol 499:147–154

Prediger RD, da Silva GE, Batista LC, Bittencourt AL, Takahashi RN (2006) Activation of adenosine A_1 receptors reduces anxiety-like behavior during acute ethanol withdrawal (hangover) in mice. Neuropsychopharmacology 31:2210–2220

Preti D, Baraldi PG, Moorman AR, Borea PA, Varani K (2015) History and perspectives of A_{2A} adenosine receptor antagonists as potential therapeutic agents. Med Res Rev 35:790–848

Puhl SL, Kazakov A, Müller A, Fries P, Wagner DR, Böhm M, Maack C, Devaux Y (2016) Adenosine A_1 receptor activation attenuates cardiac hypertrophy and fibrosis in response to α1 -adrenoceptor stimulation in vivo. Br J Pharmacol 173:88–102

Quitterer U, Lohse MJ (1999) Crosstalk between Galpha (i)- and Galpha(q)-coupled receptors is mediated by Gbetagamma exchange. Proc Natl Acad Sci U S A 96:10626–10631

Randhawa PK, Jaggi AS (2016) Unraveling the role of adenosine in remote ischemic preconditioning-induced cardioprotection. Life Sci 155:140–146

Rebola N, Porciúncula LO, Lopes LV, Oliveira CR, Soares-da-Silva P, Cunha RA (2005) Long-term effect of convulsive behavior on the density of adenosine A_1 and A_{2A} receptors in the rat cerebral cortex. Epilepsia 46:159–165

Rieg T, Steigele H, Schnermann J, Richter K, Osswald H, Vallon V (2005) Requirement of intact adenosine A_1 receptors for the diuretic and natriuretic action of the methylxanthines theophylline and caffeine. J Pharmacol Exp Ther 313:403–409

Riesenhuber A, Boehm M, Posch M, Aufricht C (2006) Diuretic potential of energy drinks. Amino Acids 31:81–83

Rittiner JE, Korboukh I, Hull-Ryde EA, Jin J, Janzen WP, Frye SV, Zylka MJ (2012) AMP is an adenosine A_1 receptor agonist. J Biol Chem 287:5301–5309

Rivera-Oliver M, Díaz-Ríos M (2014) Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review. Life Sci 101:1–9

Rivkees SA, Wendler CC (2012) Regulation of cardiovascular development by adenosine and adenosine-mediated embryo protection. Arterioscler Thromb Vasc Biol 32:851–855

Romagnoli R, Baraldi PG, Moorman AR, Iaconinoto MA, Carrion MD, Cara CL, Tabrizi MA, Preti D, Fruttarolo F, Baker SP, Varani K, Borea PA (2006) Microwave-assisted synthesis of thieno[2,3-c]pyridine derivatives as a new series of allosteric enhancers at the adenosine A_1 receptor. Bioorg Med Chem Lett 16:5530–5533

Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Iaconinoto MA, Preti D, Shryock JC, Moorman AR, Vincenzi F, Varani K, Borea PA (2008) Synthesis and biological evaluation of 2-amino-3-(4-chlorobenzoyl)-4-[N-(substituted) piperazin-1-yl]thiophenes as potent allosteric enhancers of the A1 adenosine receptor. J Med Chem 51:5875–5879

Romagnoli R, Baraldi PG, Tabrizi MA, Gessi S, Borea PA, Merighi S (2010) Allosteric enhancers of A_1 adenosine receptors: state of the art and new horizons for drug development. Curr Med Chem 17:3488–3502

Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Salvador MK, Preti D, Tabrizi MA, Moorman AR, Vincenzi F, Borea PA, Varani K (2012a) Synthesis and biological evaluation of 2-amino-3-(4-chlorobenzoyl)-4-[(4-arylpiperazin-1-yl)methyl]-5-substituted-thiophenes. Effect of the 5-modification on allosteric enhancer activity at the A_1 adenosine receptor. J Med Chem 55:7719–7735

Romagnoli R, Baraldi PG, Carrion MD, Cara CL, Cruz-Lopez O, Salvador MK, Preti D, Tabrizi MA, Shryock JC, Moorman AR, Vincenzi F, Varani K, Borea PA (2012b) Structure-activity relationships of 2-amino-3-aroyl-4-[(4-arylpiperazin-1-yl)methyl]thiophenes. Part 2: Probing the influence of diverse substituents at the phenyl of the arylpiperazine moiety on allosteric enhancer activity at the A_1 adenosine receptor. Bioorg Med Chem 20:996–1007

Romagnoli R, Baraldi PG, Carrion MD, Lopez Cara C, Kimatrai Salvador M, Preti D, Aghazadeh Tabrizi M, Moorman AR, Vincenzi F, Borea PA, Varani K (2013) Synthesis and biological effects of novel 2-amino-3-(4-chlorobenzoyl)-4-substituted thiophenes as allosteric enhancers of the A_1 adenosine receptor. Eur J Med Chem 67:409–427

Romagnoli R, Baraldi PG, Carrion MD, Cruz-Lopez O, Cara CL, Saponaro G, Preti D, Tabrizi MA, Baraldi S, Moorman AR, Vincenzi F, Borea PA, Varani K (2014a) Synthesis and biological evaluation of novel 2-amino-3-aroyl-4-neopentyl-5-substituted thiophene derivatives as allosteric enhancers of the A_1 adenosine receptor. Bioorg Med Chem 22:148–166

Romagnoli R, Baraldi PG, IJzerman AP, Massink A, Cruz-Lopez O, Lopez-Cara LC, Saponaro G, Preti D, Aghazadeh Tabrizi M, Baraldi S, Moorman AR, Vincenzi F, Borea PA, Varani K (2014b) Synthesis and biological evaluation of novel allosteric enhancers of the A_1 adenosine receptor based on 2-amino-3-(4-′-chlorobenzoyl)-4-substituted-5-arylethynyl thiophene. J Med Chem 57(18):7673–7686

Romagnoli R, Baraldi PG, Moorman AR, Borea PA, Varani K (2015) Current status of A_1 adenosine receptor allosteric enhancers. Future Med Chem 7:1247–1259

Roman V, Keijser JN, Luiten PG, Meerlo P (2008) Repetitive stimulation of adenosine A_1 receptors in vivo: changes in receptor numbers, G-proteins and A_1 receptor agonist-induced hypothermia. Brain Res 1191:69–74

Ross GW, Abbott RD, Petrovitch H, Morens DM, Grandinetti A, Tung KH, Tanner CM, Masaki KH, Blanchette PL, Curb JD, Popper JS, White LR (2000) Association of coffee and caffeine intake with the risk of Parkinson disease. JAMA 283:2674–2679

Rudolphi KA, Schubert P (1997) Modulation of neuronal and glial cell function by adenosine and neuroprotection in vascular dementia. Behav Brain Res 83:123–128

Ruiz MÁ, León DA, Albasanz JL, Martín M (2011) Desensitization of adenosine A_1 receptors in rat immature cortical neurons. Eur J Pharmacol 670:365–371

Sabbah HN, Gupta RC, Kohli S, Wang M, Rastogi S, Zhang K, Zimmermann K, Diedrichs N, Albrecht-Küpper BE (2013) Chronic therapy with a partial adenosine A_1 receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure. Circ Heart Fail 6:563–571

Sachdeva S, Gupta M (2013) Adenosine and its receptors as therapeutic targets: an overview. Saudi Pharm J 21:245–253

Sadigh-Lindell B, Sylvén C, Berglund M, Eriksson BE (2003) Role of adenosine and opioid-receptor mechanisms for pain in patients with silent myocardial ischemia or angina pectoris: a double-blind, placebo-controlled study. J Cardiovasc Pharmacol 42:757–763

Salazar-Martinez E, Willett WC, Ascherio A, Manson JE, Leitzmann MF, Stampfer MJ, Hu FB (2004) Coffee consumption and risk for type 2 diabetes mellitus. Ann Intern Med 140:1–8

Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG (1993) Molecular cloning and characterization of the human A_3 adenosine receptor. Proc Natl Acad Sci U S A 90:10365–10369

Santiago FE, Fior-Chadi DR, Carrettiero DC (2015) Alpha2-adrenoceptor and adenosine A_1 receptor within the nucleus tractus solitarii in hypertension development. Auton Neurosci 187:36–44

Sawynok J (2016) Adenosine receptor targets for pain. Neuroscience 338:1–18

Sawynok J, Reid AR (2012) Caffeine inhibits antinociception by acetaminophen in the formalin test by inhibiting spinal adenosine A_1 receptors. Eur J Pharmacol 674:248–254

Scheiff AB, Yerande SG, El-Tayeb A, Li W, Inamdar GS, Vasu KK, Sudarsanam V, Müller CE (2010) 2-amino-5-benzoyl-4-phenylthiazoles: development of potent and selective adenosine A_1 receptor antagonists. Bioorg Med Chem 18:2195–2203

Schenone S, Brullo C, Musumeci F, Bruno O, Botta M (2010) A_1 receptors ligands: past, present and future trends. Curr Top Med Chem 10:878–901

Schepp CP, Reutershan J (2008) Bench-to-bedside review: adenosine receptors--promising targets in acute lung injury? Crit Care 12(5):226

Schnurr M, Toy T, Shin A, Hartmann G, Rothenfusser S, Soellner J, Davis ID, Cebon J, Maraskovsky E (2004) Role of adenosine receptors in regulating chemotaxis and cytokine production of plasmacytoid dendritic cells. Blood 103:1391–1397

Schoelch C, Kuhlmann J, Gossel M, Mueller G, Neumann-Haefelin C, Belz U, Kalisch J, Biemer-Daub G, Kramer W, Juretschke HP, Herling AW (2004) Characterization of adenosine A_1 receptor-mediated antilipolysis in rats by tissue microdialysis, 1H-spectroscopy, and glucose clamp studies. Diabetes 53:1920–1926

Scholl DJ, Wells JN (2000) Serine and alanine mutagenesis of the nine native cysteine residues of the human A_1 adenosine receptor. Biochem Pharmacol 60:1647–1654

Schulte G, Fredholm BB (2002a) Diverse inhibitors of intracellular signalling act as adenosine receptor antagonists. Cell Signal 14:109–113

Schulte G, Fredholm BB (2002b) Signaling pathway from the human adenosine A_3 receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. Mol Pharmacol 62:1137–1146

Serchov T, Clement HW, Schwarz MK, Iasevoli F, Tosh DK, Idzko M, Jacobson KA, de Bartolomeis A, Normann C, Biber K, van Calker D (2015) Increased signaling via adenosine A_1 receptors, sleep deprivation, imipramine, and ketamine inhibit depressive-like behavior via induction of Homer1a. Neuron 87:549–562

Shearer J, Severson DL, Su L, Belardinelli L, Dhalla AK (2009) Partial A_1 adenosine receptor agonist regulates cardiac substrate utilization in insulin-resistant rats in vivo. J Pharmacol Exp Ther 328:306–311

Shen J, Halenda SP, Sturek M, Wilden PA (2005) Cell-signaling evidence for adenosine stimulation of coronary smooth muscle proliferation via the A_1 adenosine receptor. Circ Res 97:574–582

Shirley DG, Walter SJ, Noormohamed FH (2002) Natriuretic effect of caffeine: assessment of segmental sodium reabsorption in humans. Clin Sci (Lond) 103:461–466

Shryock JC, Travagli HC, Belardinelli L (1992) Evaluation of N-0861, (+−)-N6-endonorbornan-2-yl-9-methyladenine, as an A_1 subtype-selective adenosine receptor antagonist in the guinea pig isolated heart. J Pharmacol Exp Ther 260:1292–1299

Snowdy S, Liang HX, Blackburn B, Lum R, Nelson M, Wang L, Pfister J, Sharma BP, Wolff A, Belardinelli L (1999) A comparison of an A_1 adenosine receptor agonist (CVT-510) with diltiazem for slowing of AV nodal conduction in guinea-pig. Br J Pharmacol 126:137–146

Sodhi P, Hartwick AT (2014) Adenosine modulates light responses of rat retinal ganglion cell photoreceptors througha cAMP-mediated pathway. J Physiol 592:4201–4220

Spicuzza L, Di Maria G, Polosa R (2006) Adenosine in the airways: implications and applications. Eur J Pharmacol 533:77–88

Srinivas M, Shryock JC, Dennis DM, Baker SP, Belardinelli L (1997) Differential A_1 adenosine receptor reserve for two actions of adenosine on guinea pig atrial myocytes. Mol Pharmacol 52:683–691

Staehr PM, Dhalla AK, Zack J, Wang X, Ho YL, Bingham J, Belardinelli L (2013) Reduction of free fatty acids, safety, and pharmacokinetics of oral GS-9667, an A_1 adenosine receptor partial agonist. J Clin Pharmacol 53:385–392

Stone TW, Ceruti S, Abbracchio MP (2009) Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. Handb Exp Pharmacol 193:535–587

Sun X, Barnes S, Baldridge WH (2002) Adenosine inhibits calcium channel currents via A_1 receptors on salamander retinal ganglion cells in a mini-slice preparation. J Neurochem 81:550–556

Sun CX, Young HW, Molina JG, Volmer JB, Schnermann J, Blackburn MR (2005) A protective role for the A_1 adenosine receptor in adenosine-dependent pulmonary injury. J Clin Invest 115:35–43

Sundarama U, Hassanainb H, Suntresc Z, Yud JG, Cookee HJ, Guzmand J, Christofid FL (2003) Rabbit chronic ileitis leads to up-regulation of adenosine A_1/A_3 gene products, oxidative stress, and immune modulation. Biochem Pharmacol 65:1529–1538

Svenningsson P, Hall H, Sedvall G, Fredholm BB (1997) Distribution of adenosine receptors in the postmortem human brain: an extended autoradiographic study. Synapse 27:322–335

Świąder MJ, Kotowski J, Łuszczki JJ (2014) Modulation of adenosinergic system and its application for the treatment of epilepsy. Pharmacol Rep 66:335–342

Szkudelski T, Szkudelska K (2015) Regulatory role of adenosine in insulin secretion from pancreatic β-cells--action via adenosine A_1 receptor and beyond. J Physiol Biochem 71:133–140

Tang Z, Diamond MA, Chen JM, Holly TA, Bonow RO, Dasgupta A, Hyslop T, Purzycki A, Wagner J, McNamara DM, Kukulski T, Wos S, Velazquez EJ, Ardlie K, Feldman AM (2007) Polymorphisms in adenosine receptor genes are associated with infarct

size in patients with ischemic cardiomyopathy. Clin Pharmacol Ther 82:435–440

Tanner GA, Tanner JA (2001) Chronic caffeine consumption exacerbates hypertension in rats with polycystic kidney disease. Am J Kidney Dis 38:1089–1095

Tateyama M, Kubo Y (2016) Stabilizing effects of G protein on the active conformation of adenosine A_1 receptor differ depending on G protein type. Eur J Pharmacol 788:122–131

Tendera M, Gaszewska-Żurek E, Parma Z, Ponikowski P, Jankowska E, Kawecka-Jaszcz K, Czarnecka D, Krzemińska-Pakuła M, Bednarkiewicz Z, Sosnowski M, Ochan Kilama M, Agrawal R (2012) The new oral adenosine A_1 receptor agonist capadenoson in male patients with stable angina. Clin Res Cardiol 101:585–591

Thompson GW, Horackova M, Armour JA (2002) Role of P1 purinergic receptors in myocardial ischemia sensory transduction. Cardiovasc Res 53:888–901

Tofovic SP, Kost CK Jr, Jackson EK, Bastacky SI (2002) Long-term caffeine consumption exacerbates renal failure in obese, diabetic, ZSF1 (fa-fa(cp)) rats. Kidney Int 61:1433–1444

Tofovic SP, Salah EM, Jackson EK, Melhem M (2007) Early renal injury induced by caffeine consumption in obese, diabetic ZSF1 rats. Ren Fail 29:891–902

Tomé AR, Feng D, Freitas RM (2010) The effects of alpha-tocopherol on hippocampal oxidative stress prior to in pilocarpine-induced seizures. Neurochem Res 35:580–587

Tranberg CE, Zickgraf A, Giunta BN, Luetjens H, Figler H, Murphree LJ, Falke R, Fleischer H, Linden J, Scammells PJ, Olsson RA (2002) 2-amino-3-aroyl-4,5-alkylthiophenes: agonist allosteric enhancers at human A_1 adenosine receptors. Med Chem 45:382–389

Tupone D, Cetas JS, Morrison SF (2016) Hibernation, hypothermia and a possible therapeutic "shifted homeostasis" induced by central activation of A_1 adenosine receptor (A_1AR). Jpn J Psychopharmacol 36:51–54

Vallon V, Osswald H (2009) Adenosine receptors and the kidney. Handb Exp Pharmacol 193:443–470

Vallon V, Miracle C, Thomson S (2008) Adenosine and kidney function: potential implications in patients with heart failure. Eur J Heart Fail 10:176–187

van Dam RM, Hu FB (2005) Coffee consumption and risk of type 2 diabetes: a systematic review. JAMA 294:97–104

van der Klein PA, Kourounakis AP, IJzerman AP (1999) Allosteric modulation of the adenosine A1 receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. J Med Chem 42:3629–3635

van Galen PJ, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, IJzerman AP, Stiles GL, Jacobson KA (1994) A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. Mol Pharmacol 45:1101–1111

van Tilburg EW, van der Klein PA, de Groote M, Beukers MW, IJzerman AP (2001) Substituted 4-phenyl-2-(phenylcarboxamido)-1,3-thiazole derivatives as antagonists for the adenosine A1 receptor. Bioorg Med Chem Lett 11:2017–2019

van Veldhoven JP, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Struensee-Link R, Beukers MW, Brussee J, IJzerman AP (2008) A new generation of adenosine receptor antagonists: from di- to trisubstituted aminopyrimidines. Bioorg Med Chem 16:2741–2752

Vincenzi F, Targa M, Romagnoli R, Merighi S, Gessi S, Baraldi PG, Borea PA, Varani K (2014) TRR469, a potent A_1 adenosine receptor allosteric modulator, exhibits anti-nociceptive properties in acute and neuropathic pain models in mice. Neuropharmacology 81:6–14

Vincenzi F, Ravani A, Pasquini S, Merighi S, Gessi S, Romagnoli R, Baraldi PG, Borea PA, Varani K (2016) Positive allosteric modulation of A_1 adenosine receptors as a novel and promising therapeutic strategy for anxiety. Neuropharmacology 111:283–292

Vizi E, Huszár E, Csoma Z, Böszörményi-Nagy G, Barát E, Horváth I, Herjavecz I, Kollai M (2002) Plasma adenosine concentration increases during exercise: a possible contributing factor in exercise-induced bronchoconstriction in asthma. J Allergy Clin Immunol 109:446–448

Von Lubitz DK, Lin RC, Melman N, Ji XD, Carter MF, Jacobson KA (1994) Chronic administration of selective adenosine A1 receptor agonist or antagonist in cerebral ischemia. Eur J Pharmacol 256:161–167

Von Lubitz DK, Lin RC, Jacobson KA (1995) Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A_{2A} receptor agonist and antagonist. Eur J Pharmacol 287:295–302

Vyas FS, Hargreaves AJ, Bonner PL, Boocock DJ, Coveney C, Dickenson JM (2016) A_1 adenosine receptor-induced phosphorylation and modulation of transglutaminase 2 activity in H9c2 cells: a role in cell survival. Biochem Pharmacol 107:41–58

Wan WJ, Cui DM, Yang X, Hu JM, Li CX, Hu SL, Trier K, Zeng JW (2011) Expression of adenosine receptors in human retinal pigment epithelium cells in vitro. Chin Med J 124:1139–1144

Wardas J (2002) Neuroprotective role of adenosine in the CNS. Pol J Pharmacol 54:313–326

Weaver DR, Reppert SM (1992) Adenosine receptor gene expression in rat kidney. Am J Physio 263:F991–F995

Welch WJ (2015) Adenosine, type 1 receptors: role in proximal tubule Na+ reabsorption. Acta Physiol (Oxf) 213:242–248

Wider KJ, Dittrich H, Thomson S, Blantz R (2004) Method of improved diuresis in individuals with impaired renal function WO patent 2004096228, 11 nov 2004

Wilson CN (2006) Methods and pharmaceutical compositions for treating sepsis. US patent 20060276378, 27 apr 2006

Wilson CN, Batra VK (2002) Lipopolysaccharide binds to and activates A_1 adenosine receptors on human pulmonary artery endothelial cells. J Endotoxin Res 8:263–271

Wilson CN, Partridge JJ (2007) Compositions and methods for treating respiratory disorders. US patent 20070219223, 27 apr 2007

Wood JD (2004) Enteric neuroimmunophysiology and pathophysiology. Gastroenterology 127:635–657

Wu WP, Hao JX, Halldner L, Lövdahl C, DeLander GE, Wiesenfeld-Hallin Z, Fredholm BB, Xu XJ (2005) Increased nociceptive response in mice lacking the adenosine A_1 receptor. Pain 113:395–404

Xi L, Das A, Zhao ZQ, Merino VF, Bader M, Kukreja RC (2008) Loss of myocardial ischemic postconditioning in adenosine A_1 and bradykinin B_2 receptors gene knockout mice. Circulation 118:S32–S37

Yamaguchi D, Terayama R, Omura S, Tsuchiya H, Sato T, Ichikawa H, Sugimoto T (2014) Effect of adenosine A_1 receptor agonist on the enhanced excitability of spinal dorsal horn neurons after peripheral nerve injury. Int J Neurosci 124:213–222

Yang GK, Fredholm BB, Kieffer TJ, Kwok YN (2012) Improved blood glucose disposal and altered insulin secretion patterns in adenosine A_1 receptor knockout mice. Am J Physiol Endocrinol Metab 303:E180–E190

Yang T, Gao X, Sandberg M, Zollbrecht C, Zhang XM, Hezel M, Liu M, Peleli M, Lai EY, Harris RA, Persson AE, Fredholm BB, Jansson L, Carlström M (2015) Abrogation of adenosine A_1 receptor signalling improves metabolic regulation in mice by modulating oxidative stress and inflammatory responses. Diabetologia 58:1610–1620

Yavuz T, Bertolet B, Bebooul Y, Tunerir B, Aslan R, Ocal A, Ybribim E, Kutsal A (2004) Role of endogenous adenosine in atrial fibrillation after coronary artery bypass graft. Clin Cardiol 27:343–346

Yip L, Fathman CG (2014) Type 1 diabetes in mice and men: gene expression profiling to investigate disease pathogenesis. Immunol Res 58:340–350

Yip L, Taylor C, Whiting CC, Fathman CG (2013) Diminished adenosine A_1 receptor expression in pancreatic α-cells may contribute to the pathology of type 1 diabetes. Diabetes 62:4208–4219

Yoshioka K, Hosoda R, Kuroda Y, Nakata H (2002) Hetero-oligomerization of adenosine A_1 receptors with P2Y1 receptors in rat brains. FEBS Lett 531:299–303

Zahler S, Becker BF, Raschke P, Gerlach E (1994) Stimulation of endothelial adenosine A_1 receptors enhances adhesion of neutrophils in the intact guinea pig coronary system. Cardiovasc Res 28:1366–1372

Zeron MM, Hansson O, Chen N, Wellington CL, Leavitt BR, Brundin P, Hayden MR, Raymond LA (2002) Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33:849–860

Zhang J, Belardinelli L, Jacobson KA, Otero DH, Baker SP (1997) Persistent activation by and receptor reserve for an irreversible A_1-adenosine receptor agonist in DDT1 MF-2 cells and in guinea pig heart. Mol Pharmacol 52:491–498

Zhang M, Budak MT, Lu W, Khurana TS, Zhang X, Laties AM, Mitchell CH (2006) Identification of the A_3 adenosine receptor in rat retinal ganglion cells. Mol Vis 12:937–944

Zhang S, Li H, Li B, Zhong D, Gu X, Tang L, Wang Y, Wang C, Zhou R, Li Y, He Y, Chen M, Huo Y, Liu XL, Chen JF (2015) Adenosine A_1 receptors selectively modulate oxygen-induced retinopathy at the Hyperoxic and hypoxic phases by distinct cellular mechanisms. Invest Ophthalmol Vis Sci 56:8108–8119

Zhong Y, Yang Z, Huang WC, Luo X (2013) Adenosine, adenosine receptors and glaucoma: an updated overview. Biochim Biophys Acta 1830:2882–2890

Zizzo MG, Mulè F, Serio R (2006) Inhibitory responses to exogenous adenosine in murine proximal and distal colon. Br J Pharmacol 148:956–963

Zuccato C, Cattaneo E (2014) Huntington's disease. Handb Exp Pharmacol 220:357–409

Adv Exp Med Biol - Protein Reviews (2017) 19:233–253
DOI 10.1007/5584_2017_110

Published online: 25 October 2017

Molecular Mechanism of Plant Recognition of Extracellular ATP

Sung-Hwan Cho, Cuong The Nguyen, Jeongmin Choi, and Gary Stacey

Abstract

Adenosine 5′-triphosphate (ATP), a ubiquitously dispersed biomolecule, is not only a major source of biochemical energy for living cells, but also acts as a critical signaling molecule through inter-cellular communication. Recent studies have clearly shown that extracellular ATP is involved in various physiological processes in plants, including root growth, stomata movement, pollen tube development, gravitropism, and abiotic/biotic stress responses. The first plant purinergic receptor for extracellular ATP, DORN1 (the founding member of the P2K family of purinergic receptors), was identified in *Arabidopsis thaliana* by a forward genetic screen. DORN1 consists of an extracellular lectin domain, transmembrane domain, and serine/threonine kinase, intracellular domain. The predicted structure of the DORN1 extracellular domain revealed putative key ATP binding residues but an apparent lack of sugar binding. In this chapter, we summarize recent studies on the molecular mechanism of plant recognition of extracellular ATP with specific reference to the role of DORN1.

Keywords

Purinergic receptor • DORN1 • P2K1 • Extracellular ATP • Lectin receptor kinase

S.-H. Cho, C.T. Nguyen, and G. Stacey (✉)
Divisions of Plant Science and Biochemistry, Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA
e-mail: chosunghw@missouri.edu; ctn5y7@mail.missouri.edu; staceyg@missouri.edu

J. Choi
Divisions of Plant Science and Biochemistry, Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA

Department of Plant Sciences, University of Cambridge, Downing street, Cambridge CB2 3EA, UK
e-mail: jc913@cam.ac.uk

Abbreviations

ACS6	1-aminocyclopropane-1-carboxylic acid synthase 6
ADP	Adenosine 5′-diphosphate
ADPβS	Adenosine 5′-[β-thio]diphosphate
AMP	Adenosine 5′-monophosphate
AMP-PCP	Adenosine 5′-[beta,gamma]-methylenetriphosphate
ATP	Adenosine 5′-triphosphate
ATPγS	Adenosine 5′-[γ-thio]triphosphate
BEA	Beauvericin
Bz-ATP	Benzoylbenzoyl-adenosine 5′-triphosphate
2me-ATP	2-Methylthio-adenosine 5′-triphosphate
CBD	cellulose binding domain
CTP	Cytidine 5′-triphosphate
CW-PM	Cell wall-plasma membrane
DAMP	Damage-associated molecular pattern
DORN1	Does not respond to nucleotide 1
ECM	Extracellular matrix
EGTA	Ethylene-bis(oxyethylenenitrilo) tetraacetic acid
EMS	Ethyl methanesulfonate
ERFs	Ethylene response factors
GTP	Guanosine 5′-triphosphate
IPI-O	In planta induced-O
ITP	Inosine 5′-triphosphate
LecRK	Lectin receptor kinase
LOX2	Lipoxygenase 2
MAPK	mitogen-activated protein kinase
MEKK1	Mitogen-activated protein kinase kinase kinase 1
NADPH	nicotinamide adenine dinucleotide phosphate
NO	Nitrogen oxide
P2K	kinase family of purinergic receptors
PA	phosphatidic acid
PAL1	Phe-ammonia lyase 1
PK19	serine/threonine protein kinase 19
PPADS	pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
RBOHC	Respiratory burst oxidase homolog protein C
RBOHD	Respiratory burst oxidase homolog protein D
RBOHF	Respiratory burst oxidase homolog protein F
RD	Arginine-aspartate
RGD	Arginine-gylcine-aspartic acid
RGE	Arginine-gylcine-glutamic acid
RLKs	Receptor-like kinases
ROS	Reactive oxygen species
TTP	Thymidine 5′-triphosphat
UDP	Uridine 5′-diphosphate

1 Introduction

Adenosine 5′-Triphosphate (ATP) is the universal, cellular energy source (Knowles 1980). In addition, ATP can also be released into the extracellular matrix during growth by wounding or stimulation where it acts as a signaling molecule to affect a variety of cellular processes (Clark and Roux 2011; Ralevic and Burnstock 1998; Lustig et al. 1993; Burnstock 1972). Purinergic signaling (via ATP) has been extensively studied in animals. Indeed, most mammals contain multiple purinergic receptors derived from two protein families; that is, P2X, ligand-gated ion channels, and P2Y, G-protein-coupled receptors (Khakh and Burnstock 2009). There is a multi-billion dollar, pharmaceutical industry that targets various aspects of purinergic signaling in humans.

In contrast, studies of purinergic signaling in plants are still in their infancy and the full impact of these pathways on plant growth and development remains to be determined. Perhaps the first studies to show an effect of ATP on plants was the demonstration that exogenous ATP treatment promoted closure of Venus flytrap trap leaves (Jaffe 1973). Thereafter, studies showed that plant gene expression could be significantly affected by the addition of ATP (Choi et al. 2014a; Roux and Steinebrunner 2007).

As is the case in mammals, extracellular ATP acts in plants by triggering the production of

secondary messenger molecules [e.g., elevation of cytosolic Ca^{2+}, nitrogen oxide (NO), reactive oxygen species (ROS)]. These effects have been coupled to changes in plant cell growth (Chivasa et al. 2005), root hair growth (Kim et al. 2006), root growth (Yang et al. 2015), pollen tube growth (Wu et al. 2007; Bernard et al. 2011), abiotic/biotic stress responses (Chivasa et al. 2005, 2009; Dark et al. 2011; Sun et al. 2012a; Weerasinghe et al. 2009), and gravitropism (Tang et al. 2003). Since ATP, as a strongly charged molecule, is thought incapable of freely diffusing across the plant plasma membrane, the results of these studies, as well as the precedence of the animal research, indicated that plants must possess a membrane-associated receptor for extracellular ATP (Tanaka et al. 2010a; Bodin and Burnstock 2001a). However, plant genome database searches based on sequence similarity to the animal P2X or P2Y receptors failed to identify any possible orthologs.

In 2014, the first plant receptor for extracellular ATP, Does Not Respond to Nucleotide 1 (DORN1), was identified in *Arabidopsis* by a forward genetic screen approach (Choi et al. 2014b). The aim of this chapter is to review recent progress on the general responses of plants to extracellular ATP with specific reference to the role of DORN1.

2 Physiological Function of Extracellular ATP

As was the case in animals, early studies on the role of extracellular ATP in plants focused on studying the effects of exogenous addition of ATP (Table 1). These studies were conducted with the knowledge that ATP could be hydrolyzed after application, perhaps giving rise to other biologically active compounds (e.g., adenosine). Hence, some studies made use of poorly hydrolyzable ATP analogs (e.g. ATPγS, Bz-ATP, and 2me-ATP) in order to ascertain if the intact ATP molecule was responsible for whatever response was seen. The mammalian P2X and P2Y receptors show some specificity with regard to which nucleotide is recognized (Abbracchio and Ceruti 2006; Puchalowicz et al. 2014), in addition to ATP. Therefore, plant studies also need to take into account the chemical specificity of the response through testing other nucleotides, including ADP, AMP, GTP, CTP, and TTP.

Studies in mammals demonstrated that ATP can be released from cells either by wounding, direct transport or vesicular fusion with the plasma membrane (Bodin and Burnstock 2001a, b; Sawada et al. 2008; Lazarowski et al. 2003). There is some evidence to suggest that all three of these pathways may also function in plants (Choi et al. 2014a; Roux and Steinebrunner 2007; Kim et al. 2006; Tanaka et al. 2010a; Thomas et al. 2000; Cao et al. 2014) (Fig. 1).

Extracellular ATP affects hypocotyl growth. Roux et al. (2006) showed that exogenous ATP (0.1–0.2 mM) increased hypocotyl length by 10–20%. However, higher concentrations of extracellular ATP reduced hypocotyl length by 10–55%. Therefore, as might be expected for a potent, extracellular signal, plant cells need mechanisms to maintain the appropriate homeostasis. This occurs through hydrolysis of ATP mediated by specific apoplastic nucleotidases and apyrases (ATP-diphosphatase) (Riewe et al. 2008). Plants constantly monitor extracellular ATP levels and regulate growth rates. Roux and Steinebrunner (2007) proposed a model of optimal concentration of extracellular ATP for growth and development. Subsequently, Choi et al. (2014a) expanded this concept to include the plant response to various stresses. In the model, plants normally maintain extracellular ATP levels by balancing ATP hydrolysis or secretion for plant growth and development. Moderate changes of extracellular ATP levels can trigger stress defense responses. For example, published studies support the idea that both very low and very high extracellular ATP levels can lead to plant cell death (Chivasa et al. 2005). However, in contrast to our knowledge of extracellular ATP homeostasis in animals, our understanding of the molecular mechanisms that maintain optimal extracellular ATP levels in plants are still rudimentary.

Table 1 Summary of publications demonstrating the effects of extracellular ATP on plant growth, development, and stress responses

Organism	Effect	Stimulus and concentrations	References
Arabidopsis	Promote plant growth	ATP (300 μM), apyrase (unknown)	Thomas et al. (1999)
	Promote root hair growth	ATP (400 μM), ADP (10 μM)	Lew and Dearnaley (2000)
	Inhibit root gravitropism and polar auxin transport	Inhibit gravitropism (5 mM ATP) and root curling (2 mM ATP), Stimulate lateral root growth (3 mM ATP)	Tang et al. (2003), Liu et al. (2012)
	Induce calcium influx	ATP (500 μM)	Jeter et al. (2004), Demidchik et al. (2003)
	Inhibit pollen germination	ATP (2–4 mM), ADP (2–4 mM)	Wu et al. (2007, Steinebrunner et al.(2003)
	Decrease cell viability of cell cultures	AMP-PCP (0.5, 1, 1.5 mM), glucose (100 mM)-hexokinase (20, 50, 100, 200 unit/ml), apyrase (50, 100 units/ml)	Chivasa et al. (2005)
	Activate cell death in leaves	AMP-PCP (5 mM), glucose (50 mM)-hexokinase (1.85 units/ml), apyrase (0.5 units/ml)	
	Activate cell death in root/shoot	Glucose (50 mM)-hexokinase (1.85 units/ml)	
	Trigger cell death in seedling plants	AMP-PCP (1 mM)	
	Induce formation of ROS	ATP (0.5–100μM)	Song et al. (2006)
	Hypocotyl growth	Promotion: ATPγS (100–200 μM)	Roux et al. (2006)
		Inhibition: ATPγS (0.4–1 mM)	
	Regulate hypocotyl growth	ATPγS (0.2, 0.3, 1 mM)	Tonon et al. (2010)
	Regulate Root hair growth	ATP (1 mM)	Terrile et al. (2010)
	Regulate stomatal opening and closing	Opening: ATPγS (5–15 μM)	Clark et al. (2011)
		Closing: ATPγS (150–250 μM)	
	Promote stomata opening	ATP (0.1, 1, 2 mM), ATPγS (0.3 mM), 2meATP (0.3, 1 mM), Bz-ATP (0.3 mM)	Hao et al. (2012)
	Modulation of root skewing	ATP (2, 3, 4 mM), ATPγS (100 μM), ADP (4 mM)	Yang et al. (2015)
Avena sativa	Stimulate formation of nucleases and chlorophyll breakdown	ATP (0.5–2 mM)	Udvardy and Farkas (1973)
Chara corallina	Activate cytoplasmic streaming	ATP (1 mM)	Williamson (1975)
Commelina communis	Increase stomatal guard cell aperture	ATP (5–20 mM)	Nejidat et al. (1983)
Cotton	Fiber elongation	Promotion: ATPγS (30 μM), ADPβS (30 μM)	Clark et al. (2010a)
		Inhibition: ATPγS (150 μM), ADPβS (150 μM)	
Dionaea muscipula	Stimulate Venus fly trap closure	ATP (100 μM)	Jaffe (1973)
Medicago truncatula	Root hair growth	ATP (1 mM), potato apyrase (25 units/ml)	Kim et al. (2006)
Lilium longiflorum	Increase of mitotic activity in pollen tubes	ATP (0.18–1.8 mM)	Kamizyo and Tanaka (1982)

(continued)

Table 1 (continued)

Organism	Effect	Stimulus and concentrations	References
Nicotiana tabacum	Induce cell death in leaves	AMP-PCP (0.4–0.8, 1, 2 mM), glucose (50, 100 mM)-hexokinase (0.1, 0.5 unit/ml), apyrase (0.5 units/ml)	Chivasa et al. (2005), Chivasa et al. (2009), Chivasa et al. (2010)
Phaseolus vulgaris	Induce cell death in leaves	Glucose (50 mM)-heokinase (1.85 units/ml), apyrase (0.5 units/ml)	Chivasa et al. (2005)
Picea meyeri	Inhibit pollen germination and tube elongation	ATP (0.2–5 mM), ADP (5 mM), AMP (5 mM), apyrase (0–60 unit/ml)	Zhou et al. (2015)
Populus euphratica	Induce cell death in cell cultures	ATP (0.5, 1, 2 mM)	Sun et al. (2012a)
Solanum tuberosum	Change in tuber growth	ATP (1 mM), Apyrase	Riewe et al. (2008)
Vicia faba	Promote stomata opening	ATP (0.3–1 mM), ATPγS (0.3 mM), 2meATP (0.3 mM), Bz-ATP (0.3 mM)	Wang et al. (2014)
Zea mays	Decrease cell viability of cell cultures	AMP-PCP (1 mM), glucose (50 mM)-heokinase (200 units/ml), apyrase (100 units/ml)	Chivasa et al. (2005)

The applied agents used include extracellular ATP, AMP-PCP (specific competitive inhibitor of extracellular ATP), glucose-hexokinase (catalyses a reaction that consumes ATP), and apyrase (hydrolase that breaks down ATP to AMP via ADP)

Disruption of normal plant homeostasis of extracellular ATP can be detrimental to plant growth. For example, Riewe et al. (2008) reported that an apoplastic potato apyrase played a crucial role in regulating growth and development. The authors showed that suppression of potato apyrase expression led to phenotypic changes, such as increased tuber number and reduced tuber size. Clark et al. (2010a) also demonstrated that inhibition of apyrase activity blocked fiber growth in cotton. Down regulation of the expression of specific, apoplastic apyrases in both soybean and *Lotus japonicus* resulted in a reduction in nodulation; a key developmental step in symbiotic nitrogen fixation (Govindarajulu et al. 2009; Roberts et al. 2013). Subsequently, Tanaka et al. (2011) reported that the soybean apyrase GS52 targeted in these earlier studies plays a critical role in maintaining an optimal concentration of extracellular nucleotides for rhizobial infection.

2.1 Growth and Development

Effects of extracellular ATP addition on plant growth and development are well supported by a variety of studies. For example, in *Arabidopsis*, an early study reported that exogenous ATP could be hydrolyzed by apyrase, resulting in the release of phosphate that could serve as a nutrient source to enhance plant growth (Thomas et al. 1999).

Several studies found that plant roots respond to extracellular ATP. Exogenous application of ATP and ADP can promote root hair growth and depolarize the plasma membrane potential of growing root hairs (Lew and Dearnaley 2000). Tang et al. (2003) found that exogenous treatment of ATP (2–3 mM) induced root curling and stimulated lateral root growth. In addition, application of a higher concentration of exogenous ATP (5 mM) was found to inhibit gravitropic growth of seedling roots. These effects may be the result of the ability of extracellular ATP to disturb normal auxin distribution in root cells by blocking auxin transport (Liu et al. 2012). More recently, the same group reported that exogenous application of ATP or ATPγS increased primary root skewing and induced curling in *apyrase1* mutant plants (Yang et al. 2015). Blocking the expression of apyrase, which could increase the concentration of extracellular ATP, also inhibits auxin transport in *Arabidopsis* roots (Liu et al.

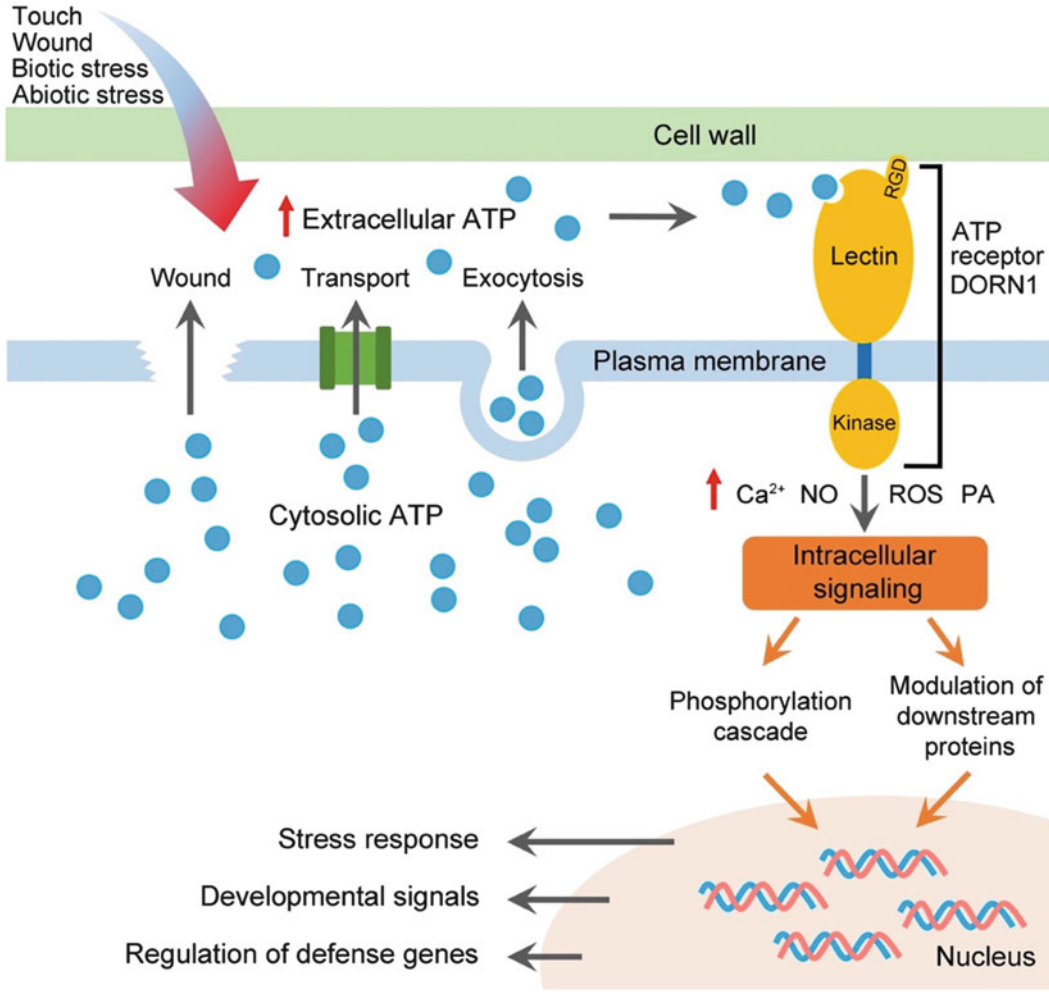

Fig. 1 Overview of ATP perception by DORN1 in plants Wounding, touch and various biotic and abiotic stresses induce ATP release into the extracellular matrix from the cytosol (Kim et al. 2006; Choi et al. 2014b; Thomas et al. 2000). *Arabidopsis* ATP receptor, DORN1, can recognize extracellular ATP via its extracellular lectin domain. The intracellular DORN1 kinase domain is activated and induces in cytoplasmic calcium concentration, generation of ROS, nitric oxide (NO), and phosphatidic acid (PA). This leads subsequently to downstream protein phosphorylation and induction of specific gene expression. The genes induced include those involved in developmental responses, stress responses and pathogen defense. In addition, DORN1 maintains cell integrity through mediating adhesion between RGD-motif-containing cell wall proteins and the plasma membrane. Figure redrafted from (Choi et al. 2014a; Roux and Steinebrunner 2007; Cao et al. 2014; Tanaka et al. 2014)

2012). Terrile et al. (2010) demonstrated that etiolated *Arabidopsis* seedlings showed inhibition of root hair growth in the presence of reducing agent, such as glutathione. This inhibition of root hair growth was restored by extracellular ATP and NO, suggesting a role for ATP and NO in the regulation of redox balance associated with plant morphogenesis.

As stated above, the exogenous addition of ATP is a common method to look for effects of purinergic signaling in plants. However, this evidence does not show unequivocally that plants possess extracellular ATP. Therefore, Kim et al.

(2006) undertook a study to demonstrate directly the presence of extracellular ATP on the surface of *Medicago truncatula* roots. In this case, the ATP-requiring luciferase protein was fused to a cellulose binding domain (CBD) that anchored the luciferase to the extracellular surface of the plant cell wall. Luminescence was apparent on the roots at the interface of adjacent epidermal cells and on the surface of the root hairs indicating sites of ATP release. The addition of exogenous apyrase eliminated light production while treatment with an elicitor (i.e., chitin) strongly increased light production, presumably by stimulating ATP release. Subsequently, this same CBD-luciferase reporter was use to show significant ATP release upon touching of the root surface, implicating extracellular ATP in the root avoidance response (Weerasinghe et al. 2009).

Extracellular ATP is also involved in pollen tube growth. In *Lilium longiflorum* pollen, addition of ATP (2.7 mM) inhibited pollen tube growth. However, the presence of exogenous ATP increased mitotic activity in pollen tubes (Kamizyo and Tanaka 1982). Steinebrunner et al. (2003) showed that addition of ATP inhibited both pollen germination and tube elongation in *Arabidopsis*. Subsequently, Reichler et al. (2009) reported that suppression of pollen germination and pollen tube elongation by extracellular ATP was mediated via NO signaling. Until recently, most studies of extracellular ATP in plants utilized angiosperms. Interestingly, a recent study examined the effect of extracellular ATP on gymnosperm pollen grains. Generally, pollen germination and growth in gymnosperms is relatively slow (Chen et al. 2008). This could be due to the fact that the pollen grains of the gymnosperm, *Picea meyeri*, were shown to release ATP to the extracellular matrix before germination and during tube elongation (Zhou et al. 2015). Similar to the studies in *Arabidopsis*, exogenous addition of ATP was shown to inhibit germination and pollen tube elongation. However, unlike angiosperms, addition of exogenous AMP also significantly inhibited pollen elongation. Therefore, further study might reveal some interesting differences in purinergic signaling among various plants species; such as, comparing angiosperms and gymnosperms.

Extracellular ATP is also a key signal involved in the control of stomatal aperture. These early studies interpreted the effects of extracellular ATP on stomatal opening to be due to an increase in cellular energy supply, such as in *Commelina* (Nejidat et al. 1983; Raghavendra 1981). However, more recent studies have viewed these effects as due to purinergic signaling. For example, in *Arabidopsis* and *Vicia faba*, extracellular ATP, as well as other nucleotides (ADP, GTP, ATPγS, Bz-ATP, and 2me-ATP), were shown to promote stomatal opening (Hao et al. 2012; Wang et al. 2014). However, perhaps reflecting issues related to ATP homeostasis, experiments showed that low concentrations of applied ATPγS (5–15 μM) induced stomatal opening, whereas higher concentrations (150–250 μM) induced closure (Clark et al. 2011). The exact mechanism of how purinergic signaling is coupled to the control of stomatal aperture remains to be elucidated.

Plant cell viability is also affected by extracellular ATP. Chivasa et al. (2005) showed that the addition of exogenous ATP protected cells against toxin application, while reduction of extracellular ATP by addition of apyrase or glucose-hexokinase led to cell death in leaves or cell cultures of *Arabidopsis*, *Zea mays*, *Phaseolus vulgaris*, and *Nicotiana tabacum*. Sun et al. (2012a) showed that this cell death response was clearly the result of ATP concentration. Relatively high levels (0.5–2 mM) of extracellular ATP increased cell death in callus cells of *Populus euphratica*.

2.2 Stress Responses

Unlike animals, sessile plants cannot move to avoid or escape stress. Therefore, plants must sense and respond in place to the various biotic or abiotic stresses they face. The key role that extracellular ATP plays in the plant response to stress was previously reviewed by Choi et al. (2014a) and Tanaka et al. (2014). Both abiotic

and biotic stresses trigger ATP release as a damage-associated molecular pattern (DAMP). Perception of extracellular ATP as a DAMP initiates plant defense responses.

Jeter et al. (2004) reported that mechanical stress increased extracellular ATP levels in *Arabidopsis* seedlings. Weerasinghe et al. (2009) also showed that mild touching of *Arabidopsis* roots induced the release of nanomolar levels of extracellular ATP. Mechanical stimulation of plant cells during pathogen infection is associated with nuclear repositioning and cytoplasmic streaming (Hardham et al. 2008). Generally, mechanical stress induces cytoskeletal rearrangements (Hardham et al. 2008). These types of effects, such as cytoplasmic streaming, are also found upon exogenous addition of ATP (Williamson 1975).

Both abiotic and biotic stress agents trigger extracellular ATP release. Jeter et al. (2004) reported that addition of salt induced the release of ATP in *Arabidopsis* seedlings. Dark et al. (2011) also showed that treatment with sorbitol, salt, abscisic acid or L-glutamate caused ATP release in *Arabidopsis* roots. Sun et al. (2012b) proposed a model for extracellular ATP mediated salinity stress signaling in *Populus euphratica* cells. Application of NaCl (200 mM) to suspension cultured cells induced a transient elevation in extracellular ATP and triggered salinity tolerance responses. Depletion of ATP by suramin (an ATP hydrolase) and PPADS (blocker of animal purinergic signaling) caused significant viability reduction in the NaCl treated cells. These data suggest that extracellular ATP contributes to salinity tolerance to maintain cell viability. Deng et al. (2015) reported that low concentrations of ATP treatment promoted cold tolerance, whereas higher concentrations inhibited cold tolerance in *Populus euphratica*. Again, underlying the importance of ATP homeostasis.

Infection by a variety of pathogens, bacteria, fungi or oomycetes, also leads to ATP release associated with the activation of innate immunity pathways. Kim et al. (2006) found addition of chitin, a known activator of pathogen-associated molecular pattern immunity (PTI) led to a rapid and significant release of ATP in *Medicago truncatula* roots. Wu et al. (2008) reported that application of yeast extract to *Salvia miltiorrhiza* roots also induced ATP release. Srobarova et al. (2009) showed that cells treated with beauvericin (BEA), a cyclic hexadepsipeptide antibiotic and mycotoxin, led to secretion of ATP into the apoplast of wheat.

Taken together, these findings suggest that various stresses induce the release of extracellular ATP in plants (Fig. 1), which correlates with the induction of stress responsive pathways in the plant. The data are clear that, similar to the situation in animals, extracellular ATP acts as a central danger signaling molecule to trigger various stress responses in plants (Tanaka et al. 2014).

3 Signal Transduction of Extracellular ATP

In animal cells, two types of purinoceptor (P2X, P2Y) recognize extracellular ATP as well as other nucleotides and activate intracellular, purinergic signaling cascades. These signaling processes include the elevation of cytoplasmic Ca^{2+} and production of ROS and NO (Bours et al. 2006; Shen et al. 2005), which are also responses to ATP that are found in plants. An interesting and still largely unaddressed question is 'to what extent do plant and animal purinergic signaling pathways converge?'

3.1 Perception of Extracellular ATP

Early studies with extracellular ATP antagonists or inhibitors suggested that plant cells have extracellular ATP receptors (Chivasa et al. 2005; Demidchik et al. 2003). However, these studies have to be viewed with some skepticism since they made use of purinergic signaling inhibitors that, although verified in animal studies, have unknown specificity in plants.

As mentioned previously, database searches for plant genes showing significant sequence similarity to animal P2X and P2Y receptors failed to identify any obvious candidates. However, Fountain et al. (2008) reported a P2X-like

receptor in green algae. However, this protein appears to function intracellularly and is not localized on the plasma membrane. The lack of apparent homology of plant proteins to known animal purinergic receptors was explained by the identification of the first plant purinergic receptor, DORN1, which was proposed to represent the founding member of a new, kinase family of purinergic receptors, P2K, in keeping with the animal nomenclature (Choi et al. 2014b). DORN1 (P2K1) was identified in *Arabidopsis* as the result of a forward genetic screen for mutant plants that failed to show an increase in intracellular calcium concentration upon the addition of ATP.

3.2 Second Messengers in Extracellular ATP Signaling

Exogenous application of ATP triggers a transient Ca^{2+} increase or Ca^{2+} oscillation in plant cells (Demidchik et al. 2003; Hanley et al. 2004). Tanaka et al. (2010b) reported that extracellular ATP induces a biophasic increase in intracellular Ca^{2+} in *Arabidopsis* root cells. These effects were seen within 30 seconds of ATP addition (Choi et al. 2014b; Tanaka et al. 2010b). The authors suggested that the first peak of ATP-induced cytosolic Ca^{2+} elevation may due to stimulation of external Ca^{2+} entry, whereas the second peak may result from both external Ca^{2+} entry and release of Ca^{2+} from intracellular stores. The addition of either a Ca^{2+} chelator (EGTA) or Ca^{2+} channel blockers (e.g. nifedipine) inhibited the induction of intracellular Ca^{2+} increases upon exogenous ATP treatment (Demidchik et al. 2003).

The application of exogenous ATP induces accumulation of ROS in various plant tissues (Kim et al. 2006; Wu et al. 2008; Demidchik et al. 2009; Song et al. 2006). The detection of ROS production is within 30–45 min after exogenous ATP treatment (Clark et al. 2010b). The enzyme that produces ROS is NADPH oxidase. In the *Arabidopsis* NADPH oxidase null mutants (*rbohC*, *rbohD*, and *rbohF*), ROS generation by extracellular ATP did not occur (Demidchik et al. 2009; Tonon et al. 2010). Exogenous ATP treatment to the *atrbohC* mutant failed to induce the expression of stress related MAP kinase genes, suggesting that the production ROS by NADPH oxidase is downstream and important for triggering subsequent events in purinergic signaling (Demidchik et al. 2009). In addition, ROS production induced by extracellular ATP is involved in various plant growth, development and stress responses such as root hair growth, defense response, stomatal movement, and programmed cell death (Kim et al. 2006; Sun et al. 2012a; Hao et al. 2012; Tonon et al. 2010).

Extracellular ATP also stimulates production of NO as an important second messenger (Reichler et al. 2009; Tonon et al. 2010; Wu and Wu 2008). The earliest detection of NO production is at 10 min and less than 30–45 min after exogenous ATP application (Reichler et al. 2009; Clark et al. 2010b). NO production stimulated by extracellular ATP was shown in algae, *Salvia miltiorrhiza* hairy roots, tomato suspension cells, and during pollen germination and hypocotyl growth (Reichler et al. 2009; Tonon et al. 2010; Wu and Wu 2008; Torres et al. 2008; Foresi et al. 2007). Wu et al. (2008) found that extracellular ATP induced increased cytosolic Ca^{2+} levels and subsequently stimulated NO production in *Salvai miltiorrhiza* hairy root cells. In *Arabidopsis*, nitrate reductase mutants (*nia1nia2*) have reduced NO production and did not show growth or stomatal aperture responses to applied ATP (Reichler et al. 2009; Clark et al. 2010b). These results suggest that NO formation is associated with extracellular ATP action.

In addition to Ca^{2+}, ROS, and NO, phosphatidic acid has emerged as a second messenger in plants. In animals, phosphatidic acid (PA) has been associated with purinergic signaling (Ralevic and Burnstock 1998). Sueldo et al. (2010) investigated the effect of extracellular ATP on PA production in *Solanum lycopersicum*. Exogenous ATP (0.1 or 1 mM) treatment induced the formation of phosphatidic acid (PA) within minutes via the activation of phospholipase D (PLD) and phospholipase C (PLC)/diacylglycerol kinase (DGK) in suspension-

cultured tomato cells. In addition, the results showed that extracellular ATP-mediated NO production downstream of PLC/DGK activation.

Extracellular ATP-induced Ca^{2+}, ROS, NO, and PA molecules appear to be linked in the plant signaling pathway. Stresses cause ATP release, then extracellular ATP induces NADPH oxidase-dependent ROS production (Demidchik et al. 2009). Both Ca^{2+} and PA are involved in the activation of NADPH oxidase (McPhail et al. 1999). PA can also induce an oxidative burst, suggesting a similar activation of NADPH oxidase (Park et al. 2004). In addition, NO accumulation induced by extracellular ATP required the activation of Ca^{2+} channels in tomato cells and hairy roots (Wu and Wu 2008; Sueldo et al. 2010). The data suggest that an increase in cytosolic Ca^{2+} is among the first responses to ATP addition, subsequently activating the production of PA, ROS, and NO.

3.3 Gene and Protein Expression Induced by Extracellular ATP

Exogenous ATP treatment increases cytosolic Ca^{2+} levels, production of PA, ROS, and NO, and these second messengers activate mitogen-activated protein kinase (MAPK) phosphorylation (Reichler et al. 2009; Jeter et al. 2004; Tanaka et al. 2010b; Demidchik et al. 2009; Song et al. 2006; Clark et al. 2010b; Sueldo et al. 2010). Downstream of these responses, addition of ATP induces the expression of a variety of genes.

Jeter et al. (2004) showed that exogenous ATP increased the expression level of MAPK (*MEKK1*, *PK19*, *MPKs*), ethylene biosynthesis and a variety of other genes involved in signal transduction (*ACS6*, *ERFs*). These findings suggest that extracellular ATP can trigger downstream reactions related to stress responses through ethylene signaling and MAPK cascades.

Song et al. (2006) reported that exogenous ATP treatment increased the expression levels of wound and defense response genes, such as *PAL1* (wound and defense response gene), *LOX2* (jasmonic acid synthesis related gene), and *ACS6* (ethylene biosynthesis related gene).

Chivasa et al. (2010) investigated the molecular and physiological effects of extracellular ATP-mediated signaling in tobacco by 2-DE (2-dimensional protein electrophoresis) coupled with mass spectrometry analysis. They found differentially expressed proteins involved in cellular processes such as photosynthesis, cell wall metabolism, ATP synthesis, pathogen defense, maintenance of cellular redox state, and protection against oxidative stress. These findings suggest that extracellular ATP-mediated signaling on the cell surface regulates important primary metabolic processes, including photosynthesis and intracellular ATP production.

As mentioned above, apoplastic apyrase functions to control extracellular ATP homeostasis in *Arabidopsis* (Wu et al. 2007). Lim et al. (2014) found that *apyrase* mutants showed an increase in the extracellular ATP levels and severely reduced growth. They expanded on these observations by performing a DNA microarray analysis of genes differentially expressed upon addition of exogenous ATP. Reduction of apyrase led to increased expression of genes involved in systemic acquired resistance, defense response to fungal pathogens, MAP kinase cascade, biotic stress responses, but also decreased expression of genes involved in root hair differentiation, iron, nitrate, and metal ion transport, UDP-glycosyltransferase and sterol biosynthetic process.

Choi et al. (2014b) conducted a DNA microarray analysis comparing gene expression of wild-type and *dorn1* mutant plants upon the addition of exogenous ATP. This analysis identified 332 up and 242 down-regulated genes in response to ATP in wild-type plants but none of these genes responded to exogenous ATP in *dorn1* mutant plants (Choi et al. 2014b). ATP-induced genes were classified into categories known to respond to biotic and abiotic stresses, including immune response, protein modification, programmed cell death, photosynthesis, and carbohydrate biosynthesis (Cao et al. 2014). In addition, Choi et al. (2014b) found a 60% overlap between genes responding to ATP

and those reported in the literature to respond to wounding, consistent with the observation that significant levels of ATP are released by wounding. These results support a role for plant purinergic signaling during stress-related response processes.

4 Identification of Plant Extracellular ATP Receptor

As mentioned previously, the first plant purinergic receptor was identified from a forward genetic screen of a *Arabidopsis* EMS (ethyl methanesulfonate) mutagenized population based on the ability of exogenous ATP to stimulate an increase in cytoplasmic calcium levels (Choi et al. 2014b), as measured using aequorin-expressing transgenic seedlings (Knight and Knight 1995). Initial screening of the EMS mutagenized *Arabidopsis* population identified two allelic mutants (*dorn1–1* and *dorn1–2*), which were then used to identify a T-DNA insertion line (*dorn1–3*) (Choi et al. 2014b) (Fig. 2). Additional screening led to the identification of six additional EMS-induced alleles and two additional T-DNA insertion mutations (Choi et al. 2014a) (Fig. 2). The locations of these mutations cover the full length of the *dorn1* gene indicating that both the extracellular lectin domain and intracellular kinase domain are essential for function. Each of these mutants failed to show the ATP-induced cytosolic calcium increase, MAPK activation and ATP-induced gene expression (Choi et al. 2014b). The *Arabidopsis DORN1* gene encodes a lectin receptor kinase (LecRK-I.9) comprising an extracellular legume (L)-type lectin domain, a transmembrane domain and an intracellular serine/threonine kinase domain (Fig. 2). Interestingly, the lectin receptor kinase protein family is found only in plants, based on sequence-based database searches (Lehti-Shiu et al. 2009). In keeping with the animal nomenclature for purinergic ATP receptors, Choi et al. (2014b) proposed the name P2K1 as a founding member of the new family of kinase, purinergic receptors in plants.

4.1 Expression Patterns and Functions of DORN1

DORN1 (*P2K1*) was initially identified as LecRK I.9 based on its interaction with the *Phytophthora insfestans* (oomycete pathogen) effector protein

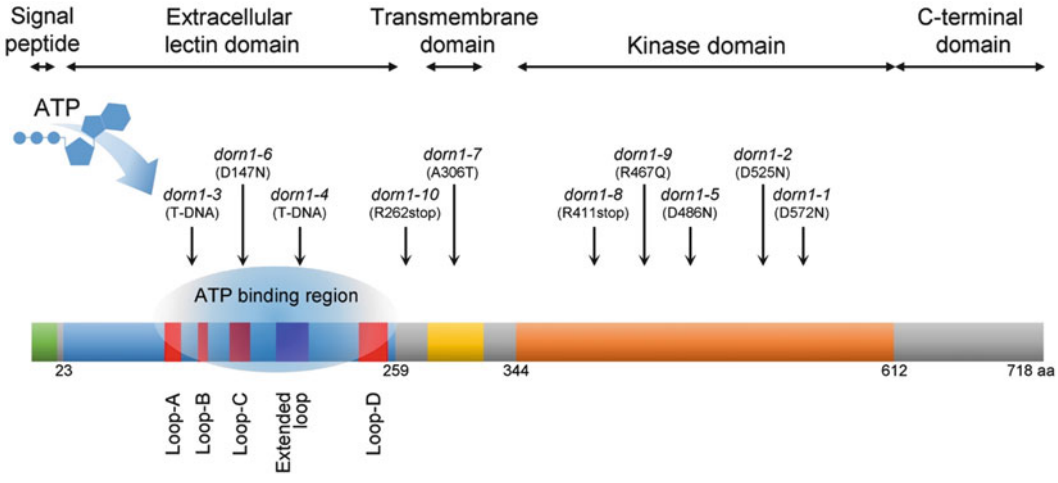

Fig. 2 Schematic of the DORN1 protein showing the specific location of the loss-of-function mutant alleles in the *DORN1* gene
The DORN1 sequence is composed of extracellular lectin domain, transmembrane domain and kinase domain. The extracellular lectin domain binds ATP at high affinity. The diagram shows eight EMS mutant alleles and two T-DNA insertion lines

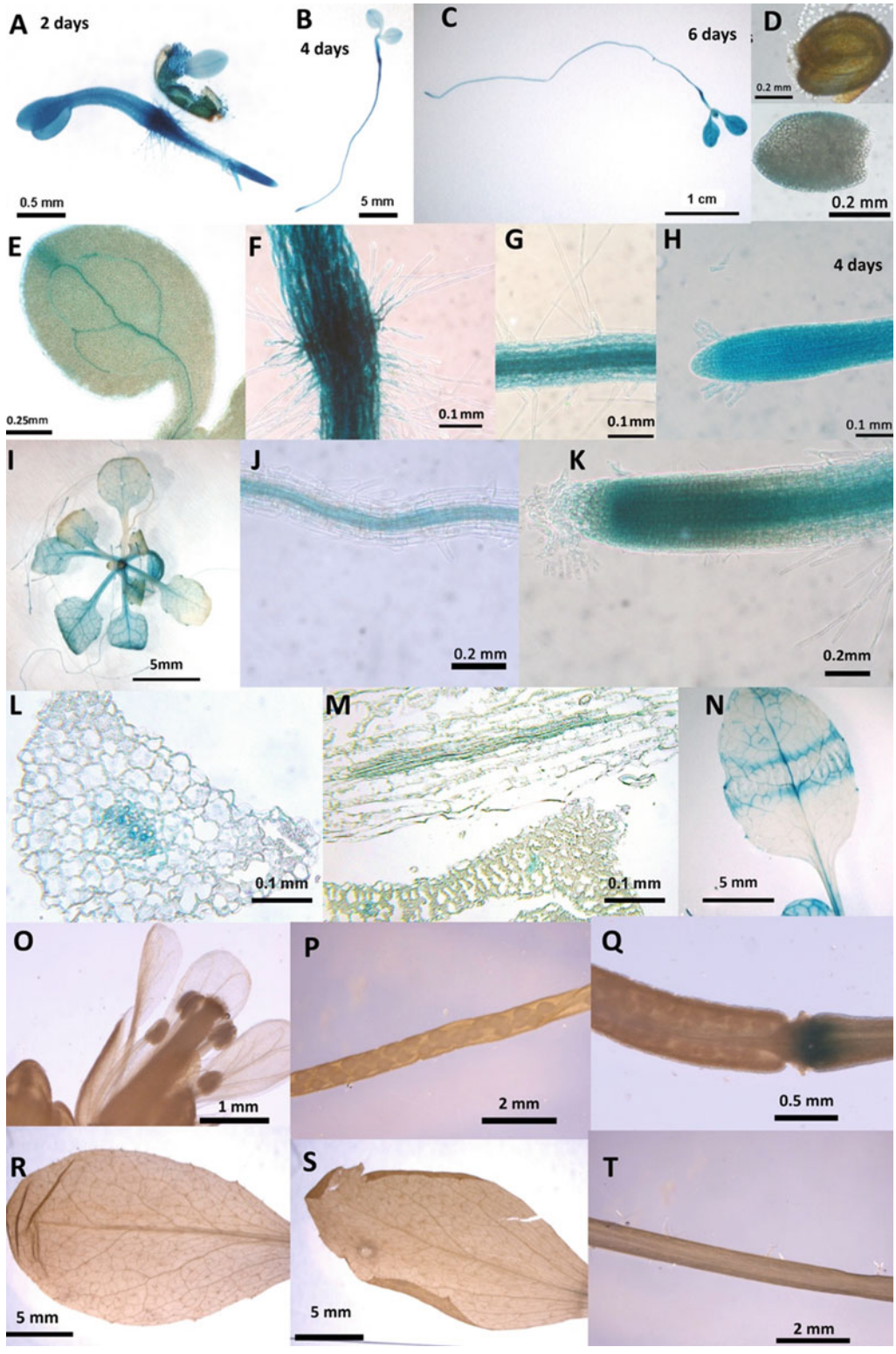

Fig. 3 Expression patterns of the *DORN1* gene at various developmental stages
The remaining pictures (**a**–**t**) correspond to *ProDORN1::GUS* transgenic plants. (**a**) 2 day-old seedling, (**b**) 4 day-old seedling, (**c**) 6 day-old seedling, (**d**) imbibed seeds, (**e**–**h**) 4 day-old seedlings in cotyledon (**e**), hypocotyl/root junction (**f**), root (**g**) and root tip (**h**). (**i**–**k**) 22 day-old plant (**i**) and root (**j**) and root tip (**k**). (**l**–**m**) 16 day-old plant leaf sections, (**n**) 29 day-old plant leaf wounded with hemostatic forceps, (**o**–**t**) 40 days-old mature plant in flower (**o**), silique (**p**), abscission zone in floral organ (**q**), rosette leaves (**r**, **s**), and stem (**t**)

IPI-O (*in planta* induced-O) via the cell attachment RGD (arginine-glycine-aspartic acid) motif (Gouget et al. 2006). The maintenance of attachment between the cell wall (CW) and plasma membrane (PM) is a critical factor that governs the plant response to a variety of stimuli, including those involved in pathogen defense (Bouwmeester et al. 2011; Bouwmeester and Govers 2009). It was suggested that DORN1 (LecRK I.9) mediates this CW-PM interaction via association with RGD domain containing proteins (Gouget et al. 2006). DORN1 contains two, proposed RGD binding domains, ASYY (residues 151–154) and PHPR (residues 257–260) located in the extracellular lectin domain (Gouget et al. 2006; Bouwmeester et al. 2011). Binding of DORN1 to the *Phytophthora* effector protein IPI-O was shown to reduce CW-PM connections, as did the addition of other RGD motif peptides (Senchou et al. 2004). Plant mutants lacking DORN1 (LecRK I.9) were found to be most susceptible to *Phytophthora* infection, while ectopic expression of DORN1 led to enhanced resistance (Bouwmeester et al. 2011, 2014). This enhanced resistance could be transferred by expressing the *Arabidopsis* DORN1 (LecRK I.9) protein in tobacco and potato. The authors of these studies interpreted their results in the context of the importance of DORN1 in mediating CW-PM interaction. However, with the subsequent identification of DORN1 as the receptor for extracellular ATP, one must consider that these phenotypes could also reflect changes in purinergic signaling.

In humans, RGD binding integrin proteins mediated PM-extracellular matrix (ECM) contact via binding to a RGD motif contained within the P2Y2 receptor (Erb et al. 2001). Thus, at least with regard to P2Y2 and DORN1, there appears to be a conserved role in mediating PM-CW/ECM interaction. Mutants of P2Y2 with a mutated RGD domain (RGE) show a slight reduction in ATP binding (Peterson et al. 2010), but more profound defects in cellular signaling, attributed to an inability to interact with integrin. Indeed, cells expressing the RGE form of P2Y2 are blocked in signaling through G_0 and G_{12}, which prevents subsequent activation of RAC and RHO and associated changes in the cytoskeleton important for cell movement (Erb et al. 2001). The exact role that the DORN1 RGD-binding domains play in its cellular function remain to be determined. *ProDORN1::GUS* expressing transgenic plants showed that *DORN1* is expressed in almost all tissues of *Arabidopsis* young seedling plants, including roots and leaves, and is highly expressed in the vasculature, shoot base and root tip region (Fig. 3). In 3-week old plants, the expression of *DORN1* in both old leaves and roots was weaker than developing tissues, such as newly emerging leaves and roots (Fig. 3). *DORN1* expression in 6-week-old plants disappeared in most tissues except the abscission zone of siliques (Fig. 3O-T). However, *DORN1* expression was strongly induced by wounding in mature leaves (Fig. 3N). Choi et al. (2014a) also showed, using a DORN1-GFP translational fusion driven by the native *DORN1* promoter, that the protein was localized to the plasma membrane.

4.1.1 Extracellular L-Type Lectin Domain

DORN1 has an extracellular L-type lectin domain, so named because of its similarity to the legume-lectin protein domain (Vaid et al. 2013). The L-type lectin domain contains a typical β-sandwich fold consisting of flattened six-stranded β-sheets and in-curved seven-stranded β-sheet (Barre et al. 2002). Computational modeling suggests that DORN1 has a similar structure (Nguyen et al. 2016) (Fig. 4).

As the name implies, normally one would expect a lectin domain to bind to carbohydrates (Herve et al. 1999). However, DORN1 lacks key amino acid residues known to be essential for monosaccharide binding (Gouget et al. 2006; Barre et al. 2002). Indeed, published efforts with the LecRK I.9 protein (Gouget et al. 2006), as well as our own unpublished efforts, failed to find any interaction between the DORN1 extracellular domain and a variety of sugars. Instead, the DORN1 extracellular lectin domain shows high affinity binding to ATP ($K_d = 45.7 \pm 3.1$ nM) (Choi et al. 2014b).

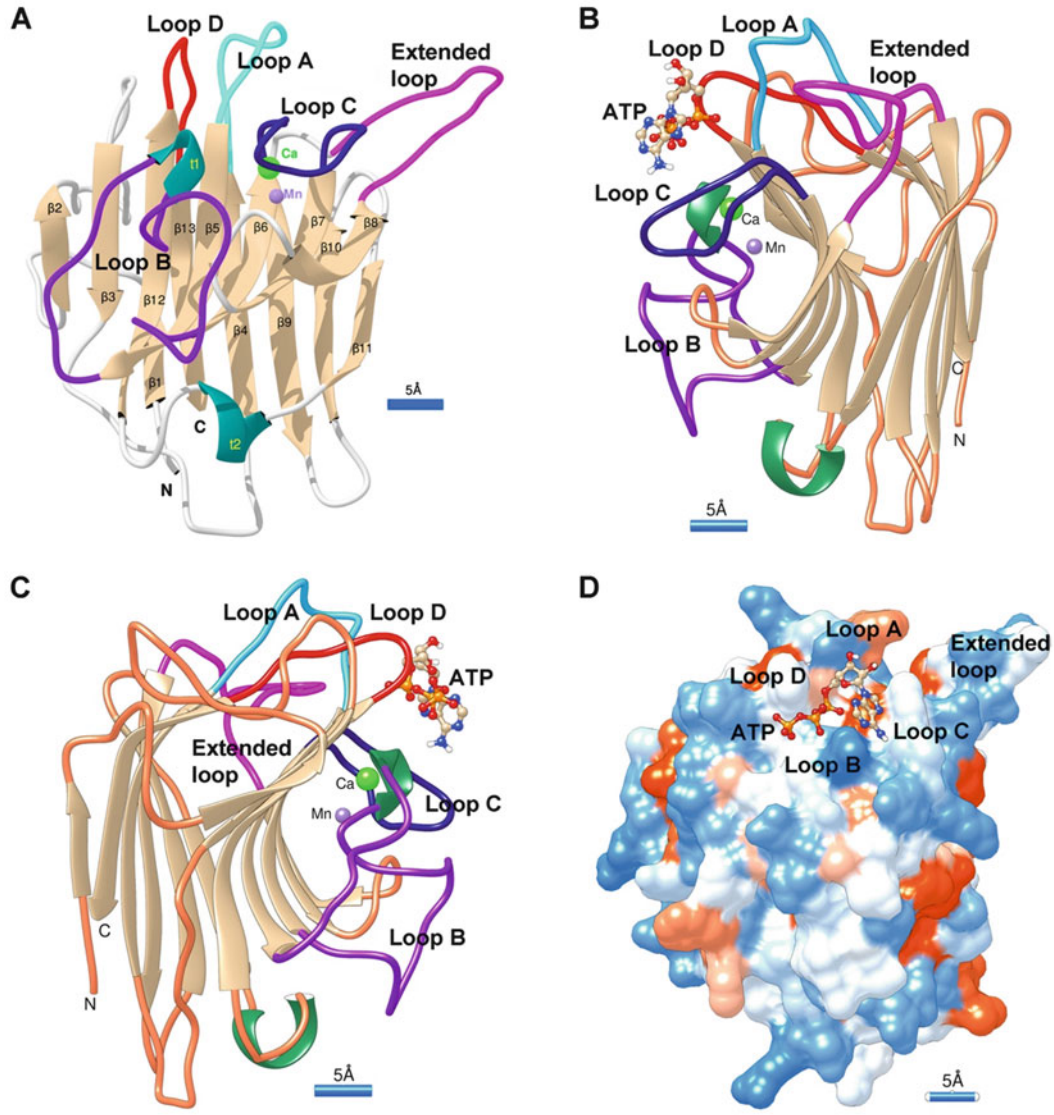

Fig. 4 Molecular modeling of the DORN1 protein structure
(**a**–**c**) Front (**a**) and right (**b**) and left (**c**) side view representation of the ribbon diagram of the DORN1 protein. The ribbon diagrams illustrate the docking form model. Each loop is colored in blue (Loop A), violet (Loop B), navy (Loop C), red (Loop D), and purple (Extended loop). Docking form model with ATP, Ca^{2+} (green ball), and Mn^{2+} (violet ball). (**d**) Electrostatic potential molecular surface of the DORN1 binding pocket and ATP molecule in ball and stick mode. Blue shading indicates electro-positive and red shading indicates electro-negative protein surfaces. Blue bars next to the figures denote relative scale of the model in Angstrom unit (1 Å = 10^{-10} m)

DORN1 prefers to bind with purine nucleotides (Choi et al. 2014b). The competitive binding affinity of various nucleotides showed that ATP and ADP were the most active agonists, followed by ITP, GTP and UTP. Various pyrimidine nucleotides did not bind (Choi et al. 2014b). The relative binding affinities of these nucleotides is roughly consistent with their biological activity

when applied to *Arabidopsis* seedlings (Choi et al. 2014b; Tanaka et al. 2010b).

Choi et al. (2014a) reported several allelic mutations in DORN1 (Fig. 2), including the *dorn1–6* (D147N) mutation. Interestingly, this residue is located in a distorted sugar-binding pocket, which appears to be involved in ATP binding (Nguyen et al. 2016).

4.1.2 Transmembrane Domain

As mentioned previously, DORN1 was localized via microscopy to the plasma membrane consistent with the presence of a single, predicted transmembrane domain (TM) (Choi et al. 2014a) (Fig. 2). One mutation, *dorn1–7* (A306T), is located in the transmembrane domain and plants with this mutation do not show an increase in cytoplasmic calcium upon ATP addition, suggesting that the TM domain is essential for function (Choi et al. 2014a) (Fig. 2).

4.1.3 Serine/Threonine Kinase Domain

The intracellular kinase domains found in various members of the LecRK protein family are between 250 and 300 amino acids in length, e.g. DORN1 kinase domain (268 amino acids) (Vaid et al. 2013). The intracellular domain of DORN1 has the sequence of a typical serine/threonine kinase (Fig. 2), consisting of 12 subdomains (Navarro-Gochicoa et al. 2003). *In vitro* kinase assays demonstrated that the DORN1 kinase domain was active in both autophosphorylation and transphosphorylation (Choi et al. 2014b). The *dorn1–1* mutations (D572N; subdomain X) and *dorn1–2* (D525N; subdomain IX) disrupt DORN1 kinase activity and also negate any purinergic signaling activity (Choi et al. 2014b) (Fig. 2). Studies have shown that LecRK kinase activity is dependent on divalent metal cations (Nishiguchi et al. 2002) and Mn^{2+} and Mg^{2+} strongly stimulate kinase activity (Nishiguchi et al. 2002). The *dorn1–5* (D486N; subdomain VII) mutation disrupts DORN1 signaling (Choi et al. 2014a) (Fig. 2). Computational modeling suggests that this aspartate residue is critical for binding Mg^{2+} in association with the β and γ phosphates of ATP. It is known that arginine–aspartate (RD) kinases carry a conserved arginine (R) immediately preceding the catalytic aspartate (D) that is required for autophosphorylation within the activation loop in subdomain VI (Johnson et al. 1996; Dardick and Ronald 2006). DORN1 belongs to the RD kinase family and contains these conserved arginine and aspartate residues in the activation loop (Choi et al. 2014a; Johnson et al. 1996). The *dorn1–9* mutation (R467Q; subdomain VIb; RD motif) in this arginine residue also disrupts ATP signaling via DORN1 (Fig. 2).

5 Computational Modeling of the DORN1 Extracellular Lectin Domain

Recently, computational modeling was used to predict the structure of the DORN1 extracellular lectin domain, as well as to predict key residues involved in ATP binding (Nguyen et al. 2016). The authors used a 'threading' approach making good use of the fact that 54 L-type lectins, ~ 250 tertiary structures and quaternary complexes have been crystallized and deposited into the protein data bank (http://www.rcsb.org) (Rose et al. 2013).

The DORN1 sequence was aligned with that of the three closest homologs [i.e., *Dolichos biflorus* (1BJQ), *Phaseolus vulgaris* (1FAT), and *Spatholobus parviflorus* (3IPV)] showing 47.3%, 52.2%, and 50.5% amino acid similarity, respectively (Nguyen et al. 2016). Interestingly, the DORN1 lectin domain has an insertion of 13 residues between β8 and β9-strands, and this insertion is only found in the lectin receptor kinases present in the *Brassicaceae* family (Nguyen et al. 2016). The sequence alignment generated was used to construct a 3D structural model of the DORN1 extracellular domain. This model predicts a β-sandwich architecture with β-jelly roll topology, one of the β-sheets consists of 7 anti-parallel β-strands (β2, β3, β5, β6, β7, β8, and β12) in the middle of the monomer, and 6 β-strands (β1, β4, β9, β10, β11, and β13) on one side of the subunit (Nguyen et al. 2016) (Fig. 4).

Given this predicted structure, additional computational methods were used to predict the specific ATP binding site and also to compare binding with other nucleotides, as well as sugars. As mentioned above, the lack of key residues indicate that DORN1 does not bind sugars and this was supported by the modeling. The putative ATP binding sites were predicted to be in the loops of the DORN1 extracellular lectin domain, including Gly98, Thr117, Arg118, Ile143, Asn145, Gly245, Thr246, and Ala247, except His99 (Nguyen et al. 2016). In addition, a putative metal ion binding site was found to be composed of five residues (Glu139, Asp141, Asn145, Asp150, and His155). Interestingly, this predicted ATP binding location is structurally similar to the carbohydrate binding pocket of the canonical L-type lectins (Nguyen et al. 2016).

Subsequent, structure-function studies utilizing site-directed mutagenesis largely supported the computational DORN1 model. For example, deletion of loop-B (116–118) and loop-C (140–145) impaired ATP binding, while deletions of loop-A (94–97), loop-D (245–247), and extended loop did not affect the binding ability of DORN1 (Nguyen et al. 2016) (Fig. 2). In addition, several residues (Glu96, Gly98, His99, Thr117, Arg118, Ile143, Trp144, Pro146, Thr246, Gly245, and Ala247) in these four loop regions for ATP binding were shown to possess a higher number of H bonds and hydrophobic interactions than to other ligands, such as di-, monosaccharides (Nguyen et al. 2016). These data suggest that this shallow pocket surrounded by the four loops in DORN1 is suitable for ATP binding, by forming hydrogen bonds and hydrophobic interactions (Figs. 2 and 3).

Recently, Li et al. (2016) reported a low-resolution (4.6 Å) X-ray crystallographic structure of the *Arabidopsis* DORN1 extracellular domain and also of the orthologous protein purified from *Camelina sativa*. As predicted from the sequence (Choi et al. 2014a), DORN1 purified by expression in planta showed significant levels of glycosylation. Potential sites of glycosylation are residues Asn56, Asn124, Asn128, Asn181, Asn204, Asn225, and Asn232 (Li et al. 2016). However, subsequent site-directed mutagenesis revealed that mutation in only four of these residues (Asn124, Asn204, As129, and Asn225) significantly affected glycosylation levels (Li et al. 2016). Nguyen et al. (2016) reported that mutations in these same residues did not affect ATP binding. In general, the data presented by Li et al. (2016), are consistent with the computational model generated by Nguyen et al. (2016). Further analysis awaits the publication of a high-resolution crystal structure.

6 Future Perspectives

Purinergic signaling in plants and animals shows clear similarities; for example, similar cellular responses (elevated cytoplasmic Ca^{2+} levels, ROS, NO production and gene expression) but these responses are mediated by unique receptors; that is, P2X and P2Y in animals and P2K1 (DORN1) in plants. This unique feature of plants is perhaps explained by the fact that the LecRK family is found only in plants. The *Arabidopsis* genome is predicted to encode over 600 receptor-like kinases (RLKs), of which 45 genes are within the LecRK family (Bouwmeester and Govers 2009). Most of these LecRK genes remain to be characterized but those that have been studied clearly implicate this protein family in recognition of a wide range of stimuli (Bouwmeester et al. 2011; Shiu and Bleecker 2001; Riou et al. 2002; Deng et al. 2009), especially those involved in plant stress.

Mammals possess multiple P2X and P2Y receptors and, therefore, it will not be surprising to find that plants also have multiple P2K receptors (Burnstock 2001, 2007). The various expression patterns of other LecRKs in different tissues and developmental stages suggest the possibility of other purinergic receptors (Bouwmeester and Govers 2009). However, the total lack of a response to ATP in *dorn1* mutant plants suggest that, at least under the conditions tested, DORN1 is the major purinergic receptor in *Arabidopsis* seedlings. Thus, other receptors likely play more specific roles at defined developmental stages (e.g. mature plant), specific

tissues (e.g. pollen) or in response to unique stimuli.

It is hoped that the identification of DORN1 as the first plant purinergic receptor will stimulate other research groups to enter this interesting area of research. Many questions remain to be answered:

- What is the key mechanism involved in modulation of ion channel activity in response to extracellular ATP (e.g. how DORN1 affect Ca^{2+} influx)?
- Do plants contain multiple purinergic receptors and, if so, what are their respective functions?
- What can be learned through comparative studies of purinergic signaling in a variety of plant species?
- What are the downstream signaling events involved in purinergic signaling and how are they integrated in the overall response pathways that plants use for growth, development and stress response?
- What can be learned from a comparative study of plant and animal purinergic signaling; where do these pathways converge and diverge? What aspects are evolutionarily conserved?
- Given the large pharmaceutical industry that has developed to exploit human purinergic signaling, are there also approaches that can be used to exploit plant purinergic signaling to improve crop productivity and sustainability?

Our hope is that this review will stimulate researchers to address these and other questions to expand our knowledge of purinergic signaling in plants.

Acknowledgement We thank Dr. Katalin Tóth and Dr. Daewon Kim (University of Missouri, USA) for valuable comments on the manuscript. This work was supported by the Next-Generation BioGreen 21 Program, Systems and Synthetic Agrobiotech Center, Rural Development Administration, Republic of Korea (grant no. PJ01116604). This work was also supported by The National Institute of General Medical Science of the National Institutes of Health (grant no. RO1GM 121445 to GS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abbracchio MP, Ceruti S (2006) Roles of P2 receptors in glial cells: focus on astrocytes. Purinergic Signal 2 (4):595–604

Barre A, Hervé C, Lescure B, Rougé P (2002) Lectin Receptor Kinases in Plants. Crit Rev Plant Sci 21 (4):379–399

Bernard C, Traub M, Kunz HH, Hach S, Trentmann O, Mohlmann T (2011) Equilibrative nucleoside transporter 1 (ENT1) is critical for pollen germination and vegetative growth in Arabidopsis. J Exp Bot 62 (13):4627–4637

Bodin P, Burnstock G (2001a) Purinergic signalling: ATP release. Neurochem Res 26(8–9):959–969

Bodin P, Burnstock G (2001b) Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 38(6):900–908

Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112 (2):358–404

Bouwmeester K, Govers F (2009) Arabidopsis L-type lectin receptor kinases: phylogeny, classification, and expression profiles. J Exp Bot 60(15):4383–4396

Bouwmeester K, de Sain M, Weide R, Gouget A, Klamer S, Canut H, Govers F (2011) The lectin receptor kinase LecRK-I.9 is a novel Phytophthora resistance component and a potential host target for a RXLR effector. PLoS Pathog 7(3):e1001327

Bouwmeester K, Han M, Blanco-Portales R, Song W, Weide R, Guo LY, van der Vossen EA, Govers F (2014) The Arabidopsis lectin receptor kinase LecRK-I.9 enhances resistance to Phytophthora infestans in Solanaceous plants. Plant Biotechnol J 12(1):10–16

Burnstock G (1972) Purinergic nerves. Pharmacol Rev 24 (3):509–581

Burnstock G (2001) Purine-mediated signalling in pain and visceral perception. Trends Pharmacol Sci 22 (4):182–188

Burnstock G (2007) Purine and pyrimidine receptors. Cell Mol Life Sci 64(12):1471–1483

Cao Y, Tanaka K, Nguyen CT, Stacey G (2014) Extracellular ATP is a central signaling molecule in plant stress responses. Curr Opin Plant Biol 20:82–87

Chen KM, GL W, Wang YH, Tian CT, Samaj J, Baluska F, Lin JX (2008) The block of intracellular

calcium release affects the pollen tube development of Picea wilsonii by changing the deposition of cell wall components. Protoplasma 233(1–2):39–49

Chivasa S, Ndimba BK, Simon WJ, Lindsey K, Slabas AR (2005) Extracellular ATP functions as an endogenous external metabolite regulating plant cell viability. Plant Cell 17(11):3019–3034

Chivasa S, Murphy AM, Hamilton JM, Lindsey K, Carr JP, Slabas AR (2009) Extracellular ATP is a regulator of pathogen defence in plants. Plant J 60(3):436–448

Chivasa S, Simon WJ, Murphy AM, Lindsey K, Carr JP, Slabas AR (2010) The effects of extracellular adenosine 5′-triphosphate on the tobacco proteome. Proteomics 10(2):235–244

Choi J, Tanaka K, Liang Y, Cao Y, Lee SY, Stacey G (2014a) Extracellular ATP, a danger signal, is recognized by DORN1 in Arabidopsis. Biochem J 463(3):429–437

Choi J, Tanaka K, Cao Y, Qi Y, Qiu J, Liang Y, Lee SY, Stacey G (2014b) Identification of a plant receptor for extracellular ATP. Science 343(6168):290–294

Clark G, Roux SJ (2011) Apyrases, extracellular ATP and the regulation of growth. Curr Opin Plant Biol 14 (6):700–706

Clark G, Torres J, Finlayson S, Guan X, Handley C, Lee J, Kays JE, Chen ZJ, Roux SJ (2010a) Apyrase (nucleoside triphosphate-diphosphohydrolase) and extracellular nucleotides regulate cotton fiber elongation in cultured ovules. Plant Physiol 152(2):1073–1083

Clark G, Wu M, Wat N, Onyirimba J, Pham T, Herz N, Ogoti J, Gomez D, Canales AA, Aranda G, Blizard M, Nyberg T, Terry A, Torres J, Wu J, Roux SJ (2010b) Both the stimulation and inhibition of root hair growth induced by extracellular nucleotides in Arabidopsis are mediated by nitric oxide and reactive oxygen species. Plant Mol Biol 74(4–5):423–435

Clark G, Fraley D, Steinebrunner I, Cervantes A, Onyirimba J, Liu A, Torres J, Tang W, Kim J, Roux SJ (2011) Extracellular nucleotides and apyrases regulate stomatal aperture in Arabidopsis. Plant Physiol 156(4):1740–1753

Dardick C, Ronald P (2006) Plant and animal pathogen recognition receptors signal through non-RD kinases. PLoS Pathog 2(1):e2

Dark A, Demidchik V, Richards SL, Shabala S, Davies JM (2011) Release of extracellular purines from plant roots and effect on ion fluxes. Plant Signal Behav 6 (11):1855–1857

Demidchik V, Nichols C, Oliynyk M, Dark A, Glover BJ, Davies JM (2003) Is ATP a signaling agent in plants? Plant Physiol 133(2):456–461

Demidchik V, Shang Z, Shin R, Thompson E, Rubio L, Laohavisit A, Mortimer JC, Chivasa S, Slabas AR, Glover BJ, Schachtman DP, Shabala SN, Davies JM (2009) Plant extracellular ATP signalling by plasma membrane NADPH oxidase and Ca^{2+} channels. Plant J 58(6):903–913

Deng K, Wang Q, Zeng J, Guo X, Zhao X, Tang D, Liu X (2009) A Lectin receptor kinase positively regulates ABA response during seed germination and is involved in salt and osmotic stress response. J Plant Biol 52(6):493

Deng S, Sun J, Zhao R, Ding M, Zhang Y, Sun Y, Wang W, Tan Y, Liu D, Ma X, Hou P, Wang M, Lu C, Shen X, Chen S (2015) Populus euphratica APYRASE2 enhances cold tolerance by modulating vesicular trafficking and extracellular ATP in Arabidopsis plants. Plant Physiol 169(1):530–548

Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Perez LI, Gonzalez FA, Gresham HD, Turner JT, Weisman GA (2001) An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G(o)-mediated signal transduction. J Cell Biol 153(3):491–501

Foresi NP, Laxalt AM, Tonon CV, Casalongue CA, Lamattina L (2007) Extracellular ATP induces nitric oxide production in tomato cell suspensions. Plant Physiol 145(3):589–592

Fountain SJ, Cao L, Young MT, North RA (2008) Permeation properties of a P2X receptor in the green algae Ostreococcus tauri. J Biol Chem 283 (22):15122–15126

Gouget A, Senchou V, Govers F, Sanson A, Barre A, Rouge P, Pont-Lezica R, Canut H (2006) Lectin receptor kinases participate in protein-protein interactions to mediate plasma membrane-cell wall adhesions in Arabidopsis. Plant Physiol 140(1):81–90

Govindarajulu M, Kim SY, Libault M, Berg RH, Tanaka K, Stacey G, Taylor CG (2009) GS52 ecto-apyrase plays a critical role during soybean nodulation. Plant Physiol 149(2):994–1004

Hanley PJ, Musset B, Renigunta V, Limberg SH, Dalpke AH, Sus R, Heeg KM, Preisig-Muller R, Daut J (2004) Extracellular ATP induces oscillations of intracellular Ca^{2+} and membrane potential and promotes transcription of IL-6 in macrophages. Proc Natl Acad Sci U S A 101 (25):9479–9484

Hao LH, Wang WX, Chen C, Wang YF, Liu T, Li X, Shang ZL (2012) Extracellular ATP promotes stomatal opening of Arabidopsis thaliana through heterotrimeric G protein alpha subunit and reactive oxygen species. Mol Plant 5(4):852–864

Hardham AR, Takemoto D, White RG (2008) Rapid and dynamic subcellular reorganization following mechanical stimulation of Arabidopsis epidermal cells mimics responses to fungal and oomycete attack. BMC Plant Biol 8:63

Herve C, Serres J, Dabos P, Canut H, Barre A, Rouge P, Lescure B (1999) Characterization of the Arabidopsis lecRK-a genes: members of a superfamily encoding putative receptors with an extracellular domain homologous to legume lectins. Plant Mol Biol 39(4):671–682

Jaffe MJ (1973) The role of ATP in mechanically stimulated rapid closure of the venus's flytrap. Plant Physiol 51(1):17–18

Jeter CR, Tang W, Henaff E, Butterfield T, Roux SJ (2004) Evidence of a novel cell signaling role for extracellular adenosine triphosphates and diphosphates in Arabidopsis. Plant Cell 16(10):2652–2664

Johnson LN, Noble ME, Owen DJ (1996) Active and inactive protein kinases: structural basis for regulation. Cell 85(2):149–158

Kamizyo A, Tanaka N (1982) Studies on the generative nuclear division III. Effect of exogenous ATP on the generative nuclear division in Lilium longiflorum. Cytologia 47:195–205

Khakh BS, Burnstock G (2009) The double life of ATP. Sci Am 301(6):84–90, 92

Kim SY, Sivaguru M, Stacey G (2006) Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. Plant Physiol 142(3):984–992

Knight H, Knight MR (1995) Recombinant aequorin methods for intracellular calcium measurement in plants. Methods Cell Biol 49:201–216

Knowles JR (1980) Enzyme-catalyzed phosphoryl transfer reactions. Annu Rev Biochem 49:877–919

Lazarowski ER, Boucher RC, Harden TK (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol 64(4):785–795

Lehti-Shiu MD, Zou C, Hanada K, Shiu SH (2009) Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes. Plant Physiol 150(1):12–26

Lew RR, Dearnaley JDW (2000) Extracellular nucleotide effects on the electrical properties of growing Arabidopsis thaliana root hairs. Plant Sci 153(1):1–6

Li Z, Chakraborty S, Xu G (2016) X-ray crystallographic studies of the extracellular domain of the first plant ATP receptor, DORN1, and the orthologous protein from Camelina sativa. Acta Crystallogr F Struct Biol Commun 72(Pt 10):782–787

Lim MH, Wu J, Yao J, Gallardo IF, Dugger JW, Webb LJ, Huang J, Salmi ML, Song J, Clark G, Roux SJ (2014) Apyrase suppression raises extracellular ATP levels and induces gene expression and cell wall changes characteristic of stress responses. Plant Physiol 164 (4):2054–2067

Liu X, Wu J, Clark G, Lundy S, Lim M, Arnold D, Chan J, Tang W, Muday GK, Gardner G, Roux SJ (2012) Role for apyrases in polar auxin transport in Arabidopsis. Plant Physiol 160(4):1985–1995

Lustig KD, Shiau AK, Brake AJ, Julius D (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci U S A 90 (11):5113–5117

McPhail LC, Waite KA, Regier DS, Nixon JB, Qualliotine-Mann D, Zhang WX, Wallin R, Sergeant S (1999) A novel protein kinase target for the lipid second messenger phosphatidic acid. Biochim Biophys Acta 1439(2):277–290

Navarro-Gochicoa MT, Camut S, Timmers AC, Niebel A, Herve C, Boutet E, Bono JJ, Imberty A, Cullimore JV (2003) Characterization of four lectin-like receptor kinases expressed in roots of Medicago truncatula. Structure, location, regulation of expression, and potential role in the symbiosis with Sinorhizobium meliloti. Plant Physiol 133(4):1893–1910

Nejidat A, Itai C, Roth-Bejerano N (1983) Stomatal response to ATP mediated by phytochrome. Physiol Plant 57(3):367–370

Nguyen CT, Tanaka K, Cao Y, Cho SH, Xu D, Stacey G (2016) Computational analysis of the ligand binding Site of the extracellular ATP Receptor, DORN1. PLoS One 11(9):e0161894

Nishiguchi M, Yoshida K, Sumizono T, Tazaki K (2002) A receptor-like protein kinase with a lectin-like domain from lombardy poplar: gene expression in response to wounding and characterization of phosphorylation activity. Mol Gen Genomics 267 (4):506–514

Park J, Gu Y, Lee Y, Yang Z, Lee Y (2004) Phosphatidic acid induces leaf cell death in Arabidopsis by activating the Rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation. Plant Physiol 134(1):129–136

Peterson TS, Camden JM, Wang Y, Seye CI, Wood WG, Sun GY, Erb L, Petris MJ, Weisman GA (2010) P2Y2 nucleotide receptor-mediated responses in brain cells. Mol Neurobiol 41(2–3):356–366

Puchalowicz K, Tarnowski M, Baranowska-Bosiacka I, Chlubek D, Dziedziejko V (2014) P2X and P2Y receptors-role in the pathophysiology of the nervous system. Int J Mol Sci 15(12):23672–23704

Raghavendra AS (1981) Energy supply for stomatal opening in epidermal strips of Commelina benghalensis. Plant Physiol 67(2):385–387

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50(3):413–492

Reichler SA, Torres J, Rivera AL, Cintolesi VA, Clark G, Roux SJ (2009) Intersection of two signalling pathways: extracellular nucleotides regulate pollen germination and pollen tube growth via nitric oxide. J Exp Bot 60(7):2129–2138

Riewe D, Grosman L, Fernie AR, Zauber H, Wucke C, Geigenberger P (2008) A cell wall-bound adenosine nucleosidase is involved in the salvage of extracellular ATP in Solanum tuberosum. Plant Cell Physiol 49 (10):1572–1579

Riou C, Hervé C, Pacquit V, Dabos P, Lescure B (2002) Expression of an Arabidopsis lectin kinase receptor gene, lecRK-a1, is induced during senescence, wounding and in response to oligogalacturonic acids. Plant Physiol Biochem 40(5):431–438

Roberts NJ, Morieri G, Kalsi G, Rose A, Stiller J, Edwards A, Xie F, Gresshoff PM, Oldroyd GE, Downie JA, Etzler ME (2013) Rhizobial and mycorrhizal symbioses in Lotus japonicus require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling. Plant Physiol 161(1):556–567

Rose PW, Bi C, Bluhm WF, Christie CH, Dimitropoulos D, Dutta S, Green RK, Goodsell DS, Prlic A, Quesada M, Quinn GB, Ramos AG, Westbrook JD, Young J, Zardecki C, Berman HM, Bourne PE (2013) The RCSB Protein Data Bank: new resources for research and education. Nucleic Acids Res 41(D1):D475–D482

Roux SJ, Steinebrunner I (2007) Extracellular ATP: an unexpected role as a signaler in plants. Trends Plant Sci 12(11):522–527

Roux SJ, Song C, Jeter C (2006) Regulation of plant growth and development by extracellular nucleotides. In: Communication in plants. Springer, Berlin/Heidelberg, pp 221–234

Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H, Yamamoto A, Moriyama Y (2008) Identification of a vesicular nucleotide transporter. Proc Natl Acad Sci U S A 105(15):5683–5686

Senchou V, Weide R, Carrasco A, Bouyssou H, Pont-Lezica R, Govers F, Canut H (2004) High affinity recognition of a Phytophthora protein by Arabidopsis via an RGD motif. Cell Mol Life Sci 61(4):502–509

Shen J, Harada N, Nakazawa H, Yamashita T (2005) Involvement of the nitric oxide-cyclic GMP pathway and neuronal nitric oxide synthase in ATP-induced Ca^{2+} signalling in cochlear inner hair cells. Eur J Neurosci 21(11):2912–2922

Shiu SH, Bleecker AB (2001) Plant receptor-like kinase gene family: diversity, function, and signaling. Sci STKE 2001(113):re22

Song CJ, Steinebrunner I, Wang X, Stout SC, Roux SJ (2006) Extracellular ATP induces the accumulation of superoxide via NADPH oxidases in Arabidopsis. Plant Physiol 140(4):1222–1232

Srobarova A, da Silva JA, Kogan G, Ritieni A, Santini A (2009) Beauvericin decreases cell viability of wheat. Chem Biodivers 6(8):1208–1215

Steinebrunner I, Wu J, Sun Y, Corbett A, Roux SJ (2003) Disruption of apyrases inhibits pollen germination in Arabidopsis. Plant Physiol 131(4):1638–1647

Sueldo DJ, Foresi NP, Casalongue CA, Lamattina L, Laxalt AM (2010) Phosphatidic acid formation is required for extracellular ATP-mediated nitric oxide production in suspension-cultured tomato cells. New Phytol 185(4):909–916

Sun J, Zhang CL, Deng SR, CF L, Shen X, Zhou XY, Zheng XJ, ZM H, Chen SL (2012a) An ATP signalling pathway in plant cells: extracellular ATP triggers programmed cell death in Populus euphratica. Plant Cell Environ 35(5):893–916

Sun J, Zhang X, Deng S, Zhang C, Wang M, Ding M, Zhao R, Shen X, Zhou X, Lu C, Chen S (2012b) Extracellular ATP signaling is mediated by H(2)O(2) and cytosolic Ca(2)(+) in the salt response of Populus euphratica cells. PLoS One 7(12):e53136

Tanaka K, Gilroy S, Jones AM, Stacey G (2010a) Extracellular ATP signaling in plants. Trends Cell Biol 20(10):601–608

Tanaka K, Swanson SJ, Gilroy S, Stacey G (2010b) Extracellular nucleotides elicit cytosolic free calcium oscillations in Arabidopsis. Plant Physiol 154(2):705–719

Tanaka K, Nguyen CT, Libault M, Cheng J, Stacey G (2011) Enzymatic activity of the soybean ecto-apyrase GS52 is essential for stimulation of nodulation. Plant Physiol 155(4):1988–1998

Tanaka K, Choi J, Cao Y, Stacey G (2014) Extracellular ATP acts as a damage-associated molecular pattern (DAMP) signal in plants. Front Plant Sci 5:446

Tang W, Brady SR, Sun Y, Muday GK, Roux SJ (2003) Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. Plant Physiol 131(1):147–154

Terrile MC, Tonon CV, Iglesias MJ, Lamattina L, Casalongue CA (2010) Extracellular ATP and nitric oxide signaling pathways regulate redox-dependent responses associated to root hair growth in etiolated Arabidopsis seedlings. Plant Signal Behav 5(6):698–701

Thomas C, Sun Y, Naus K, Lloyd A, Roux S (1999) Apyrase functions in plant phosphate nutrition and mobilizes phosphate from extracellular ATP. Plant Physiol 119(2):543–552

Thomas C, Rajagopal A, Windsor B, Dudler R, Lloyd A, Roux SJ (2000) A role for ectophosphatase in xenobiotic resistance. Plant Cell 12(4):519–533

Tonon C, Cecilia Terrile M, Jose Iglesias M, Lamattina L, Casalongue C (2010) Extracellular ATP, nitric oxide and superoxide act coordinately to regulate hypocotyl growth in etiolated Arabidopsis seedlings. J Plant Physiol 167(7):540–546

Torres J, Rivera A, Clark G, Roux SJ (2008) Participation of extracellular nucleotides in the wound response of Dasycladus vermicularis and Acetabularia acetabulum (Dasycladales, Chlorophyta)(1). J Phycol 44(6):1504–1511

Udvardy J, Farkas GL (1973) ATP stimulates the formation of nucleases in excised Avena leaves. Z Pflanzenphysiol 69(5):394–401

Vaid N, Macovei A, Tuteja N (2013) Knights in action: lectin receptor-like kinases in plant development and stress responses. Mol Plant 6(5):1405–1418

Wang F, Jia J, Wang Y, Wang W, Chen Y, Liu T, Shang Z (2014) Hyperpolization-activated Ca(2+) channels in guard cell plasma membrane are involved in extracellular ATP-promoted stomatal opening in Vicia faba. J Plant Physiol 171(14):1241–1247

Weerasinghe RR, Swanson SJ, Okada SF, Garrett MB, Kim SY, Stacey G, Boucher RC, Gilroy S, Jones AM (2009) Touch induces ATP release in Arabidopsis roots that is modulated by the heterotrimeric G-protein complex. FEBS Lett 583(15):2521–2526

Williamson RE (1975) Cytoplasmic streaming in Chara: a cell model activated by ATP and inhibited by cytochalasin B. J Cell Sci 17(3):655–668

Wu SJ, Wu JY (2008) Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in Salvia miltiorrhiza hairy roots. J Exp Bot 59(14):4007–4016

Wu J, Steinebrunner I, Sun Y, Butterfield T, Torres J, Arnold D, Gonzalez A, Jacob F, Reichler S, Roux SJ (2007) Apyrases (nucleoside triphosphate-diphosphohydrolases) play a key role in growth control in Arabidopsis. Plant Physiol 144(2):961–975

Wu SJ, Liu YS, JY W (2008) The signaling role of extracellular ATP and its dependence on Ca^{2+} flux in elicitation of Salvia miltiorrhiza hairy root cultures. Plant Cell Physiol 49(4):617–624

Yang X, Wang B, Farris B, Clark G, Roux SJ (2015) Modulation of root skewing in arabidopsis by apyrases and extracellular ATP. Plant Cell Physiol 56 (11):2197–2206

Zhou J, Fan C, Liu K, Jing Y (2015) Extracellular ATP is involved in the initiation of pollen germination and tube growth in Picea meyeri. Trees 29(2):563–574

Adv Exp Med Biol - Protein Reviews (2017) 19:255–256
DOI 10.1007/5584_2017

Index