C terminus of the P2X7 receptor: treasure hunting

Helio Miranda Costa-Junior · Flávia Sarmiento Vieira ·
Robson Coutinho-Silva

Received: 24 September 2010 / Accepted: 5 January 2011 / Published online: 27 January 2011
© Springer Science+Business Media B.V. 2011

Abstract P2X receptor (P2XR) is a family of the ATP-gated ion channel family and can permeabilize the plasma membrane to small cations such as potassium, sodium, and calcium, resulting in cellular depolarization. There are seven P2XR that have been described and cloned, with 45% identity in amino acid sequence. Each P2X receptors has two transmembrane domains that are separated by an extracellular loop and an intracellular N and C terminus. Unlike the other P2X receptors, the P2X7R has a larger C terminus with an extra 200 amino acid residues compared with the other receptors. The C terminus of the P2X7R has been implicated in regulating receptor function including signaling pathway activation, cellular localization, protein–protein interactions, and post-translational modification (PTM). In the present review, we discuss the role of the P2X7R C terminus in regards to receptor function, describe the specific domains and motifs found therein and compare the C terminus sequence with others proteins to discover predicted domains or sites of PTM.

Keywords P2X7 · C terminus · Intracellular signaling · Motif · Protein–protein interaction

Introduction

Classically, researchers focus on one protein and study its function within a linear pathway, on a single context or by minimizing the possibilities of its interactions. However, sequencing the genome has revealed a small number of genes that correspond to a large number of proteins, and the same protein can be presented in different ways, such as phosphorylated or nitrosylated proteins. This discovery changed the classic way of thinking about proteins and their interactions, and in addition, many studies have focused on studying isolated proteins outside of the cell or biological context, which limits identifying their true actions and interactions within a cell. Cells and biological systems are dynamic entities, and one protein can be involved in many interactions and pathways that should not be studied in isolation; we must consider multipoint effects/interactions. These multi-interacting proteins have important roles in cellular signaling pathways with two or more biological functions, initiating a cascade of events that will shape cell identity or define a biological context.

A good example for utilizing this new multipoint view is the P2X7 receptor (P2X7R). As a member of the adenosine triphosphate (ATP)-gated ion channel family, P2X7R has a unique long C terminus with an extra 200 amino acid residues compared with the other P2X receptors. The sequence identity with other P2X receptors is of approximately 40–50 % [1]. The first receptor was cloned from rat brains and then from monocytes and microglia cells in humans and mice. P2X7R activation was initially described as a low conductance ion channel [2, 3] associated with

H. M. Costa-Junior (✉)
Instituto de Biofísica Carlos Chagas Filho-UFRJ, Av. Carlos
Chagas Filho n 373, Bloco G do CCS, Cidade Universitária,
21941-902, Ilha do Fundão,
Rio de Janeiro, Brazil
e-mail: heliomcjr@gmail.com

H. M. Costa-Junior
CryoBio - Centro de Criogenia e Terapia Celular,
Av. Carlos Chagas Filho 791,
Rio de Janeiro, Brazil

H. M. Costa-Junior · F. Sarmiento Vieira · R. Coutinho-Silva
Laboratório de Imunofisiologia, Universidade Federal do Rio de
Janeiro, Instituto de Biofísica Carlos Chagas Filho, CCS,
Rio de Janeiro, Brazil

pore-formation activity [2, 4, 5], maturation, and release of pro-inflammatory cytokines [6–8], killing of pathogens [9], activation of caspases [10–12], membrane blebbing formation [13, 14], activation of different phospholipases [9, 15, 16], production of free radicals [17], cell cycle regulation [18], T cell maturation [19], and many other functions. P2X7R is a typical one-protein receptor that has different functions in different or even the same cells.

One situation that illustrates the complexity of P2X7R is the apparently progressive increase in pore permeability to big molecules associated with this receptor. Pore-formation activity is one of the most studied characteristics of P2X7R. It classically defined the prolong millimolar ATP stimulation, leading to the opening of membrane pores and permitting the transport of large molecules under 900 Da [20, 21]. Two main mechanisms have been proposed to explain the increase in permeability attributed to P2X7R activation in time-dependent manners; (a) a prolonged ATP stimulation due to P2X7R conformational changes and recruitment of new P2X7 sub-units, resulting in dilatation of the ion channel and increase of permeability [22, 23]; (b) ATP stimulation could induce P2X7R changes, resulting in the exposure of new protein linear sequences or motifs that recruited/interacted with other channel/ pore proteins, which are permeated to larger molecules [24], such as connexins and pannexins families. The pathway related with P2X7R pore formation is still unclear and more experimental data are necessary to better understand it. The connexin family is a pore-forming candidate that could be induced by P2X7R. In 2004, Fortes et al. demonstrated that connexin 43 (Cx43) co-localized with P2X7R by confocal imaging, bypassing the dye in ATP-sensitive macrophage cultures of Cx43 but not in ATP-insensitive macrophage [25]. Recently, some authors have suggested that the protein responsible for P2X7R pore formation is pannexin 1 (Panx1) [26]. Panx1 is structurally, but not sequentially, related to connexins [27–29]. Different researchers have demonstrated that Panx1 disruption by small interfering RNA could inhibit some functions of P2X7R, including pore formation [30–33]. Schachter et al. have also made a significant contribution that can help to identify the pore molecule and distinguish the mechanisms of ionic dye capture in macrophages [34]. They showed that macrophages could uptake both positively charged dyes, such as ethidium bromide, and negatively charged dyes, such as Lucifer yellow; however, P2X7R-transfected HEK 293 cells only uptake positive dyes, indicating that different mechanisms are used to capture different dyes. One possible explanation to these data could be that these differences might be associated with Cx- or Panx1-dependent pathways, but it needs to be proved.

How can one single protein maintain such a variety of different functions? To answer this and other questions,

some studies have focused on the C terminus of P2X7R, which is larger than other P2X C termini. It has been suggested that the P2X7R C terminus interacts with different proteins, as demonstrated using proteomic assays by Kim et al. [35] and Gu et al. [36], and that some amino acids or motifs have an important role on P2X7R activity. Here, we will discuss the role of the C terminus domain in the context of different biological functions of P2X7R and analyze the C terminus linear sequence with some predictive tools to try to understand the global functions of P2X7R.

Examining the P2X7R C terminus

Despite the great interest in P2X7R and the huge advances in this field, there is not much information about its structure and motif composition. Only recently, the crystal structure of the zebrafish P2X4 orthologue receptor was resolved [37], adding to our understanding of the general P2X structure. There are data indicating that the P2X7R N terminus has some important residues related to selectivity and activity of the P2X7R ion channel and interaction with MAPKs [38] and on the extracellular loop, some residues in the ATP-interacting sequence are glycosylated [39]. The P2X7R C terminus is involved in the majority of functions related to P2X7R, and thus, our review will focus on the C terminus of P2X7R.

In 2001, Denlinger et al. were the first to conduct an alignment comparison of the C terminus of P2X7R with the domains of other proteins using the BLAST program [40]. In Denlinger's report, the authors identified several regions of interest in murine P2X7R: a SH3 motif present within residues 441–460; region similar to the TNFR1 death domain at residues 436–531; a very similar region (50% identity) to the high molecular weight protein 3 (from *Mycoplasma genitalium*) and to a hypothetical protein C18H2.1 (from *Caenorhabditis elegans*) at residues 389–405 and 494–508, respectively; and of all motifs identified by the authors, the most well characterized was a lipopolysaccharide (LPS)-binding domain corresponding to amino acids 573–590.

Looking closer at Denlinger's data, we noticed that the SH3 domain was located inside the death domain of the P2X7R C terminus. In fact, Adriouch et al. [41] and Stunff et al. [42] described a single-coding mutation (T1352C) in C57Bl/6 mice in comparison to Balb/c mice that changes a single amino acid, the proline residue at position 451, to a leucine residue (P451L) [41]. This mutation resulted in an impairment in cell death and pore formation in C57Bl/6 mice, but not in Balb/c mice that shared this region with rat and human P2X7R [41]. However, the P451L mutation did not affected other P2X7R functions, as reported to the ion

channel current [43] and phospholipase D activation [42]. The loss of function on P451L has been associated with the fact that P451 is in the SH3 domain (PxxP, x=any amino acid), which interacts with Src tyrosine kinase and leads to activation of Panx1 [24, 44]. Panx1 was suggested to be the real pore opening controlled by P2X7 for the transport of ions [26, 34]. The most important evidence that supports this idea was the use of a peptide based on the SH3 domain that binds to P451 on P2X7, leading to the inhibition of the interaction between this protein and Src tyrosine kinase proteins in J774 macrophage, a Balb/c derived cell lineage [24], impairing the pore opening in a way that is independent of Panx1 or others proteins. In neural progenitor cell from C57Bl/6 mice, Delarasse et al. showed a pore formation by ATP stimulation that allowed the entry of ethidium bromide in these cells, but the cell death observed was independent of the pore opening [45]

In comparison to other proteins, the P2X7R C terminus has two consensus motifs (488–492 and 570–574) similar to the SH3 domain (PxxxR) of Grb2 (Table 1), a protein involved in the MAPK pathway and one motif consensus (576–579, consensus conserved in mouse, rat, and human P2X7R) similar to the SH3 domain of GADS. By proteomic analysis, Gu et al. described that a nonmuscle myosin interacted with P2X7R and could modulate negatively the P2X7 pore formation in THP-1 monocytes and P2X7R transfected HEK 293 [36]. The nonmuscle myosin IIA heavy chain (myosin-9) identified in human monocyte THP-1 shared the same predicted SH3 domain (PxxxR) predicted in P2X7R and similar to Grb2, but the real interacting motif between them is unknown and only espouses in non ATP stimulated P2X7R. The P451L mutation had a similar effect to another mutation, changing a glutamic acid residue at position 496 to alanine (E496A) [46, 47]. This mutation is frequently found in individuals suffering from chronic lymphocytic leukemia [46], and E496A causes a defect in B lymphocyte apoptosis [47] by abolition of pore-formation and lytic functions of the P2X7R. However, neither mutation affects the surface expression of P2X7R. Together with E496A, another two polymorphisms in P2X7R were found: an isoleucine residue at position 568 that was changed to asparagine (I568N) and a threonine at position 357 that was changed to serine (T357S), both described in human leukocytes [48–50]. T357S was described to cause an impairment in ATP-induced apoptosis, mycobacterial killing in macrophages, and a loss-function of P2X7R in lymphocytes, monocytes, and macrophages [49]. Interestingly, the T357 residue was not predicted to be a phosphorylation site because, in this case, the change of threonine to serine probably did not affect the phosphorylation; however, this change possibly interferes with the reduction reaction carried out by P2X7 due to the loss of a methyl radical from the threonine

residue. T357 (T357–S360) was also shown to be involved in a potential beta-arrestin binding motif, along with T508–S510 and S540–S543, as reported by Feng et al. who also proposed that one of these sequences is involved in P2X7R internalization and recycling triggered by ATP [51]. The third polymorphism identified on the P2X7R C terminus is I568N, which is found in patients with lymphocytic leukemia [48] and located inside the putative P2X7R LPS-binding site (551–581). I568N is critical for correct trafficking of P2X7R and, when expressed on monocytes and macrophages, decreases P2X7R function to a value around 50% below normal.

At the LPS-binding site identified by Denlinger et al. [40, 52], the authors described that the arginine and lysine residues at positions 578 and 579, respectively (R578 and K579), were essential to locate P2X7R on the surface of cells, despite normal levels of total protein expression [52]. Mutations on these residues did not affect protein folding or membrane insertion [52]. The authors suggested that R578 and K579 motifs in the C terminus P2X7R could control receptor trafficking to the membrane. This defect was partially restored at 24–27°C and appears to be involved with recycling events regulated by P2X7R. Mutations of R578 and K579 abolished the interaction with LPS in vitro, lipid-dependent endocytosis, and provoked impairment of potassium currents and pore openings that could lead to improper IL-1 β release [52]. Inside the LPS-binding site, one n-myristoylation site is present (residues 586 to 591—predicted by Proscan (<http://www.bimas.cit.nih.gov/molbio/proscan/>) [53]), emphasizing the importance of this sequence to lipid interactions.

Along with R578 and K579, other amino acid residues were described to be involved in cell surface expression or membrane sub-localization. These residues include the cysteine at position 572 (C572), arginine at 574 (R574), and phenylalanine at 581 (F581), identified by point mutation as unique P2X7R pore-enabling domains [54]; and twelve C terminus cysteine residues of P2X7R [55], including C572. The P2X7R pore-enabling domain is a specific P2X7R region overlapping with a previously identified LPS-binding site [40, 54]. Point mutation experiments have revealed that C572, R574, and F581 are the major amino acid residues related to the complete lack of cell surface expression and also participate in defects in pore opening [54]. Both domains interact with protein members of the epithelial membrane protein family (EMP-1, EMP-2, and EMP-3) [56] involved in cell blebbing, which is one downstream effect of P2X7R triggering activity.

The P2X7R C terminus presents 12 cysteine residues (Fig. 1) that participate in membrane localization [55]. Palmitoylation is a post-translational modification related to the reversible membrane localization signal [57] and is

Table 1 Analysis of putative motifs in the murine P2X7R C terminus (C57B1/6-Q91M0) using Minimotif Miner to compare with the described protein motif and comparison with rat (Q64663) and human (Q99572) P2X7R

AA positions	Mouse P2X7 consensus	Rat P2X7 consensus	Human P2X7 consensus	Motif consensus	Minimotif miner consensus motif
381	NEY	NEY	NEY	[DE]YY	Jak2 is phosphorylated by JAK2; first Tyr is phosphorylated
381	NEY	NEY	NEY	E[FDY]Y	peptide is dephosphorylated by PTP1B; Tyr is dephosphorylated
397	TLKYVSF	TLKYVSF	TLKYVSF	[IVLM]kY[TVAK][VLF]	peptide binds the #1 SH2 domain of SHP-2; Tyr must be phosphorylated
398	LKYVSF	LKYVSF	LKYVSF	Y[AEV][YFESNV][PFIH]	Lymphocyte cytosolic protein 2 binds the #1 SH2 domain of Itk; Tyr must be phosphorylated
478	CGNC	CGNC	CGSC	CxxC	binds Tim and is trafficked to mitochondrial inner membrane
497	LCCR	LCCR	LCCR	LxxRR	Rsk binds Erk1
588	YSGF	YSGF	YSGF	YxxF	Peptide binds the #1 SH2 domain of SHP-1; Tyr must be phosphorylated
528	LYQDP	LYQEP	LYQEP	[IVL]Yx(1,5)[PF]	p73 is phosphorylated by Abl; phosphorylated on Tyr
413, 458 450	DQQL, EIQL PLTP	DQQL, EIQL PLIP	NQQL, EMQL PLIP	[DE]xxLL Px[ST]P	Binds the VHS domain of GGA proteins MBP is phosphorylated by Erk; phosphorylation Ser
450	PLTP	PLIP	PLIP	PxTP	Phosphorylated by CDK1/CyclinB1 complex; phosphorylated on Thr
387	KCES	KCEP	KCES	[KRHDE]C[DE]	NMDA receptor is nitrosylated by an unknown target; Cys is nitrosylated
398	LKYVSF	LKYVSF	LKYVSF	Y[ILV]x[FPYW]	Peptide binds the #1 SH2 domain of Rasa ; Tyr must be phosphorylated
521	DTLQL	EALQL	HVLQFL	[DE]xxxL[L]	LIMP_II binds the Trunk domain of AP1,AP2,AP3 beta subunits
523	LQLL	LQLL	LQLL	LxxLL	Nuclear receptor coactivator 1 isoform 3 binds Oxysterol receptor LXR beta
470, 510, 560 550	SGD, SKL, SQD YRCY	SRD, SEL, SQD YRSY	SRD, SEL, SQD YRCY	SxD Yxx[FY]L	Phosphorylated by CamKII; phosphorylation AP1, AP2, AP3, AP4, and trafficked by internalization
582	PKTE	PKTQG	PKSEG	[VILAF]Kxε	PIASX is sumoylated by SUMO-1; sumoylated
528	LYQDPLL	LYQEPLL	LYQEPLL	YxxP	Crk binds the #1 SH2 domain of Crk; Tyr must be phosphorylated
528	LYQDPLL	LYQEPLL	LYQEPLL	Y[RKHHQED][RKHHQED][IP]	Peptide binds the SH2 domain of Src; Y residues must be phosphorylated
258	LYQDPLL	LYQEPLL	LYQEPLL	Yxx[ILMP]	Peptide binds the #1 SH2 domain of Blnk; Tyr must be phosphorylated
380, 381	NEY, EYYY	NEY, EYYY	NEY, EYYY	[EDY]Y	Peptide is dephosphorylated by TC-PTP; Tyr is dephosphorylated
488, 570 364	PEQRR, PSCCR RSGV	PENRR, PSCCR RSRV	PESHRR, PSCCR RSHI	PxxxR RxGx	Binds the #2 SH3 domain of Grb2 InsP3R1 binds the phosphatase domain of PP1alpha
376, 450, 468, 582	PCT, PLT, PKS, PKT	PCA, PPI, PRS, PKT	PCV, PPI, PRS, PKS	Px[ST]	Sic1 is phosphorylated by Iml1; phosphorylation Ser/Thr

400, 550, 588	YVSE, YRCY, YSGF	YVSE, YRSY, YSGF	YVSF, YRCY, YSGF	Yxx[VILFWYm]	ITAM peptide motif binds the SH2 domain of ZAP70; Tyr must be phosphorylated
437, 449, 470, 519	SDLs, SPLT, SGDS, SRDT	LELS, SPPI, SRDS, SREA	TDLS, TPPI, SRDS, SRHV	Sxx[ST]	Phosphorylated by Casein Kinase I; phosphorylation Ser/Thr
449, 452, 473	SP, TP, SP	SP, IP, SP	TP, IP, SP	[ST]P	Phosphorylated by Erk
358, 553, 588	YSS, YAT, YSG	YAS, YAT, YSG	YSS, YAT, YSG	Y[A/G/S/T/D/E]	Phosphorylated by Src
576	RIRK	RIRK	RIRK	RxxK	Binds the SH3 domain of GADS
491, 500	RRR, RRR	RRR, RRR	HRC, RRR	[KR]R	Factor is proteolyzed by Kexin2; cleaves after C-terminal Arg
539	EAT	EAI	DST	ExT	p27(Kip1) binds Skp2; Thr must be phosphorylated
509, 522	TSKL, TLQL	TSKL, ALQL	TSKL, VLQF	Txx[IL]	Binds the #2 FHA domain of Rad53; Thr must be phosphorylated
387, 399, 505, 557	KCES, KYVS, RCIT, RFGS	KCEP, KYVS, QCIT, RFVS	KCES, KYVS, ACIT, RFGS	[KR]xx[ST]	Rsk is phosphorylated by RSK; phosphorylation Ser/Thr
378, 390, 402, 445	TVNE, SIME, SFVD, SLHD	AVNE, PIVE, SFVD, SLHH	VVNE, SIVE, SFVD, ALHD	[ST]xx[DE]	Phosphorylated by Casein Kinase II; phosphorylation Ser/Thr
370, 400	YC, YV	SC, YV	WC, YV	Y[TLVFIC]	Growth hormone receptor binds the SH2 domain of Stat5; Tyr must be phosphorylated
445, 452	SLHDS, TPGQS	SLHH, IPGQP	ALHD, IPGQP	[ST]xxxS	Glycogen synthase is phosphorylated by GSK3 alpha; phosphorylation Ser/Thr

involved in the clustering and re-location of diverse receptors and membrane proteins through plasma membrane and lipid raft microdomains [57]. Garcia-Marcos et al. demonstrated two different P2X7R populations, one which was associated in clusters on lipid rafts [58]. P2X7R is palmitoylated in peritoneal macrophages, and Gonnord et al. recently showed that ATP stimulations do not affect this palmitoylated state or the distribution of P2X7R in lipid rafts [55], indicating that palmitoylation is important to the location of P2X7R and not to their mobilization after ATP stimulation. Mutations on these twelve cysteine residues compromised the presence of P2X7R on the cell surface and induced receptor expression on the endoplasmic reticulum and lysosomes, whereas the absence of palmitoylated cysteines abolished P2X7R from the Golgi apparatus [55], and the C572 was described as a residue involved in membrane/traffic signal [54]. All of these amino acid residue modifications lead to an impairment of receptor localization on the cell surface, and are consequently related to the lack of pore opening activity. However, we could observed three groups of palmitoylated cysteines (C371–C377, C477–C506, and C572–C573), one possibility is that each cysteine-rich group could be involved with different P2X7R locations, or the sequence of 100 amino acid residues between the first and second cysteine-rich group could interact with different proteins even that these two cysteine-rich groups have been attached to the membrane lipids.

Recently, Roger et al. described that an 18 amino acid sequence rich in cysteine residues (CRSHIYPWCK CCQPCVV—human P2X7R, Fig. 1) contributed to P2X7R Ca^{2+} -independent facilitation current [59]. Animals in which this segment has been deleted showed no Ca^{2+} -independent facilitation current and slower deactivation [59]. Focusing on this linear sequence, we could observe the same cysteine-rich region that was described by Gonnord et al. [55] as a palmitoylation site, and it suggests a possible relationship between cysteine palmitoylation with current properties. However, there is no conclusive evidence that these amino acid residues act directly on pore activity, or if pore activity is compromised by the lack of surface receptors or misplacing localization on plasma membrane. These data suggest an important molecular interaction between P2X7R and phospholipids in the cell membrane, to correct synthesis, trafficking, localization, and subsequent pore activity.

Smart et al. have also demonstrated an important role for the P2X7R C terminus in ethidium uptake requiring over 95% of the C terminus [54]. In *Xenopus laevis* oocyte protein expression, the expression of a P2X7R tail truncated at residue 436 abolished retention in the endoplasmic reticulum and increased the levels of the receptor on the cell surface. However, in HEK-293 cells, P2X7R protein cell surface expression was compromised when a

Fig. 1 Review of motif alignment with the linear amino acid sequence of the P2X7 C terminus and comparison between rat (Q64663), mouse (C57Bl/6-Q91M0 and Balb/c-Q8CHP3), and human (Q99572) P2X7R. X=any amino acid

single amino acid on the C terminus was mutated. To explain this difference between mammalian cells and *Xenopus* oocytes, it is important to remember that these two types of organisms perform post-translational processing differently. If we analyze data by Feng et al. on studies of P2X7R internalization, we observe three bands for P2X7R: 85, 65, and 18 kDa. As suggested by the authors, these variant forms of P2X7R represent different post-translational modifications [51]. The 65-kDa P2X7R, the most common isoform found, is a naïve receptor, whereas the 85-kDa protein is a mature receptor, and the 18-kDa protein is the degraded form. It is possible that receptor maturation is involved in glycosylation events and that there are some substantial differences between human and *Xenopus* glycosylation.

Independent of protein system expression, Becker et al. described two fragments of the P2X7R C terminus related to regulatory gating activity and sensibility [60, 61]. In this report, the authors demonstrated by electrophysiology experiments that the expression of truncated P2X7R (t-P2X7R, 1–436) on the oocyte surface produces a response of 5% of the full-length P2X7R current, while when this t-P2X7R was co-expressed with the C terminus, the response

to ATP-gated current amplitudes had a 10–20% increase [60], reaching levels that were similar to P2X7R wild type. Sub-fragmenting the C terminus in association with electrophysiological experiments, Becker et al. restricted the amino acid residue sequence that was related with gate sensibility and activity to a specific domain: I409–L494. This sequence contains three of the twelve cysteine residues that are palmitoylated and the most conserved SH3 domains described for the P2X7R C terminus [41, 44, 55]. We can predict nine amino acid residues that undergo glycosylation or correlate with modifications in the P2X7R C terminus (K425, T452, and W476). However, the actual importance of these residues on P2X7R has yet to be determined, and until the current moment no suggestions occur related to C terminus glycosylation.

On the most distal portion of the P2X7R C terminus, Adinolfi et al. described a tyrosine phosphorylation site that could act as a negative regulatory domain to receptor activity [62]. The authors showed a protein–protein interaction between human P2X7R and heat shock protein 90 (HSP90) in macrophages, where the HSP90 association with P2X7R occurs at phosphorylated-tyrosine position 550 [62]. The direct interaction of a point mutation in this

residue to a phenylalanine, Y550F, increased the association between HSP90 and decreased the sensitivity to the agonist 15-fold, which was reversed by application of an HSP90 inhibitor, geldanamycin. It was also shown that, when triggered by ATP, the interaction of P2X7R and Src tyrosine kinase occurs in the SH3 domain (described above). In parallel, Kin et al. performed an over expression followed by proteomics analysis of P2X7R using transfected HEK-293 cells, and identified an interaction between P2X7R and protein tyrosine phosphatase beta (RPTPbeta—[35]. In a hypothetical situation, such as a macrophage infection by some microorganism such as *Chlamydia* [63], *Mycobacterium* [64], or *Leishmania amazonensis* [65], when an up-regulation of P2X7R function occurs, it is possible that the infection activates RPTPbeta, which dephosphorylates Y550; this decreases the interaction with HSP90 and increases the agonist sensitivity to P2X7R. The P2X7R induces a mechanism that can kill pathogen microorganisms, but if the microorganism induces the recruitment of Src tyrosine kinase to interact with the SH3 domain of P2X7R, this will cause an up-regulation of the levels of phosphorylated-Y550, consequently attracting more HSP90, and inhibiting the microorganism death.

Amino acid phosphorylation and dephosphorylation are key events in signaling pathways. Phosphorylating Y550 with a tyrosine kinase induces a down regulation of receptor function. At the same time, Gorodeski's group has shown that an unknown amino acid residue is responsible for the up-regulation of P2X7R recycling [51, 66, 67]. Using three different prediction programs (NetPhos, NetPhosK, and MotifScan—Table 2) [68, 69] and http://myhits.isb-sib.ch/cgi-bin/motif_scan, we predicted the existence of other putative phosphorylated residues with threshold levels at 0.70. We found the threonines at 397, 509, and 555 (T397, T509, and T555)

to be predicted PKC phosphorylation sites. It is well-known that P2X7R can recruit different types of protein kinases, such as PKCs [70–73]. PKCs have been described in P2X7 signaling pathways in some cellular contexts. In the astrocyte RBA-2 cell line, ATP and BzATP activate a signaling pathway that stimulates TGF- β 1 RNA expression which is partially blocked by the PKC inhibitors GF109203X and Gö6976, as well as the MAPK inhibitor PD98059 [71]. The P2X7R stimulated by BzATP in rat parotid acinar cells activates PKC and ERK [72]. In the rat submandibular gland, P2X7 activates PKC and stimulates PLD; this effect is blocked by a generic inhibitor of PKC and is absent in P2X7R^{-/-} mice [73]. In the apoptotic pathway, our group has obtained some data suggesting that PKC δ is involved in cell death induced by ATP, but the significance of P2X7R triggering PKC δ is unclear.

Most residues and motifs described thus are very similar to P2X7R in humans, rats, and mice. However, the identity is low, mouse P2X7R is around 80% to human P2X7R and 85% to rat P2X7R [74], which explains some pharmacological differences between mouse (C57Bl/6 and Balb/c), rat, and human P2X7R [74, 75]. The pharmacology properties of P2X7R are not simple to be defined; most of the agonists and classical antagonists present different potential that varies by species and single-nucleotides polymorphism. The first comparisons of rat, mouse, and human P2X7R expressed in the same cell system (human astrocytoma 1321N1 cell) was made by Donnelly-Roberts et al. [75]. The authors observed that some antagonists, such as KN62, were more potent and selective to inhibited human P2X7R cation influx, but no difference was described when pore-formation activity was compared in human and mouse P2X7R [75]. Brilliant Blue G was only selective to inhibited cation influx in rat P2X7R, but no difference was observed to pore-formation activity [75].

Table 2 Putative phosphorylation sites on the mouse P2X7R C terminus (C57Bl/6–Q91M0) predicted by different bioinformatics programs and amino acid residue comparison with rat (Q64663) and human P2X7R (Q99572)

AA position in the mouse P2X7 C terminus	AA position in the rat P2X7 C terminus	AA position in the human P2X7 C terminus	Phosphorylated by	Motif scan	NetPhosK 1.0	NetPhos
S390	P390	S390	CK2 phosphorylation	x		x
T397	T397	T397	PKC phosphorylation	x	x	
S402	S402	S402	CK2 phosphorylation	x		x
S443	S443	P443	PKA phosphorylation		x	x
S445	S445	A445	CK2 phosphorylation	x		x
T452	I452	I452	CK2, p38, cdk5, GSK3		x	x
T509	T509	T509	PKC phosphorylation	x	x	x
T522	A522	V522	PKA phosphorylation		x	
S543	S543	S543	cdc2 phosphorylation		x	
T555	T555	T555	PKC phosphorylation	x	x	

x positive prediction

These single amino acids variations and different motifs sequences explain how hard it is to understand the physiological role and pharmacological differences in P2X7R. For example, BzATP, the most potent agonist to P2X7R, is stronger to stimulated human P2X7R than mouse and rat P2X7R [75]. If we look at the pore sensibility and activation domain (Fig. 1), we could find four amino acid residues present in human P2X7R (N413, R419, A433, and A445) that are not shared in the rat or mouse sequence. These differences could interfere in the YO-PRO uptake as discussed by Donnelly-Roberts et al. [75]. Other interesting data reported by Donnelly-Roberts et al. is that there is no difference in increased of intracellular calcium or Yo-Pro uptake between C57Bl/6 and Balb/c P2X7R when expressed in 1321N1 cell [75]. In both cases, the pharmacological profile was very similar. It suggests that the P451L mutation could be dependent on some protein interaction, as example the Panx1 or other pore-forming protein, and it is dependent of cellular environment.

However, most of the studies that use transfection systems to analyze P2X7R function generally perform their assays with rat-P2X7R. Even though there is significant sequence identity between mouse and human, it is important not to over-interpret data where significant amino acid differences might alter predicted function. A good case example is the calmodulin-binding site of rat-P2X7R [14]. The authors elegantly showed that isoleucine residue 541 (I541) and serine residue 552 (S552) bind to calmodulin, which regulates calcium ion channel activity and subsequent membrane blebbing formation [14]. A point mutation (human–rat) of I541 to threonine (I541T) and S552 to cysteine (S552C) reduced the calcium current by 80% and blocked the co-immunoprecipitation of calmodulin with P2X7R. Importantly, these two point mutations are the native sequence in human and mouse P2X7R (Fig. 2). This finding implies that interactions between calmodulin and P2X7R do not occur in these species. The I541 in rat-P2X7R causes a break in a polar amino acid sequence near a putative P2X7R LPS-binding site, whereas T541 in human and mouse increases the polar characteristics of this motif and may interfere with P2X7R trafficking. Recently, Roger et al. compared the properties of ATP evoked currents and the protein–protein interaction between P2X7R and calmodulin in HEK293 transfected with rat,

human, and triple mutant human P2X7R [59]. The human P2X7R current in comparison with rat P2X7 was slower and the human P2X7 did not interact with calmodulin by co-immunoprecipitation. In an elegant experiment, Roger et al. created a three point mutated human P2X7R, where this mutation corresponded to calmodulin-binding motif described in rat P2X7 (T541I, C552S, and G559V (Glycine to Valine)), the current in triple human mutated P2X7R was similar to rat P2X7R and bigger than wild-type human P2X7R and the calmodulin was found co-immunoprecipitated with the mutated receptor [59].

The link established between calmodulin and P2X7R and their native differences in rat and human suggested that the human expressing P2X7R cells could be more resistant to some ATP biological effects, and one of these effects are related with inflammatory sites. The sensibility of macrophage activation by Gram-negative bacterial endotoxin, such as LPS is central to inflammatory response and endotoxin tolerance. The pre-treatment of RAW 264.2 macrophages with oxidized-ATP inhibited the LPS-stimulated NO production and iNOS expression [76]. Oxidized ATP also inhibited the interleucine-1 maturation in human macrophage [77] or in mice isolated aorta [7]. In C57Bl/6 mice macrophages, RAW264.2 and HEK 293 it was observed the requirement of calmodulin to Toll-like receptors (TLR) 4 (LPS responsive), TLR 3 (dsRNA responsive), and TLR 9 (CpG DNA responsive) in triggering cytokine and interferon type-I production by IRF3 and TAK1 [78]. It suggests that in an inflammatory situation when the host is invaded by some Gram-negative bacteria and the LPS are stimulated, the cytokine and NO production could be dependent or partially dependent of P2X7R-calmodulin or that calmodulin also could be involved in inflammasome activation. If that occurs we could presume that in the rat macrophage the LPS response or inflammasome activation could be more efficient than human and mouse macrophages.

What is expected for the future?

Over the past thirty years, the idea of an ATP molecule has been transformed from a simple energy supply molecule to an important second messenger in many signaling pathways [79]. Since 1976, when Geoffrey Burnstock developed his purinergic co-transmission theory [20, 21] and the ATP molecule was described as the first non-adrenergic and non-cholinergic neurotransmitter in the nervous system up till now, where P1 and P2 purinergic receptors have been cloned, characterized, and targeted therapeutically, the ATP molecule has grown in importance as a second messenger. Our understanding of the biological functions of purinergic receptors are limited, and our understanding of P2X7R is

mouse P2X7r (9Z1M0)	T N S R L R H R A Y R C
human P2X7r (Q99572)	T N S R L R H C A Y R C
rat P2X7r (Q64663)	I N S K L R H C A Y R S
	** : * * * . * * * .
X nonpolar amino acids	X polar amino acids
X aromatic amino acids	X positively charged amino acids

Fig. 2 Comparison between the rat (Q64663), mouse (C57Bl/6–Q91M0), and human (Q99572) P2X7R Calmodulin-binding site as suggested in Refs. [17, 57]

even more restricted. In this review, we have focused specifically on the C terminus portion of P2X7R, a specific domain with an extra 200 amino acid residues compared with other P2X receptors. Here, we have enumerated the most relevant domains and motifs found in the P2X7R C terminus.

Classically, the activation of P2X7R results in the rapid and reversible permeability of the membrane to Ca^{2+} , Na^{+} , and K^{+} ions. This activation requires sustained millimolar concentrations of ATP to stimulate the pore opening, which allows molecules less than 900 Da to enter the cell. Associated with the K^{+} efflux, P2X7R activation results in the activation of caspase 1 [11], culminating with the maturation and release of interleukin 1-beta (IL-1beta) [8], described as part of the inflammasome pathway [80, 81]. Other downstream signaling pathways have also been related to P2X7R, including L-selectin (metalloproteases) membrane shedding [82], activation of phospholipases A_2 and D [9, 16], recruitment and activation of protein kinases such as MAPKs [72], PKC [70, 71], Src [44], and GSK-3 [83, 84], target to phosphatases [62], and membrane blebbing via ROCK [13] and EMPs [56] activation. P2X7R studies are complex because protein function is cell type-specific (e.g., macrophages, monocytes, lymphocytes, kidney cells, or epithelial cells). To understand how P2X7R can trigger multi-pathway signaling, we need to start viewing this receptor as exactly what it is: one ATP-ligand protein that activates different pathways. There is much still to learn. An analysis of the P2X7R sequence using Minimoto Miner Scan [85–87] and focusing on the C terminus has allowed us to find many new and previously undiscovered motifs and domains listed in Table 1.

The key to studying signaling pathways is to understand that protein function is completely dependent on time and space. In other words, the effects of activating one protein are entirely dependent on the timing of the event with regards to the clientele of downstream signaling targets that

are present for interactions. It is important to remember that each receptor is embedded in a microenvironment, inside a particular ordered structure (microenvironment or cellular compartment), maintaining different intermolecular interactions and communications depending on the state of post-translational modifications. P2X7R is not exempt from this paradigm, and it can be found in the plasma membrane, in microdomains such as lipid rafts, and in organelle membranes as well as on the nuclear membrane, where this receptor was predicted to be localized based on the bipartite nuclear localization signal found at R491-R505 (Bipartite nuclear localization signal by MotifScan EXPASY (http://myhits.isb-sib.ch/cgi-bin/motif_scan)). Each one of these cellular compartments is composed of different proteins that probably affect the P2X7R function, but this explains only part of the versatility of this receptor. The proximity of two proteins is no guarantee that the proteins are interacting; for this interaction to occur, it is necessary to have two complementary domains or motifs.

One motif predicted was the consensus binding to SH3 domain. Using the Minimoto Miner Scan, we predicted three proline rich sequences that bind SH3 domains similar to the Grb2 adaptor protein family. The Grb2 family of proteins are adaptor proteins that are expressed in lymphoid and myeloid lineages and are involved in tyrosine kinase signaling organization [88, 89] and binding to LAT (Linker in activated T cell). We also found two PxxxR consensus sequences (P488–R492 and P570–R574) in the SH3 domains of Grb2 and one RxxxK (R576–K579) consensus sequence in GADS. In general, Grb2 is involved in the SOS/Ras/ERK signaling pathway activation [88, 89], and GADS is involved in SLP-76 recruitment leading to tyrosine kinase activation, ending with NF-AT signaling [88, 89]. GADS-deficient animals show significant apoptotic induction in double negative thymocytes and a reduction in thymocyte differentiation [89]. Some authors have demonstrated that thymocytes during T cell maturation

Fig. 3 Influence of P2X7R on body weight gain. **a** C57BL/6 P2X7R knockout (P2X7^{-/-}) and C57BL/6 wild-type mice were fed with normocaloric diet for 3 weeks and their weight measured. The P2X7^{-/-} mice demonstrated a higher body weight gain compared with wild-type mice. **b** C57BL/6, control mice were injected with

vehicle or treated with BBG at 45.5 mg/kg every 48 h by intraperitoneal injection, fed with normocaloric diet for 3 weeks and their weight measured. The BBG-treated mice demonstrated a higher body weight gain compared with controls

respond to extracellular ATP in a P2X7R-dependent manner, which results in apoptosis [19]. At least one author has described that apoptosis induced by P2X7R had an ERK dependent component [10, 90], and during infection by *Trypanosoma cruzi*, double positive thymocytes were more susceptible to apoptosis induced by P2X7R [91]. The significance of these putative motifs to SH3 of the Grb2 family is unclear because no one has studied this interaction; however, it may be important to thymocytes and T cell maturation. It is possible that activated P2X7R recruits Grb2 to trigger ERK signaling or that apoptosis in thymocytes occurs by P2X7R sequestering GADS, which impairs the GADS/SLP76/tyrosine kinase pathway and leads to thymocyte cell death.

The activation of tyrosine kinases by the Grb2 family is dependent on SH2 domains. This domain interacts with phosphorylated tyrosine and coordinates the actions of different tyrosine kinases. SH2 domains are present in the adaptor protein family, such as the Grb2 family, and within tyrosine kinases itself [92, 93]. In the P2X7R C terminus, we found some tyrosines that can be phosphorylated and have consensus binding, such as SH2 motifs, to several tyrosine kinases, such as Src tyrosine kinase described above, and others such as JAK2, Itk, RASA, ZAP70, Crk, and Blnk (for more detail see Table 1). The consensus peptide bind to SH2 motif that we predicted in P2X7R C terminus, situated at N380–K387, is similar to the auto-phosphorylated tyrosine (pY) sequence in JAK2 (pY1008), and this same pY that, binding to SH2, is the substrate to protein-tyrosine phosphatase 1B (PTP1B) [94], is also predicted in the P2X7R C terminus in the same sequence (Table 1).

Phosphorylation and dephosphorylation of JAK2 by JAK2 and PTP1B are involved in obesity and diabetes regulation [95–97] and PTP1B is related to leptin resistance and disruptions of the PTP1B gene are involved in obesity and type 2 diabetes mellitus [98]. Obesity represents a major risk factor in the development of diabetes; in beta cells of islets, a compensatory mechanism exists that upregulates P2X7R in response to an increased insulin demand and the animals knockout to P2X7R [99], suggesting that P2X7R could be involved in controlling obesity. The fact that the P2X7R C terminus has a similar consensus to JAK2, which has been described as a specific substrate to PTP1B, suggests that P2X7R is a good candidate for obesity studies. In fact, our group has preliminary data which shows that when P2X7R knockout mice and mice treated with brilliant blue G (BBG) were compared with wild type (WT) or non-treated, these animals gain more weight than controls in normocaloric diet (Fig. 3).

P2X7R is also relevant to some human diseases and pathologies. In human bone disease, mutations in the P2X7R C terminus at E496A and I568N have been

associated with the loss-of-function of the receptor, increasing the risk of fracture in post-menopausal females [100]. Dysfunction of P2X7R function causes alterations in inflammatory response [101, 102] and pain [103, 104], and the inhibition of P2X7R is involved in spinal cord regeneration [105].

There are many open questions about P2X7R function, activation, and specificity. In this review, we discussed different domains and motifs present in the P2X7R C terminus as well as their functions and importance in P2X7R biology. We also predicted some interesting putative domains and suggested some putative biological functions for each (summarized in Table 1). However, we know that there is much still to do to understand the complete role of the P2X7R C terminus and its therapeutic potential.

Acknowledgment Supported by funds from the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Programa de Apoio a Núcleos de Excelência (PRONEX), Brazil.

References

1. Erb L, Liao Z, Seye CI, Weisman GA (2006) P2 receptors: intracellular signaling. *Pflugers Arch* 452(5):552–562
2. North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82(4):1013–1067
3. 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
4. Persechini PM, Bisaggio RC, Alves-Neto JL, Coutinho-Silva R (1998) Extracellular ATP in the lymphohematopoietic system: P2Z purinoceptors off membrane permeabilization. *Braz J Med Biol Res* 31(1):25–34
5. Coutinho-Silva R, Alves LA, de Carvalho AC, Savino W, Persechini PM (1996) Characterization of P2Z purinergic receptors on phagocytic cells of the thymic reticulum in culture. *Biochim Biophys Acta* 1280(2):217–222
6. Clark AK, Staniland AA, Marchand F, Kaan TK, McMahon SB, Malcangio M (2010) P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. *J Neurosci* 30(2):573–582
7. Chiao CW, Tostes RC, Webb RC (2008) P2X7 receptor activation amplifies lipopolysaccharide-induced vascular hyporeactivity via interleukin-1 beta release. *J Pharmacol Exp Ther* 326(3):864–870
8. Qu Y, Franchi L, Nunez G, Dubyak GR (2007) Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol* 179(3):1913–1925
9. Coutinho-Silva R, Stahl L, Raymond MN, Jungas T, Verbeke P, Burnstock G, Darville T, Ojcius DM (2003) Inhibition of chlamydial infectious activity due to P2X7R-dependent phospholipase D activation. *Immunity* 19(3):403–412
10. Bulanova E, Budagian V, Orinska Z, Hein M, Petersen F, Thon L, Adam D, Bulfone-Paus S (2005) Extracellular ATP induces cytokine expression and apoptosis through P2X7 receptor in murine mast cells. *J Immunol* 174(7):3880–3890

11. Kahlenberg JM, Dubyak GR (2004) Mechanisms of caspase-1 activation by P2X7 receptor-mediated K⁺ release. *Am J Physiol Cell Physiol* 286(5):C1100–C1108
12. Kahlenberg JM, Lundberg KC, Kertesz SB, Qu Y, Dubyak GR (2005) Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF- κ B-driven protein synthesis. *J Immunol* 175(11):7611–7622
13. Morelli A, Chiozzi P, Chiesa A, Ferrari D, Sanz JM, Falzoni S, Pinton P, Rizzuto R, Olson MF, Di Virgilio F (2003) Extracellular ATP causes ROCK I-dependent bleb formation in P2X7-transfected HEK293 cells. *Mol Biol Cell* 14(7):2655–2664
14. 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
15. Alzola E, Perez-Etxebarria A, Kabre E, Fogarty DJ, Metioui M, Chaib N, Macarulla JM, Matute C, Dehaye JP, Marino A (1998) Activation by P2X7 agonists of two phospholipases A2 (PLA2) in ductal cells of rat submandibular gland. Coupling of the calcium-independent PLA2 with kallikrein secretion. *J Biol Chem* 273(46):30208–30217
16. Costa-Junior HM, Mendes AN, Davis GH, da Cruz CM, Ventura AL, Serezani CH, Faccioli LH, Nomizo A, Freire-de-Lima CG, Bisaggio C (2009) Rda, and P.M. Persechini, ATP-induced apoptosis involves a Ca²⁺-independent phospholipase A2 and 5-lipoxygenase in macrophages. *Prostaglandins Other Lipid Mediat* 88(1–2):51–61
17. Hewinson J, Mackenzie AB (2007) P2X(7) receptor-mediated reactive oxygen and nitrogen species formation: from receptor to generators. *Biochem Soc Trans* 35(Pt 5):1168–1170
18. Bianco F, Ceruti S, Colombo A, Fumagalli M, Ferrari D, Pizzirani C, Matteoli M, Di Virgilio F, Abbracchio MP, Verderio C (2006) A role for P2X7 in microglial proliferation. *J Neurochem* 99(3):745–758
19. Tsukimoto M, Maehata M, Harada H, Ikari A, Takagi K, Degawa M (2006) P2X7 receptor-dependent cell death is modulated during murine T cell maturation and mediated by dual signaling pathways. *J Immunol* 177(5):2842–2850
20. Burnstock G, Fredholm BB, North RA, Verkhratsky A (2010) The birth and postnatal development of purinergic signalling. *Acta Physiol (Oxf)* 199(2):93–147
21. Burnstock G (2009) Purinergic signalling: past, present and future. *Braz J Med Biol Res* 42(1):3–8
22. Tsukimoto M, Harada H, Ikari A, Takagi K (2005) Involvement of chloride in apoptotic cell death induced by activation of ATP-sensitive P2X7 purinoceptor. *J Biol Chem* 280(4):2653–2658
23. Virginio C, MacKenzie A, North RA, Surprenant A (1999) Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. *J Physiol* 519(Pt 2):335–346
24. Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, Scemes E (2008) P2X7 receptor-pannexin 1 complex: pharmacology and signaling. *Am J Physiol Cell Physiol* 295(3):C752–C760
25. Fortes FS, Pecora IL, Persechini PM, Hurtado S, Costa V, Coutinho-Silva R, Braga MB, Silva-Filho FC, Bisaggio RC, De Farias FP, Scemes E, De Carvalho AC, Goldenberg RC (2004) Modulation of intercellular communication in macrophages: possible interactions between GAP junctions and P2 receptors. *J Cell Sci* 117(Pt 20):4717–4726
26. Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1 β release by the ATP-gated P2X7 receptor. *EMBO J* 25(21):5071–5082
27. D'Hondt C, Ponsaerts R, De Smedt H, Bultynck G, Himpens B (2009) Pannexins, distant relatives of the connexin family with specific cellular functions? *Bioessays* 31(9):953–974
28. Scemes E, Suadicani SO, Dahl G, Spray DC (2007) Connexin and pannexin mediated cell-cell communication. *Neuron Glia Biol* 3(3):199–208
29. Yen MR, Saier MH Jr (2007) Gap junctional proteins of animals: the innexin/pannexin superfamily. *Prog Biophys Mol Biol* 94(1–2):5–14
30. 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
31. 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
32. Pelegrin P, Surprenant A (2007) Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1 β release through a dye uptake-independent pathway. *J Biol Chem* 282(4):2386–2394
33. Pelegrin P, Barroso-Gutierrez C, Surprenant A (2008) P2X7 receptor differentially couples to distinct release pathways for IL-1 β in mouse macrophage. *J Immunol* 180(11):7147–7157
34. Schachter J, Motta AP, de Souza Zamorano A, da Silva-Souza HA, Guimaraes MZ, Persechini PM (2008) ATP-induced P2X7-associated uptake of large molecules involves distinct mechanisms for cations and anions in macrophages. *J Cell Sci* 121(Pt 19):3261–3270
35. 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
36. Gu BJ, Rathsam C, Stokes L, McGeachie AB, Wiley JS (2009) Extracellular ATP dissociates nonmuscle myosin from P2X(7) complex: this dissociation regulates P2X(7) pore formation. *Am J Physiol Cell Physiol* 297(2):C430–C439
37. 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
38. 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
39. 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 P2X(7) reveals a key residue important for receptor function. *Biochemistry* 49(22):4611–4619
40. 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
41. 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
42. Le Stunff H, Auger R, Kanellopoulos J, Raymond MN (2004) The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes. *J Biol Chem* 279(17):16918–16926
43. 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
44. Suadicani, S.O., R. Iglesias, D.C. Spray, and E. Scemes (2009) Point mutation in the mouse P2X7 receptor affects intercellular calcium waves in astrocytes. *ASN Neuro* 1(1)
45. Delarasse C, Gonnord P, Galante M, Auger R, Daniel H, Motta I, Kanellopoulos JM (2009) Neural progenitor cell death is induced by extracellular ATP via ligation of P2X7 receptor. *J Neurochem* 109(3):846–857
46. 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

47. Wiley JS, Dao-Ung LP, Gu BJ, Sluyter R, Shemon AN, Li C, Taper J, Gallo J, Manoharan A (2002) A loss-of-function polymorphic mutation in the cytolytic P2X7 receptor gene and chronic lymphocytic leukaemia: a molecular study. *Lancet* 359(9312):1114–1119
48. 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
49. Shemon AN, Sluyter R, Fernando SL, Clarke AL, Dao-Ung LP, Skarratt KK, Saunders BM, Tan KS, Gu BJ, Fuller SJ, Britton WJ, Petrou S, Wiley JS (2006) A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X7 function and impairs ATP-induced mycobacterial killing by macrophages. *J Biol Chem* 281(4):2079–2086
50. 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
51. 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
52. Denlinger LC, Sommer JA, Parker K, Gudipaty L, Fiset 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
53. Combet C, Blanchet C, Geourjon C, Deleage G (2000) NPS@: network protein sequence analysis. *Trends Biochem Sci* 25(3):147–150
54. 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
55. 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
56. 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
57. Resh, M.D. (2006) Palmitoylation of ligands, receptors, and intracellular signaling molecules. *Sci STKE* (359):re14.
58. Garcia-Marcos M, Perez-Andres E, Tandel S, Fontanils U, Kumps A, Kabre E, Gomez-Munoz A, Marino A, Dehaye JP, Pochet S (2006) Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. *J Lipid Res* 47(4):705–714
59. Roger S, Gillet L, Baroja-Mazo A, Surprenant A, Pelegrin P (2010) C-terminal calmodulin-binding motif differentially controls human and rat P2X7 receptor current facilitation. *J Biol Chem* 285(23):17514–17524
60. Becker D, Woltersdorf R, Boldt W, Schmitz S, Braam U, Schmalzing G, Markwardt F (2008) The P2X7 carboxyl tail is a regulatory module of P2X7 receptor channel activity. *J Biol Chem* 283(37):25725–25734
61. Boldt W, Klapperstuck M, Buttner C, Sadtler S, Schmalzing G, Markwardt F (2003) Glu496Ala polymorphism of human P2X7 receptor does not affect its electrophysiological phenotype. *Am J Physiol Cell Physiol* 284(3):C749–C756
62. 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
63. Coutinho-Silva R, Perfettini JL, Persechini PM, Dautry-Varsat A, Ojcius DM (2001) Modulation of P2Z/P2X(7) receptor activity in macrophages infected with *Chlamydia psittaci*. *Am J Physiol Cell Physiol* 280(1):C81–C89
64. Fairbairn IP, Stober CB, Kumararatne DS, Lammas DA (2001) ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X(7)-dependent process inducing bacterial death by phagosome-lysosome fusion. *J Immunol* 167(6):3300–3307
65. Chaves SP, Torres-Santos EC, Marques C, Figliuolo VR, Persechini PM, Coutinho-Silva R, Rossi-Bergmann B (2009) Modulation of P2X(7) purinergic receptor in macrophages by *Leishmania amazonensis* and its role in parasite elimination. *Microbes Infect* 11(10–11):842–849
66. Feng YH, Li X, Zeng R, Gorodeski GI (2006) Endogenously expressed truncated P2X7 receptor lacking the C-terminus is preferentially upregulated in epithelial cancer cells and fails to mediate ligand-induced pore formation and apoptosis. *Nucleosides Nucleotides Nucleic Acids* 25(9–11):1271–1276
67. 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
68. Blom N, Gammeltoft S, Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 294(5):1351–1362
69. Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4(6):1633–1649
70. Armstrong S, Pereverzev A, Dixon SJ, Sims SM (2009) Activation of P2X7 receptors causes isoform-specific translocation of protein kinase C in osteoclasts. *J Cell Sci* 122(Pt 1):136–144
71. Wang CM, Chang YY, Sun SH (2003) Activation of P2X7 purinoceptor-stimulated TGF-beta 1 mRNA expression involves PKC/MAPK signalling pathway in a rat brain-derived type-2 astrocyte cell line, RBA-2. *Cell Signal* 15(12):1129–1137
72. Bradford MD, Soltoff SP (2002) P2X7 receptors activate protein kinase D and p42/p44 mitogen-activated protein kinase (MAPK) downstream of protein kinase C. *Biochem J* 366(Pt 3):745–755
73. Pochet S, Gomez-Munoz A, Marino A, Dehaye JP (2003) Regulation of phospholipase D by P2X7 receptors in submandibular ductal cells. *Cell Signal* 15(10):927–935
74. 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
75. Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF (2009) Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. *Br J Pharmacol* 157(7):1203–1214
76. Hu Y, Fiset PL, Denlinger LC, Guadarrama AG, Sommer JA, Proctor RA, Bertics PJ (1998) Purinergic receptor modulation of lipopolysaccharide signaling and inducible nitric-oxide synthase expression in RAW 264.7 macrophages. *J Biol Chem* 273(42):27170–27175
77. Ferrari D, Chiozzi P, Falzoni S, Dal M (1997) Susino, L. Melchiorri, O.R. Baricordi, and F. Di Virgilio, Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. *J Immunol* 159(3):1451–1458
78. Liu X, Yao M, Li N, Wang C, Zheng Y, Cao X (2008) CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. *Blood* 112(13):4961–4970
79. Khakh BS, Burnstock G (2009) The double life of ATP. *Sci Am* 301(6):84–90, 92
80. Di Virgilio F (2007) Liaisons dangereuses: P2X(7) and the inflammasome. *Trends Pharmacol Sci* 28(9):465–472
81. 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
82. Moon H, Na HY, Chong KH, Kim TJ (2006) P2X7 receptor-dependent ATP-induced shedding of CD27 in mouse lymphocytes. *Immunol Lett* 102(1):98–105
 83. Ortega F, Perez-Sen R, Morente V, Delicado EG, Miras-Portugal MT (2010) P2X7, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. *Cell Mol Life Sci* 67(10):1723–1733
 84. Ortega F, Perez-Sen R, Delicado EG, Miras-Portugal MT (2009) P2X7 nucleotide receptor is coupled to GSK-3 inhibition and neuroprotection in cerebellar granule neurons. *Neurotox Res* 15(3):193–204
 85. Balla S, Thapar V, Verma S, Luong T, Faghri T, Huang CH, Rajasekaran S, del Campo JJ, Shinn JH, Mohler WA, Maciejewski MW, Gryk MR, Piccirillo B, Schiller SR, Schiller MR (2006) MinmotifMiner: a tool for investigating protein function. *Nat Meth* 3(3):175–177
 86. Schiller, M.R. (2007) Minmotif miner: a computational tool to investigate protein function, disease, and genetic diversity. *Curr Protoc Protein Sci Chapter 2: p. Unit 2 12*
 87. Rajasekaran, S., S. Balla, P. Gradie, M.R. Gryk, K. Kadaveru, V. Kundeti, M.W. Maciejewski, T. Mi, N. Rubino, J. Vyas, and M. R. Schiller (2009) Minmotif miner 2nd release: a database and web system for motif search. *Nucleic Acids Res* 37(Database issue):D185–D190
 88. Yamasaki S, Nishida K, Sakuma M, Berry D, McGlade CJ, Hirano T, Saito T (2003) Gads/Grb2-mediated association with LAT is critical for the inhibitory function of Gab2 in T cells. *Mol Cell Biol* 23(7):2515–2529
 89. Liu SK, Berry DM, McGlade CJ (2001) The role of Gads in hematopoietic cell signalling. *Oncogene* 20(44):6284–6290
 90. Auger R, Motta I, Benihoud K, Ojcius DM, Kanellopoulos JM (2005) A role for mitogen-activated protein kinase(Erk1/2) activation and non-selective pore formation in P2X7 receptor-mediated thymocyte death. *J Biol Chem* 280(30):28142–28151
 91. Mantuano-Barradas M, Henriques-Pons A, Araujo-Jorge TC, Di Virgilio F, Coutinho-Silva R, Persechini PM (2003) Extracellular ATP induces cell death in CD4+/CD8+ double-positive thymocytes in mice infected with *Trypanosoma cruzi*. *Microbes Infect* 5(15):1363–1371
 92. Sarmay G, Angyal A, Kertesz A, Maus M, Medgyesi D (2006) The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. *Immunol Lett* 104(1–2):76–82
 93. Maus M, Medgyesi D, Kovcsdi D, Csuka D, Koncz G, Sarmay G (2009) Grb2 associated binder 2 couples B-cell receptor to cell survival. *Cell Signal* 21(2):220–227
 94. Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, Barford D, Tonks NK (2001) TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J Biol Chem* 276(51):47771–47774
 95. Rondinone CM, Trevillyan JM, Clampitt J, Gum RJ, Berg C, Kroeger P, Frost L, Zinker BA, Reilly R, Ulrich R, Butler M, Monia BP, Jirousek MR, Waring JF (2002) Protein tyrosine phosphatase 1B reduction regulates adiposity and expression of genes involved in lipogenesis. *Diabetes* 51(8):2405–2411
 96. Morrison CD, White CL, Wang Z, Lee SY, Lawrence DS, Cefalu WT, Zhang ZY, Gettys TW (2007) Increased hypothalamic protein tyrosine phosphatase 1B contributes to leptin resistance with age. *Endocrinology* 148(1):433–440
 97. Zabolotny JM, Kim YB, Welsh LA, Kershaw EE, Neel BG, Kahn BB (2008) Protein-tyrosine phosphatase 1B expression is induced by inflammation in vivo. *J Biol Chem* 283(21):14230–14241
 98. Koren S, Fantus IG (2007) Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract Res Clin Endocrinol Metab* 21(4):621–640
 99. Glas R, Sauter NS, Schulthess FT, Shu L, Oberholzer J, Maedler K (2009) Purinergic P2X7 receptors regulate secretion of interleukin-1 receptor antagonist and beta cell function and survival. *Diabetologia* 52(8):1579–1588
 100. Ohlendorff SD, Tofteng CL, Jensen JE, Petersen S, Civitelli R, Fenger M, Abrahamsen B, Hermann AP, Eiken P, Jorgensen NR (2007) Single nucleotide polymorphisms in the P2X7 gene are associated to fracture risk and to effect of estrogen treatment. *Pharmacogenet Genomics* 17(7):555–567
 101. Takenouchi T, Sekiyama K, Sekigawa A, Fujita M, Waragai M, Sugama S, Iwamaru Y, Kitani H, Hashimoto M (2010) P2X7 receptor signaling pathway as a therapeutic target for neurodegenerative diseases. *Arch Immunol Ther Exp (Warsz)* 58(2):91–96
 102. Romagnoli R, Baraldi PG, Cruz-Lopez O, Lopez-Cara C, Preti D, Borea PA, Gessi S (2008) The P2X7 receptor as a therapeutic target. *Expert Opin Ther Targets* 12(5):647–661
 103. 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
 104. Dray A (2008) Neuropathic pain: emerging treatments. *Br J Anaesth* 101(1):48–58
 105. 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 USA* 106(30):12489–12493