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Review

The participation of plasma membrane hemichannels to purinergic signaling ☆

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ABSTRACT

The field of hemichannels is closely related to the purinergic signaling and both areas have been growing in parallel. Hemichannels open in response to a wide range of stressful conditions, such as ischemia, pressure or swelling. Hemichannels represent an important mechanism for the cellular release of adenosine 5′-triphosphate (ATP), which is an agonist of the P2Y and P2X family of purinergic receptors. Therefore, hemichannels are key molecules in the regulation of purinergic receptor activation, during physiological and pathophysiological conditions. Furthermore, purinergic receptor activation can also lead to the opening of hemichannels and the subsequent amplification of purinergic signaling via a positive signaling feedback loop, giving rise to the concept of ATP-induced ATP release. Purinergic receptor signaling is involved in regulating many physiological and pathophysiological processes. P2Y receptors activate inositol trisphosphate and transiently increase intracellular calcium. This signaling opens both connexin and pannexin channels, therefore contributing to the expansion of calcium waves across astrocytes and epithelial cells. In addition, several of the P2X receptor subtypes, including the P2X2, P2X4 and P2X7 receptors, activate select cellular permeation pathways to large molecules, including the pannexin-1 channels, which are involved in the initiation of inflammatory responses and cell death. Consequently, the interplay between purinergic receptors and hemichannels could represent a novel target with substantial therapeutic implications in areas such as chronic pain, inflammation or atherosclerosis. This article is part of a Special Issue entitled: The communicating junctions, roles and dysfunctions.

Contents

Abbreviations: αβmeATP, α,β-methylene ATP; APC, Antigen presenting cell; ATP, Adenosine 5′-triphosphate; BCECs, Bovine corneal endothelial cells; BzATP, 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP; cAMP, Cyclic adenosine 5′-monophasphate; CD39, ENTPD1; CD73, NT5E; CNS, Central nervous system; Cx, Connexin; DAMP, Danger associated molecular pattern; EC, Endothelia cell; EET, Epoxyeicosatrienoic acid; ENTPD1, Ecto-nucleoside triphosphate diphosphohydrolase 1; ERK, Extracellular signal-regulated protein kinases; IHC, Inner hair cells; IP3, Inositol trisphosphate; MAPK, Mitogen activated protein kinase; MHC, Major histocompatibility complex; NMDG, *N*-methyl-D-glucamine; NO, Nitric oxide; NT5E, Ecto-5′-nucleotidase; OHC, Outer hair cells; OoC, Organ of Corti; PAMP, Pathogen associated molecular pattern; PLC, Phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; RhoA, Ras homolog gene family member A; SMC, Smooth muscle cells; TCR, T-cell receptor; TM, Transmembrane; TNP-ATP, 2′,3′-*O*-(2,4,6-Trinitrophenyl)-ATP; VCAM-1, Vascular cell adhesion molecule 1

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1. Introduction

1.1. Historic perspective of purinergic signaling pathways

In 1929, Drury and Szent-Gyorgyi described the potent actions of the extracellular application of adenine compounds on the heart [1]. Four decades later, ATP was proposed as the transmitter responsible for non-adrenergic, non-cholinergic transmission in the gut and bladder. In 1972 Burnstock introduced the concept of purinergic signaling [2], in which extracellular purines (most notably ATP, adenosine, and pyrimidine) act as extracellular signaling molecules. Both, pyrimidine and purine nucleotides, are released from cells through several physiologically relevant mechanisms, including diffusion through membrane hemichannels, activation of membrane transporters and vesicular exocytosis [3–6]. In addition, purines and pyrimidines are released from dying cells: this being an early indicator for cell damage [5,7]. Upon release, ATP (and other nucleotides) are enzymatically degraded within seconds by an extended family of ectonucleotidases [8]. This process is physiologically relevant as ATP metabolites are also agonists of purinergic receptors (Fig. 1). These purinergic signaling molecules activate three receptor classes: metabotropic P1 receptors, which are activated by adenosine, while the nucleotide receptors comprise the P2 family and are subdivided into P2Y metabotropic and P2X ionotropic sub-classes (Fig. 1) [6,9–13].

The purinergic signaling is a primitive system, ATP is an ancient and fundamentally important biological molecule. There are purinergic receptors in ancestral green algae and early in the fungi lineage [14,15]. Purinergic signaling is implicated in many neuronal and non-neuronal mechanisms, including exocrine and endocrine secretion, immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation [16,17]. In addition, purinergic signaling processes are implicated in mediating cell proliferation, differentiation and death [18,19]. ATP-mediated signaling has been identified in virtually all tissues and cell types and appears to be the most widespread and omnipresent of all known extracellular signaling molecules.

1.2. P1 or adenosine receptors

The P1 class of purinergic receptor was first described in 1989 and comprises four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fig. 1) [20–25]. The nomenclature used follows the Alexander et al. guide [26]. In common with other G protein-coupled receptors, they contain seven transmembrane (TM) domains of approximately 21 to 28 hydrophobic amino acids composing a α-helix structure. The amino-terminus of the protein is present on the extracellular side of the plasma membrane, while the protein's carboxy-terminus lies on the cytoplasmic side of the membrane. A pocket for the ligand-binding site is formed by the three-dimensional arrangement of the α-helical TM domains, and the agonist is believed to bind within the upper half of this pore [11]. Binding of the agonist to the receptor gives rise to a stable configuration as has been shown by x-ray crystallography with the A_{2A} receptor [27]. The TM domains are connected by three extracellular and three cytoplasmic hydrophilic loops [11]. P1 receptors are coupled to adenylate cyclase signaling pathways in which A_1 and A_3 receptor activations have inhibitory effects on adenylate cyclase through the Gi/o protein α-subunits. Activation of the A_{2A} and A_{2B} receptors stimulates the production of cyclic AMP (cAMP) via adenylate cyclase through the Gs protein [6,22,28].

A number of selective agonists and antagonists have been identified for several of the P1 adenosine receptor subtypes including agonists for the A_1, A_{2A} and A_{2B} receptors, and antagonists for the A_1, A_{2B} and A_3 receptors [11].

1.3. Purinergic P2Y receptors

The first P2Y receptor ($P2Y_1$) was cloned in 1993 [29,30], and since then several other subtypes have been isolated. Eight human P2Y receptor subtypes have so far been identified, the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors [12,31] (Fig. 1). A number of putative P2Y receptor subtypes have also been identified but these correspond to non-mammalian orthologs, or receptors with sequence homology to P2Y receptors, but for which there is no functional evidence of activation by nucleotides. Recently, the orphan receptor GPR_{80}/GPR_{99} was named $P2Y_{15}$ receptor on the basis that it would be activated by AMP [32].

Molecular studies have shed light upon the mechanisms of receptor activation. Site-directed mutagenesis of the $P2Y_1$ and $P2Y_2$ receptors has shown that some positively charged residues in TM3, TM6 and TM7 are crucial for receptor activation by nucleotides [33,34]. Furthermore four cysteine residues in the extracellular loops, which are conserved in P2Y receptors, are known to be essential for proper trafficking of the receptor to the cell surface [35].

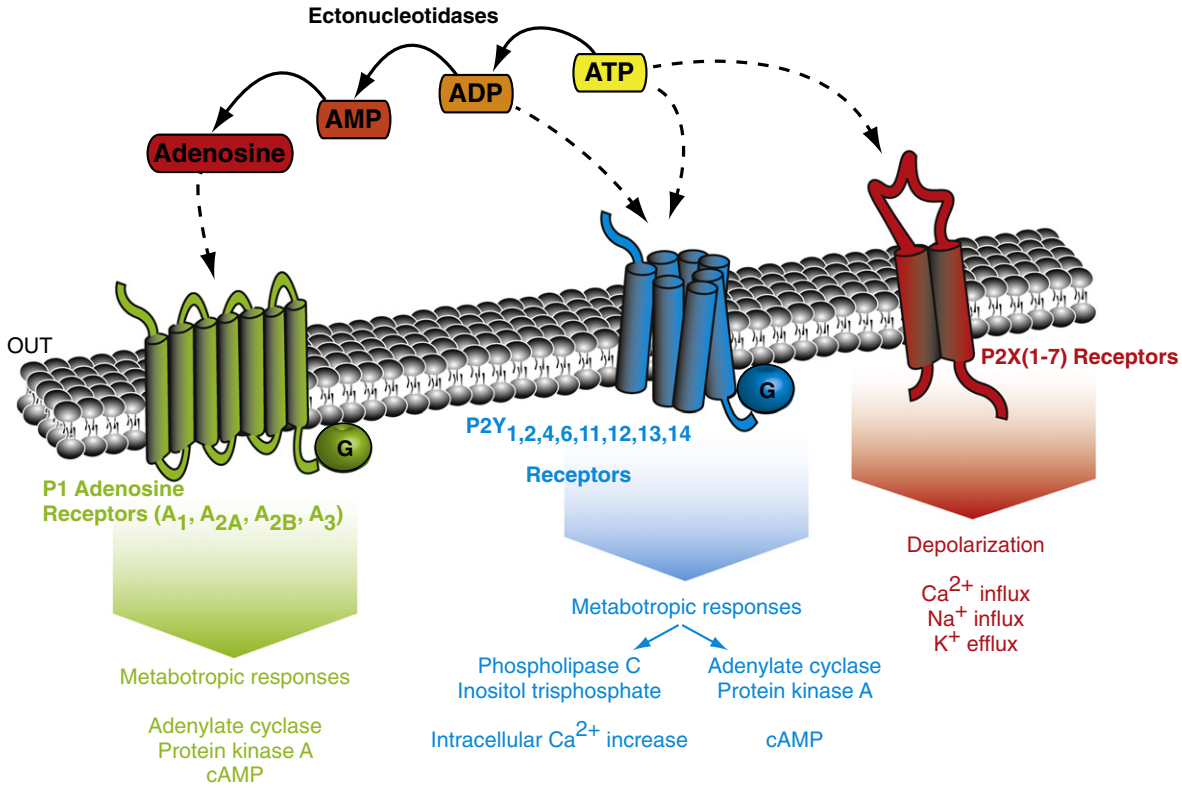

Fig. 1. The purinergic receptor family. Extracellular ATP is the agonist of both P2X and P2Y receptors and is also the substrate of ectonucleotidases, which degrade ATP to adenosine and transiently generate ADP, providing the agonist for P2Y receptors. Adenosine, the final product of adenine nucleotide hydrolysis, activates P1 or adenosine receptors. P2X receptors are ionotropic and their activation opens a cationic conduit, resulting in cellular hyperpolarization via K^+ efflux and extracellular Na^+ and Ca^{2+} influx. P2Y receptors are metabotropic G-protein coupled receptors and their activation leads to phospholipase C activation, generation of inositol trisphosphate and rise of cytosolic Ca^{2+} from intracellular stores, alternatively P2Y receptors could also couple to adenylate cyclase activation. Adenosine receptors are also G-protein coupled receptors that differentially regulate the production of cAMP; whereas A_{2A} and A_{2B} activate adenylate cyclase and increase the levels of cAMP, A_1 and A_3 receptors inhibit adenylate cyclase.

P2Y receptors couple to G proteins and activate the cAMP or inositol trisphosphate (IP3) second messenger systems [12]. The lack of potent and selective agonists and antagonists for many of the P2Y receptor subtypes has limited research in this field to focus upon studying the $P2Y_1$, $P2Y_4$, $P2Y_{13}$ and $P2Y_{14}$ receptors. The most striking difference between the P2Y receptor subtypes is their ability to use different natural nucleotides as agonists. The $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors preferentially utilize ADP, while $P2Y_{11}$ has a high affinity for ATP. The $P2Y_2$ and $P2Y_4$ receptors utilize UTP while UDP is the agonist for the $P2Y_6$ receptor. Finally UDP-sugars activate the $P2Y_{14}$ receptor [12]. The various P2Y receptors are functionally coupled to distinct G proteins and therefore their downstream signaling varies among subtypes, activating phospholipase C (PLC), IP3, intracellular calcium increase or cAMP production [12]. Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) are non-selective antagonists of the P2Y receptors, but also antagonize the P2X receptors (see later Section 1.4). The PLC inhibitor U73122 is commonly used to block the intracellular calcium rise associated with P2Y receptor mediated downstream signaling [36]. Recently, selective antagonists for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors have been developed by the pharmaceutical industry [12].

1.4. Purinergic P2X receptors

The ionotropic P2X receptors are ATP-gated cation channels, selective for Na^+, K^+ and Ca^{2+} ions [37]. The first cDNA encoding a P2X receptor (P2X1) was isolated in 1994 [38,39] and since then, seven different P2X receptor subunits have been identified (P2X1 to P2X7) [6,37,40].

The members of this receptor family show conserved subunit topology. They comprise two TM spanning regions and possess intracellular amino- and carboxy-terminus which contain consensus binding motifs for protein kinases. A large extracellular loop exits which contains 10 conserved cysteine residues and is capable of forming a series of disulfide bridges. The first TM is involved in channel gating while the second lines the ion pore in the membrane [37,41–44]. P2X receptor subunits are capable of forming functional homomeric and heteromeric trimers.

1.4.1. Homomeric P2X receptors

P2X receptor homomeric subtypes are non-specific cation channels and after activation there is an influx of calcium and sodium. The P2X1, P2X2, P2X3, P2X4 and P2X7 are the most studied P2X receptor subunits. P2X1 and P2X3 show a fast desensitization in contrast to the P2X2, P2X4 and P2X7 receptors. While α,β-methylene ATP (αβ-meATP) is a potent agonist for P2X1 and P2X3 receptors, there are no specific ligands for the P2X2 receptor [39,45,46]. Suramin, PPADS and 2′,3′-*O*-(2,4,6-Trinitrophenyl) (TNP)-ATP can be used as non-selective P2X antagonists [47], but specific antagonists for P2X1 and P2X3 receptors now exist [45,46]. Homomeric P2X4 receptors are activated by ATP but not by αβme-ATP. ATP-evoked currents at P2X4 receptors are potentiated by ivermectin, cibacron blue and zinc [46,48]. Prolonged ATP application of several seconds, to P2X2 and P2X4 receptor channels results in an increasing permeability to large organic cations such as *N*-methyl-D-glucamine (NMDG) [49]. This increase in permeability is also the main feature of the homomeric P2X7 receptor, inducing the opening of a large pore that allows large dye molecules, such as ethidium bromide or YO-PRO-1, to enter the cell (see Section 3.1). Among all the P2X receptors, P2X7 receptor stimulation induces structural changes in the cell such as microvesicle shedding, membrane blebbing, activation of MAPK and ultimately cell death [50–52]. A potent agonist for the

P2X7 receptor is 2′,3′-*O*-(benzoyl-4-benzoyl)-ATP (BzATP); however, BzATP is an effective agonist at similar or lower concentrations at other P2X receptors [37,46]. Selective antagonists for P2X7 receptor have been developed by pharmaceutical companies as potential anti-inflammatory drugs [53].

1.4.2. Heteromeric P2X receptors

Heteromeric receptor subunits were first described after their co-expression in *Xenopus* oocytes and subsequent functional analysis using voltage patch clamp. The most physiologically relevant of these heteromeric receptors are the P2X1/2, P2X2/3, P2X1/4 and P2X1/5 receptors. P2X1 and P2X2 receptors co-expression yielded a mixed population of homomeric and heteromeric receptors. The heteromeric P2X1/2 ion channels showed a novel sensitivity to extracellular pH [54]. It has been claimed that trimeric P2X1/2 receptors incorporate one P2X1 and two P2X2 subunits [54–56]. P2X2/3 heteromeric channels are characterized by a sustained current elicited by αβ-meATP [46,56,57]. They also share some properties with homomeric P2X2 receptors in that they are potentiated by low pH, and they desensitize slowly [57,58]. Voltage clamp experiments revealed functional P2X receptors with kinetic properties resembling that of homomeric P2X4 receptors, and a pharmacological profile similar to homomeric P2X1 receptors [49]. The defining phenotype of the P2X1/5 heteromer is a sustained current evoked by αβ-meATP and a higher sensitivity to ATP. This is not seen in the P2X1 and P2X5 homomeric channels [5,46,59].

1.5. Purinergic receptors and hemichannels

Gap junctions are intercellular channels that allow the passage of ions and other small molecules between neighboring cells. They are formed from two multimeric subunits called hemichannels or connexons that reside in the plasma membranes of two closely opposed cells. Connexons are composed of six transmembrane protein subunits called connexins, with intracellular amino- and carboxy-termini. Several connexins have been reported to form functional hemichannels when expressed in *Xenopus* oocytes [60], raising the intriguing possibility that connexons may function as transmembrane ion channels in addition to serving as precursors in the formation of gap junction channels [61]. The opening of hemichannels occurs in response to many physiological and pathological situations, including volume regulation, proliferation, calcium wave propagation by extracellular messengers, and cell death during metabolic inhibition [62]. It is well known that hemichannels allow permeability to diverse intracellular molecules, such as IP3, ATP, glucose, glutamate or prostaglandins [63]. Numerous connexins have been proposed to release ATP under different stimuli, including connexins 26, 32, 37 and 43 [64–67]. However, connexons are not the only hemichannels releasing ATP, and recently pannexins, mainly pannexin-1, have been implicated in this phenomenon [68]. Pannexin-1 is a member of the mammalian pannexin family of proteins, a family with homology to the invertebrate gap junction proteins, the innexins. Pannexin-1 form channels, or pannexons, that open in non-junctional surface membrane in several vertebrate cell types. Although transfected pannexin-1 can induce electrical coupling of *Xenopus* oocytes [69], presumably via gap junctions, unlike connexins, pannexin-1 may not form gap junctions in mammalian tissues and cells [70].

In 1986 Gordon hypothesized that the release process for ATP could involve changes in the topography of plasma membrane constituents, resulting in the opening of pores that allows the selective release of some low molecular weight cytoplasmic constituents [71]. Since then, there has been a significant increase in studies investigating this process and it is now known that hemichannels form pores in the plasma membrane of the cells that account for the physiological release of ATP to the extracellular milieu and are thus considered as initiators or enhancers of the purinergic signaling [72] (Fig. 2). On the other hand, several purinergic receptors have been linked to the opening of hemichannels, generating a positive feedback loop to amplify the purinergic signaling [73,74] (Fig. 2). However, it should be stressed that many aspects of the interactions between pannexin channels or connexin hemichannels and purinergic receptors have not been clarified.

1.6. Limitations of hemichannel research tools

There are several approaches to investigate the function of hemichannels, including the use of blockers, genetic knock down/out systems, and conductance or permeation measurements to different endogenous molecules such as ATP or charged fluorescent tracer molecules. They all have their disadvantages and as such, there is no conclusive technique to identify the nature of hemichannels. Therefore a combination of techniques is commonly used to characterize hemichannel opening kinetics. Hemichannel pharmacology is not specific, and different blockers inhibit different connexin and/or pannexin channels [63]. In addition, these inhibitors could have non-hemichannel targets and often blocks other channels [75,76]. Therefore extreme care must be taken to ensure that they do not affect purinergic receptor activation in a particular experimental condition. Genetic knock down/out systems could present strong compensatory effects, since more than one hemichannel subtype are expressed, and knock down of a specific subtype could lead to a compensatory response via the function of other hemichannel [77]. Unitary conductance varies among the different hemichannels and does not correlate with the size of the permeant pore [78,79]. The use of fluorescent charged trace molecules has been successfully and extensively used to report the existence of hemichannels, but they are usually accumulative dyes and data interpretation regarding to hemichannels closing is difficult to obtain.

2. How hemichannels modulate purinergic receptors

ATP release to the extracellular environment can serve as a physiological signal for intercellular communication as well as a noxious signal that promotes cell death. ATP acts mainly as an autocrine/paracrine signal, modulating a variety of cellular functions by activating ionotropic P2X or metabotropic P2Y receptors (see Section 1) [13]. Several studies propose a tight relationship between ATP release through hemichannels and activation of the purinergic signaling in the surrounding cells, with different physiological or pathophysiological roles (summarized in Fig. 3).

2.1. Connexin channels activating P2 receptors

2.1.1. Corneal endothelial cell communication

Intercellular Ca^{2+} wave propagation is a form of cell–cell communication. In corneal endothelial cells, intercellular Ca^{2+} wave propagation evoked by mechanical stimulation is partially mediated by ATP release and the subsequent activation of P2Y receptors in neighboring cells. For example, in bovine corneal endothelial cells (BCECs), ATP is released through connexin hemichannels. Experiments using hemichannel blockers such as flufenamic acid or the connexin mimetic peptide Gap26, significantly reduced ATP-mediated Ca^{2+} propagation in confluent monolayers of BCECs as well as in contact-free cells [80]. The presence of connexin (Cx) 43 has been demonstrated in BCECs [81], and in human corneal endothelial cells [82]. Suramin (a non-selective P2 receptor antagonist) and apyrase application suppressed intercellular Ca^{2+} wave propagation in BCECs after mechanical stimulation. Furthermore Ca^{2+} wave propagation was augmented after exposure to an ectonucleotidase inhibitor [83], suggesting a secondary paracrine action of extracellular ATP through purinergic receptors.

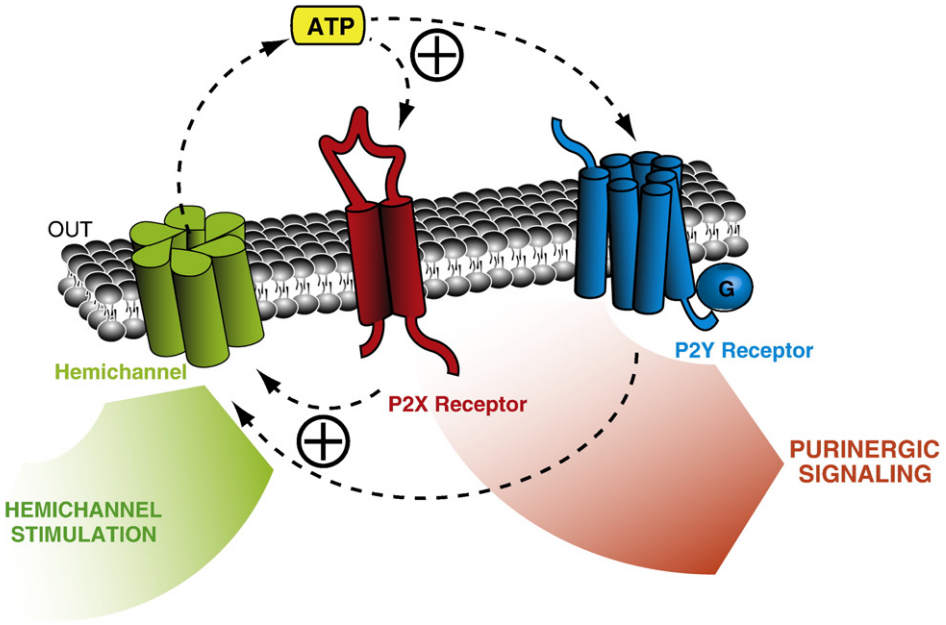

Fig. 2. ATP-induced ATP release. Hemichannel activation represents a well-established pathway for the release of ATP, which serve as an agonist for purinergic receptors. Activation of different purinergic receptors (like P2X7 or P2Y receptors) leads to the opening of hemichannels, generating a positive feedback-signaling loop. P2X7R receptors probably open hemichannels via its intracellular Ct region, whereas P2Y receptors use IP3 to open hemichannels.

2.1.2. Inner ear: hair cells electromotility

The mammalian cochlea is a snail-shaped inner ear structure divided in three chambers, namely the *scala vestibuli*, the *scala tympani*, and the *scala media*. The epithelium comprises the organ of Corti (OoC), a sensory organ which rests on the basilar membrane and is responsible for sound transduction. The OoC has the form of an epithelial ridge encompassing highly specialized sensory inner hair cells (IHCs) and outer hair cells (OHCs) [84]. Connexin hemichannels in the cochlea can release ATP at concentrations that account for the submicromolar concentrations measured in the cochlear fluids *in vivo*. Connexin hemichannels can open to release ATP by a reduction in the extracellular Ca^{2+} concentration or by mechanical stimulation. Nanomolar concentrations of extracellular ATP elicit inward currents in OHCs and also alter OHC electromotility. P2 receptor expression has been identified in cochlear cells, including OHCs [85] and P2X receptor antagonists inhibit styryl dye entry into hair cells [86]. The subcellular location of P2X receptors in hair cells and the endogenous presence of extracellular ATP in cochlear tissue, P2X receptors could partially mediate rapid styryl dye entry into hair cells, producing a spatial pattern similar to a transduction-mediated mechanism [86]. P2 receptors blockade abrogates the effect of ATP on OHC electromotility. ATP released from cochlear-supporting cells via connexin hemichannels enters into the endolymph and perilymph. This ATP consequently acts on the P2 receptors on the OHC cells to mediate electromotility [67]. In addition, it has been found that part of the ERK1/2 signal in the cochlea depends on Ca^{2+} influx, indicating a role for the damage-induced Ca^{2+} wave in this signaling and implicating ionotropic P2X receptors as potential mediators [87]. Majumder et al. [88] showed that Cx26 and Cx30 form hemichannels that allow the release of ATP from the endolymphatic surface of cochlear supporting and epithelial cells and also form gap junction channels that allow the concomitant intercellular diffusion of Ca^{2+}-mobilizing second messengers. Released ATP in turn activates G-protein coupled $P2Y_2$ and $P2Y_4$ receptors, PLC-dependent generation of IP3 and increased cytosolic Ca^{2+} release from intracellular stores, thereby instigating the regenerative propagation of intercellular Ca^{2+} signals [88].

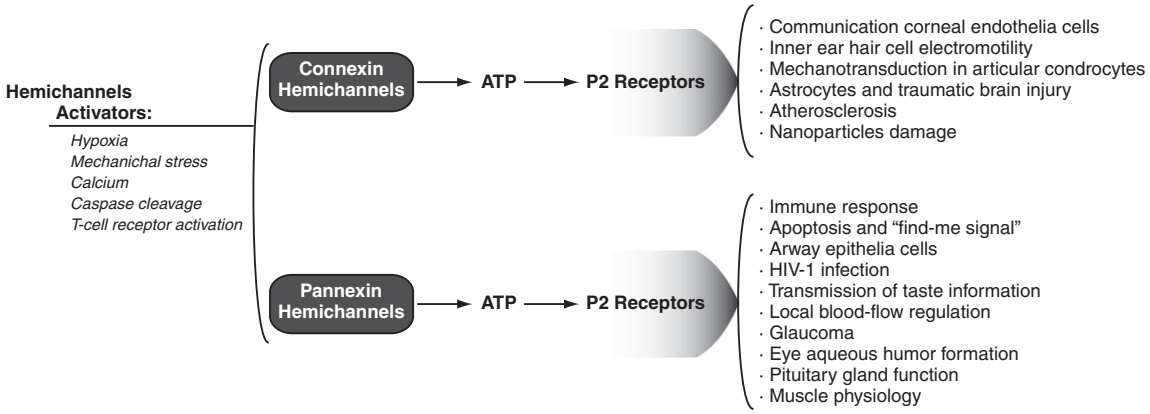

Fig. 3. Pathological and physiological roles of ATP released through hemichannels. Hemichannels are activated in response to different stimuli (hypoxia, mechanical stress or extracellular calcium decrease), inducing the release of ATP which in turns activate purinergic signaling in a wide range of pathological and physiological situations.

2.1.3. Articular chondrocytes: mechanotransduction signaling

Mechanical loading of articular chondrocytes triggers release of ATP into the pericellular microenvironment [89]. This ATP and its derivatives activate purine receptors on the cell membrane to trigger autocrine and paracrine mechanotransduction signaling cascades, involving intracellular Ca^{2+}. In various cell types, primary cilia function as mechanoreceptors and deflection of the cilium initiates intracellular Ca^{2+} signaling as part of a mechanotransduction signaling cascade [90]. The primary cilia are involved in purinergic Ca^{2+}-signaling mediated chondrocyte mechanosensitivity. The cilium acts as a mechanosensor triggering the release of ATP, probably through Cx43 hemichannels, and subsequent signaling through P2 receptors. Cx43 is expressed in bovine chondrocyte primary cilia and human chondrocytes, but its expression was confined to the upper 200 μm of the tissue closest to the articular surface [91]. The fact that these potential mechanosensitive cells are primarily in the superficial/middle zone of articular cartilage may indicate that the mechanotransduction pathways in these cells are different to those which may operate in deeper tissue zones [92]. The loss of Cx43 expression in deep tissue zones and their enhanced expression in agarose culture may be related to the oxygen levels in the cell environment, as hypoxia is known to regulate Cx43 dephosphorylation, translocation and proteosomal degradation in some cell types [93]. Recently, the expression of pannexin-3 has been found in chondrocytes [94,95] and it has been proposed as ATP release conduit, which regulates intracellular ATP and cAMP levels, promoting chondrocyte differentiation [95]. Similarly, human articular chondrocytes showed positive immunofluorescence labeling for purine receptors: $P2Y_1$, $P2Y_2$, P2X2, P2X4 and P2X7 [91], and these have been shown to be functional by the activation of intracellular Ca^{2+} rise following the addition of exogenous ATP [96] and by inhibition using non-selective purinergic antagonists such as suramin [97].

2.1.4. Astrocytes: traumatic brain injury

Traumatic brain injury leads to the development of gliosis, which is characterized by hypertrophic and hyperplastic changes of astrocytes in response to brain injury. ATP is released from astrocytes after stretch-induced injury. Cx43 is a perfect candidate to aid release of ATP from astrocytes, since Cx43 is the main gap junction protein expressed in this cell type [98] and mechanical stretch is a well-related signal to activate Cx43 hemichannel opening [74]. Although pannexin-1 has recently also been characterized as an ATP release conduit in astrocytes [99], it is unclear if pannexin-1 opens in response to mechanical stimulation [74,100]. Extracellular ATP stimulates ERK kinase in astrocytes by a signaling process mediated by P2 purinergic receptors. ERK is rapidly phosphorylated after stretch-induced injury of cultured astrocytes by a calcium-dependent pathway in which ATP release contributes to its activation by stimulating P2X2 and $P2Y_1$ purinergic receptors [101]. Furthermore, P2X7 receptor activation has also been associated with brain injury or trauma inducing neuronal death. In these situations, it has been demonstrated that ATP is released by activated microglia and astrocytes [102].

2.1.5. Pathogenesis of atherosclerosis

Atherosclerosis is a progressive disease characterized by accumulation of macrophages, T lymphocytes, smooth muscle cells (SMCs) and lipids in the vascular wall in large- and medium-sized arteries. Endothelial dysfunction leads to the increase in cell adhesion molecules and secretion of chemo-attractants. The development of the atherosclerotic plaque is correlated with changes in the pattern of vascular connexin expression [103], and depending on Cx40/Cx43 ratio, the signaling pathway will be anti- or pro-inflammatory [104]. Circulating leukocytes release ATP, mainly via Cx37 hemichannels in monocytes [105]. ATP is subsequently converted first to AMP by CD39 or ecto-nucleoside triphosphate diphosphohydrolase 1 (ENTPD1), which is located on the surface of leukocytes and endothelial cells (ECs). AMP is then converted to adenosine by CD73 or ecto-5′-nucleotidase (NT5E) (located on the surface of ECs). In physiological environments, Cx40 is expressed in ECs, and the conversion rate favors the production of adenosine, which activates adenosine A_{2B} receptors on the endothelium. A_{2B} activation triggers cAMP-dependent signaling, which further activates CD73, and maintains the expression of endothelial adhesion molecules for leukocytes (such as VCAM-1) to very low levels [106]. This prevents leukocyte adhesion along the endothelium, thus providing an anti-inflammatory mechanism. In the presence of a pro-inflammatory stimulus, the expression of Cx40 decreases while that of Cx43 increases [107]. In the absence of Cx40, CD73 activity is decreased [108], which results in an attenuation of adenosine production and a subsequent increase in leucocyte adhesion. The decrease of CD73 activity also induces an increase in extracellular ATP, which in turn activates purinergic receptors (P2Y) on ECs, triggering Ca^{2+}-dependent signaling. Ca^{2+} waves propagates through Cx43-made gap junction channels and increase the expression of endothelial adhesion molecules for leukocytes (i.e. P-selectin), therefore promoting leukocyte adhesion and providing a pro-inflammatory mechanism [104].

2.1.6. Nanoparticles and DNA damage

Nanoparticles have a specific capacity for drug loading, high superparamagnetism, efficient photoluminescence and distinctive Raman signatures, and are therefore important materials in the targeted delivery of imaging agents and anticancer drugs [109]. Although the placenta, lung, gastrointestinal tract and skin have been cited as barriers to many nanomaterials, there is some evidence that nanoparticles could translocate them [110]. Bhabra et al. [111] described how nanoparticles could cause DNA damage across a cellular barrier. They used a barrier of BeWo cells, which were grown as a monolayer on a transwell insert in order to model the placental transport of amino acids, immunoglobulin, hormones, fatty acids, transferrin and viruses [112]. CoCr nanoparticles damage mitochondria [113] and cobalt ions mimic hypoxic conditions [114] in the top layer of the BeWo barrier, both cause ATP release from BeWo cells through Cx43 hemichannels and pannexin-1 channels. The released ATP activated P2Y receptors in the second cellular layer, or passed through connexin gap junctions into the second layer of cells. Here, ATP caused a rise in intracellular calcium and subsequent ATP secretion, via connexin hemichannels and pannexin channels. This ATP then will cause DNA damage to human fibroblasts beneath the barrier through uncharacterized P2 receptor activation.

2.2. Pannexin channels activating P2 receptors

2.2.1. Immune cells: neutrophil chemotaxis and T cell activation

Pannexin-1 channels have been recently found to promote ATP release from several immune cell types [115] modulating different physiological functions, such as neutrophil chemotaxis. Neutrophils are activated by bacterial formylated peptides through formyl-peptide receptors, and these receptors induce the release of ATP through the opening of pannexin-1 channels. Released ATP activates $P2Y_2$ receptors amplifying and facilitating the signal to sense chemotactic gradients. This allows neutrophils to polarize within the chemotactic gradient field and induce the translocation of pannexin-1, ENTPD1 and adenosine A_3 receptors to the leading edge of the cell. Accumulation of these purinergic signaling components focuses ATP release and the formation of adenosine at the leading edge, resulting in an autocrine activation of A_3 receptors, promoting cell migration towards the source of formylated peptides [116]. Also C5a-induced chemotaxis in murine macrophages was greatly modulated by autocrine ATP release activating $P2Y_2$, $P2Y_{12}$ and A_3 receptor signaling, but these purinergic responses were not reduced in macrophages derived from pannexin-1-knockout mice, suggesting that pannexin-1 was not involved in ATP release in response to C5a [117].

On the other hand, recent evidence suggests that purinergic signaling at the immune synapse could serve as a signal amplification mechanism needed for antigen recognition by T cells [115]. This process involves the formation of an immune synapse between a T cell and an antigen-presenting cell (APC). The immune synapse contains a large number of signaling molecules that are required for T cell activation, including MHC molecules, co-stimulatory receptors and T cell receptors (TCR), in addition to the purinergic signaling receptors P2X1, P2X4 and P2X7 [118]. Actually, it has been revealed that TCR stimulation results in the translocation of P2X1, P2X4 and pannexin-1 to the immune synapse [119] and triggers the release of cellular ATP through pannexin-1 channels [120]. This ATP upon release into the immune synapse promotes a purinergic signal amplification through P2X1, P2X4 and P2X7 receptors in the T-cell, facilitating a Ca^{2+} influx and downstream signaling, leading to IL-2 transcription [119,121]. In this way, Woehrle et al. [122] recently revealed that hypertonic saline resuscitation induced ATP release through pannexin-1 channels and that P2X1, P2X4 and P2X7 receptor activation enhanced T cell function. Extracellular ATP also regulates numerous cellular immune responses, including differentiation of T-helper cells [123] or B cell activation [124]. Extracellular ATP is also indirectly involved in effector T cell suppression. ATP is converted to adenosine by ectonucleotidases CD39 and CD73 which are expressed on the surface of regulatory T cells, and this adenosine can activate A_{2A} receptor-mediated signaling processes to induce effector T-cell suppression [125]. Nevertheless, the role of pannexin-1 or other hemichannels in the production of this extracellular ATP has been not established.

2.2.2. Apoptosis and "find-me signals"

Apoptotic cells release 'find-me' signals at the earliest stages of death to recruit phagocytes. A recent study targeting pannexin-1 with pharmacological inhibitors or small interfering RNAs identified pannexin-1 as the conduit for ATP release from apoptotic cells. Cleavage of pannexin-1 by the executioner caspases 3 and 7 appeared to trigger its channel activity, and this was necessary for the recruitment of phagocytic cells [126]. In the same way, dying cells deficient in pannexin-1 were less effective at recruiting macrophages in a transwell assay [127]. ATP itself, when released from injured cells, has been reported to promote chemotactic and phagocytic responses in microglia through $P2Y_{12}$, $P2Y_{13}$ and $P2Y_6$ receptors [128]. Similar observations were reported for monocytes and dendritic cells [129]. It is possible that ATP increases random migration of some of these cell types rather than promoting chemotaxis. The receptor may be required for autocrine purinergic signaling that regulates chemotaxis in response to other stimuli, for example to chemoattractive peptides (as reviewed in Section 2.2.1) or to danger and pathogen associated signals (DAMPs and PAMPs) that are present at inflammatory sites. ATP and other danger signals may also induce the release of chemokines from sentinel cells, such as IL-8, that can recruit leukocytes to the inflammatory sites [130]. Thus, it seems more likely that released ATP regulates chemotaxis rather than inducing it [115]. On the other hand, ATP released through pannexin-1 as "find-me" signal could be related with anti-tumor immunity, as dying tumor cells can activate NLRP3/inflammasome-dependent signaling in tumor associated macrophages and promotes anti-tumor immunity [131].

2.2.3. Airway epithelial cells: mucociliary clearance

The mucociliary clearance process removes foreign particles and pathogens from the airways and is the primary innate defense mechanism in the lung [132]. ATP within the airway surface liquid controls key components of mucociliary clearance via activation of epithelial cell surface purinergic receptors [133]. It has been described that pannexin-1 channels account for 60% of the ATP released from airway epithelial cells in response to cell swelling and membrane stretch after hypotonic shock by a Rho-regulated pathway [134,135]. Extracellular ATP activates $P2Y_2$ receptors promoting mucin secretion, increased ciliary beat frequency, and also regulating electrolyte transport and airway surface liquid volume production by inhibiting sodium absorption [136]. Therefore, pannexin-1 induced extracellular ATP release promotes the cystic fibrosis transmembrane conductance regulator and Ca^{2+}-activated Cl^- channel activity [137].

2.2.4. HIV-1 infection

Seror et al. [138] revealed a novel signaling pathway involved in the early steps of HIV-1 infection. Binding of the HIV-1 envelope to cellular receptors leads to a rapid ATP release from host cells through pannexin-1. ATP then activates $P2Y_2$ receptors leading to phosphorylation and activation of Pyk2 kinase. $P2Y_2$ and Pyk2 control plasma membrane depolarization, hemifusion and fusion, which are required steps for HIV-1 infection. Furthermore, pannexin-1, $P2Y_2$, and Pyk2 were all recruited to the contact site between the HIV-encoded envelope glycoprotein complex and CD4/CXCR4-containing membranes, suggesting that they act within the virological synapse to transduce signals that enhance membrane-to-membrane fusion, participating in the infectious process, and facilitating the viral cell-to-cell transmission [139]. $P2Y_2$ activation is known to mediate Ca^{2+} mobilization via PLC [11] and Pyk2 detected during HIV-1 infection might be induced in response to chemokine stimulation or elevations of intracellular Ca^{2+} concentrations [140]. Potential inhibition of any of the constituents of the molecular cascade of pannexin-1-ATP release-$P2Y_2$-Pyk2 could interrupt the HIV-1 life cycle at the level of viral entry and could constitute effective novel strategies for the blockade of HIV-1 infection.

2.2.5. Taste buds: transmission of taste information

Another example of physiological pannexin-1 derived purinergic signaling can be observed in the taste bud type II receptor cells [141] and in intact taste buds transmitting taste information [142]. The binding of sapid molecules to G-protein coupled gustatory receptors leads to an increase of intracellular calcium, which can trigger the opening of the non-selective monovalent cation channel TRPM5. This pathway results in Na^+ influx and membrane depolarization, which induces pannexin-1 channel opening and ATP release, activating downstream purinergic ATP-signaling events [143]. Released ATP will act on P2X receptors of the pre-synaptic serotonergic nerve (Type III) cells, producing the release of serotonin at the pre-synaptic membrane of these cells [144]. Serotonin will activate serotonin receptors on the post-synaptic gustatory nerve fiber cells and modulate the transmission of taste information to the brain. ATP activates P2X2 and P2X3 receptors of the post-synaptic gustatory nerve fibers from the facial glossopharyngeal nerves [145]. In addition, a form of amplification loop exists where ATP and its degradation product, ADP, exert a positive feedback on the receptor cells (Type II) through $P2Y_1$ receptor activation, which mediates autocrine excitation [146].

2.2.6. Erythrocytes: local blood-flow regulation

It has been suggested that pannexin-1 channels could be involved in local blood-flow regulation [147]. Oxygen-deprived or shear-stressed erythrocytes release ATP, which then binds to P2Y receptors on erythrocytes and endothelial cells. In erythrocytes, activation of the P2Y receptor leads to further release of ATP through pannexin-1 channels (ATP-inducing ATP release, Fig. 2). In endothelial cells, binding of ATP to P2Y receptors initiates a calcium wave propagated by opening pannexin-1 channels. The calcium wave propagates backwards and reaches the precapillary sphincter region, where endothelial nitric oxide (NO) synthase is activated, and NO released from the endothelial cell relaxes the smooth muscle. In arterioles, smooth muscle covers the endothelial cells and NO-induced relaxation of smooth muscle could occur without the need of a propagated calcium wave.

The vascular response to ATP could be also amplified by the secretion of cis- and trans-epoxyeicosatrienoic acid (EET) from erythrocytes [148]. These vaso-active lipid mediators are metabolized from arachidonic acid and released by an ATP-dependent stimulation of erythrocyte P2X7 receptors. These actions require the presence of functional channels such as the cystic fibrosis transmembrane conductance regulator and pannexin-1.

2.2.7. Retina: glaucoma

Increased ocular pressure resulting from acute glaucoma leads to an elevation in extracellular ATP levels that damage the ganglion cells. This theory is strongly supported by the ability of the soluble ATPase apyrase and P2X7 receptor antagonists to reduce the ganglion cell damage induced by acute elevations in ocular pressure [149]. The demonstration that stimulation of the P2X7 receptor on retinal ganglion cells elevates intracellular calcium and kills retinal ganglion cells in vitro is also consistent with this model [150]. The rise in ATP is not due to cell rupture, while the pharmacology suggests that pannexin-1 channels contribute to some of the ATP release. The presence of a pressure-dependent rise in extracellular ATP in the inner retina provides further evidence that ATP release can transduce mechanical stimuli into neurochemical signals [151].

2.2.8. Ciliary epithelial cells: eye aqueous humor formation

Hypotonicity triggers ATP release by retinal non-pigmented (NPE) and pigmented (PE) ciliary epithelial cells through pannexin-1 channels and connexin hemichannels in a similar proportion [152]. ATP release enables the purinergic regulation of aqueous humor formation, controlling the fluid inflow from the stroma through the bilayered ciliary epithelium into the aqueous humor [152]. Chloride-channel activity at the stromal and aqueous humor surfaces of the ciliary epithelium may be of particular importance in modulating inflow [153]. Stimulation of NPE chloride channels at the aqueous humor surface is expected to increase inflow and intraocular pressure, whereas stimulation of PE chloride channels at the contralateral surface should have the opposite effect, reducing net fluid inflow and pressure [152]. Extracellular ATP acts directly on $P2Y_2$ receptors of the PE cells [154] to increase intracellular cAMP production which augments PE-cell maxi-chloride channels activity [155]. At the contralateral ciliary epithelial surface, ATP is metabolized to adenosine by ectoenzymes and can activate adenosine A_3 receptors of the NPE cells, stimulating chloride channels and fluid secretion [156].

2.2.9. Pituitary gland

In the pituitary gland, pannexin-1 mediates the release of ATP from the terminals of magnocellular hypothalamic neurons and other cells of the anterior pituitary [157]. In the anterior pituitary, pannexin-1 expression has been identified in corticotrophic and a fraction of somatotrophic cells, while in the posterior pituitary it has been localized to S100-positive pituicytes. Pannexin-1 expression has also been demonstrated in a mouse pituitary adrenocorticotropin secreting cell line (AtT-20) and in rat immortalized pituitary cells [158,159]. Overexpression of pannexin-1 in cultured pituitary cells increased ATP release under normal and hypotonic experimental conditions, while ATP release was blocked by carbenoxolone [159]. Furthermore, ATP release was attenuated by two novel isoforms of pannexin-1 (pannexin-1c and 1d) when co-expressed with full length pannexin-1 [160]. These results suggest that the pannexin-1c and 1d isoforms may act as dominant-negative effectors modulating the function of full length pannexin-1 through the formation of heteromeric channels [160]. ATP released through pannexin-1 activates P2X receptors in secretory anterior pituitary cells [158] and nerve terminals in the posterior pituitary [161], molecular evidence is indicative of P2X2, P2X3, P2X4, and P2X7 expression in this region [162]. Electrophysiological studies have established the expression of P2X2 receptors in vasopressinergic neurohypophysial terminals and gonadotrophs [163,164]. These receptors facilitate the firing of action potentials, elevate intracellular Ca^{2+}, and stimulate hormone release. Similarly, P2Y receptors, including $P2Y_1$ and $P2Y_2$, have been described in different cell populations of the pituitary gland and in the majority of pituicytes in primary culture [162]. In the pituicytes, ATP triggers a rapid rise in intracellular Ca^{2+}, which was abolished when PLC and the endoplasmic reticulum calcium pump were blocked, indicating the functional operation of Ca^{2+}-mobilizing P2Y receptors [165].

2.2.10. Skeletal muscle: muscle physiology

ATP released during skeletal muscle activity through pannexin-1 channels, act through P2X and P2Y receptors to modulate Ca^{2+} homeostasis in muscle cells and muscle physiology [166]. ATP-induced activation of P2X receptors leads to an early increase in intracellular Ca^{2+} which is linked to excitation–contraction cell coupling, while ATP-induced activation of P2Y receptors leads to second, slow and more gradual increase in intracellular Ca^{2+}, which is linked to excitation–transcription regulation [167].

3. Purinergic receptors modulating hemichannels activity

For over three decades it has been known that extracellular ATP is able to permeabilize plasma membranes of cells to molecules up to 900 Da [168,169]. We now know that ATP acts on purinergic receptors to activate different signaling cascades some of which culminate in the opening of hemichannels [73,170–173] (Fig. 2). The opening of hemichannels constitutes a direct route for cell permeabilization to different metabolites, which could flux inside or outside the cell depending on their concentration gradient, size, charge and structure [63,172]. Activation of the P2X7 receptor activation is uniquely associated with permeabilization of plasma membranes, and is currently the most well studied purinergic receptor associated with plasma membrane hemichannels [37,73,170]. However, under some conditions other purinergic receptors such as different P2Y or P2X receptors, could also couple to the opening of plasma membrane hemichannels.

3.1. P2X7 receptors and hemichannels

Before the P2X7 receptor was cloned in 1996 [174], it was known as the P2Z receptor. The most striking and unique feature of this receptor was that activation led to a rapid uptake of higher molecular weight molecules (up to approximately 900 Da). This cell permeabilization process was considered to be due to the opening of a non-selective "large pore", in contrast to the small cationic conductance to Na^+, K^+ and Ca^{2+} formed by the receptor pore itself [37]. There are two contrasting and controversial hypotheses to explain this phenomenon. One theory assumes that P2X7 receptor conductance pathways dilate over the time, thereby allowing the passage of large molecules; the other assumes that P2X7 receptors activate a second non-selective permeabilization pathway [37,170], although recent evidence points to a co-existence of both processes [170] (Fig. 4). The first attempt to characterize the P2X7 receptor associated large pore was attributed to Cx43, functioning as "half-gap junction" or pore connecting the cell cytoplasm with the extracellular environment [175]. Cx43 expression was found in J774 mouse macrophage cell line and extracellular ATP application was able to permeabilize this cell line, whereas it fails to permeabilize a cell line deficient in Cx43 [175]. However, later it was demonstrated that ATP induced cell permeabilization was due to P2Z receptors and that macrophages from Cx43 deficient mice exhibited the same rate of cell permeabilization as the wild type counterparts [176]. These early observations suggested that P2X7 receptor associated pore had similarities with the connexin hemichannels. However, the identity of P2X7 receptor associated non-selective, large-conductance pore has remained uncharacterized until the recent identification of pannexin-1 channels as partial mediators for this dye uptake [177,178].

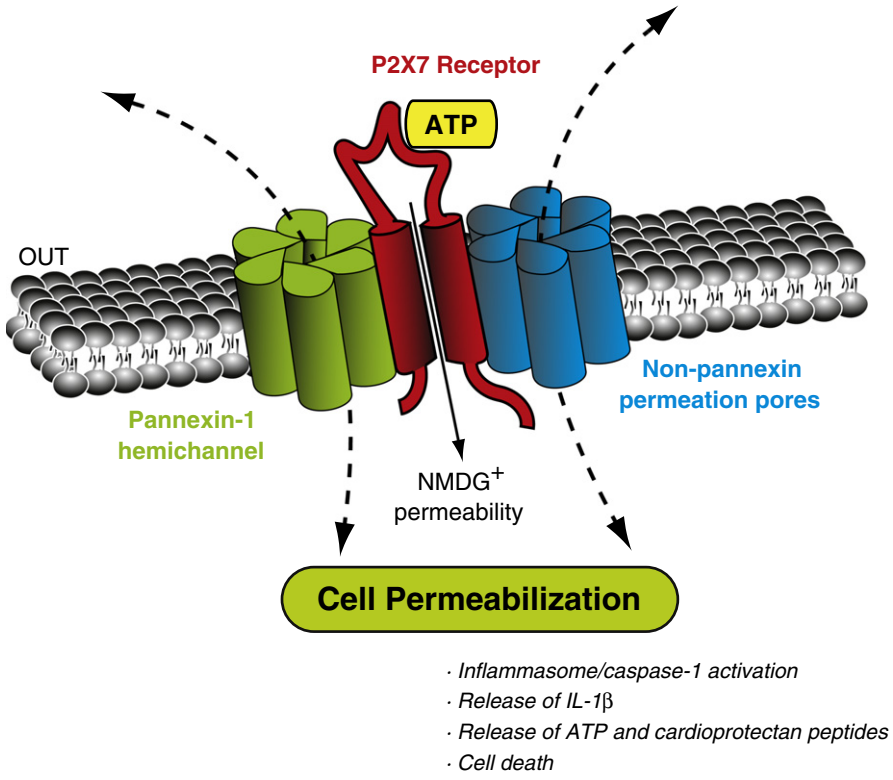

Fig. 4. P2X7 receptor stimulation couple to different cell-permeation pathways. ATP binding to P2X7 receptor opens the ion channel pore within milliseconds. With the time ATP allows the dilation of P2X7 receptor pore to a high conductance state permeable to NMDG. This new conformation of P2X7 receptor allows the opening of different hemichannel pores usually measured through dye uptake experiments (reviewed on [170]). Opening of the different permeation pores allows diffusion of molecules from inside the cell to the extracellular space and from the extracellular space to the intracellular space (represented as dotted arrows in the figure). Pannexin-1 channels are partially involved in P2X7 receptor induced cell permeabilization and are responsible for different downstream signaling events.

It was first described in the human cell line HEK293 expressing P2X7 receptors (HEK293-P2X7) and in human THP-1 monocytes, that the P2X7 receptor associated dye uptake was sensitive to carbenoxolone, a classical connexin gap-junction blocker which is more potent inhibiting pannexin-1 channels than connexin hemichannels [177]. Additional pharmacology found sensitivity to other pannexin-1 channels blockers such as mefloquine and probenecid, but not for classical connexin hemichannels blockers heptanol, lanthanum or connexin-mimetic inhibitory peptides [177–181]. However, the use of pharmacological inhibitors is inconclusive, since they also block connexins and other channels; for example carbenoxolone blocks voltage-gated calcium channels in the retina and decreased the firing rate in neuronal networks in response to a sustained depolarising current [75,76]. In addition, some studies have found P2X7 receptor induced dye uptake to be insensitive to carbenoxolone [182], although this could be due to the fact that carbenoxolone is only effective at blocking the initial rapid dye uptake after P2X7 receptor activation, without altering the final accumulation of dye inside the cell [73] and that both carbenoxolone and other drugs such as probenecid lack efficacy in inhibiting pannexin-1 when the potassium channel subunit Kvβ3 was co-expressed [183].

Experiments in which endogenous knock-down of pannexin-1 expression using siRNA in human cell lines HEK293-P2X7, THP-1 monocytes, 1321 N1 astrocytes and in mouse macrophage cell line J774, revealed a decrease of the dye uptake associated to P2X7 receptor stimulation [177–179]. The involvement of pannexin-1 is further supported by the use of a mimetic peptide identical to the ten amino acid residues of the first extracellular loop of human pannexin-1 close to the first transmembrane domain [177]. The use of small mimetic peptides with homology to the extracellular loops of connexins has been widely used to selectively characterize the involvement of different connexins in the gap-junctions [184]. The mechanism of action of these peptides is unclear, presumably they selectively bind to the docking site of two hemichannels and therefore interfere with the hemichannel coupling in the gap-junction and permeation pore formation [184]. However their specificity is also questioned and their mechanism of action could be due to physical obstruction of the pore of the hemichannel [185].

More evidences in favor of pannexin-1 mediating P2X7 dependent dye uptake, comes from co-immunoprecipitation experiments, in which P2X7 receptors can physically interact and associate with pannexin-1 in the plasma membrane of cells. Overexpression of pannexin-1 with P2X7 receptors resulted in larger ATP-induced inward currents and dye uptake compared to cells expressing P2X7 receptor with endogenous levels of pannexin-1 [160,177,186]. All these data support the role of pannexin-1 channels as part of the P2X7 receptor associated permeation conduit.

However, work from two independent groups showed that P2X7 receptor associated large-pore was more complicated than a single permeation pathway and presented different selectivity for cationic and anionic dyes [182,187]. In HEK293 cells expressing P2X7 receptors, the associated dye uptake was only observed to cationic dyes, such as Yo-Pro-1 or ethidium bromide. In contrast, in murine macrophages, P2X7 receptors activate at least two separate permeation pathways, one mainly selective for cationic dyes and the other mainly selective for anionic dyes, being the anionic pathway insensitive to temperature and through a diffusional process [182,187]. This ties in with previous studies which report two phases for P2X7 receptor mediated dye uptake in macrophages, where pannexin-1 was involved in the first fast phase (reviewed in [73]). However, in both of the studies reporting the different ion selective permeation pathways associated with the P2X7 receptor, carbenoxolone or mefloquine were ineffective in blocking cell permeabilization and pannexin-1 contribution was discarded, proposing the involvement of a transporter or

exchanger for this differential selectivity [182,187]. Recently, two other independent works employing murine macrophages derived from pannexin-1 knock-out mice, showed no alteration of P2X7 receptor associated dye uptake in macrophages, but both confirmed the involvement of pannexin-1 mediating ATP release [127,188].

From all these studies we can conclude that P2X7 receptor associated dye uptake is a cell-type selective process and a very complex mechanism involving different permeation pathways with yet to be identified hemichannels or pathways activated at different times [170] (Fig. 4). As suggested by siRNA experiments and the use of pannexin-1 knock-out mice we can postulate that pannexin-1 channels are predominantly associated with P2X7 receptor activation in human cells more than in mouse macrophages. Unpublished data from our group using primary macrophages from wild type and pannexin-1 knock-out mice also supports this idea since we found that (*i*) carbenoxolone had little effect blocking P2X7 receptor associated dye uptake in primary mouse macrophages and (*ii*) macrophages from pannexin-1 knock-out mice present a strong dye uptake insensitive to carbenoxolone. In agreement with previous studies [127,182], we hypothesize that pannexin-1 is not the main permeation pathway in primary murine macrophages associated with P2X7 receptor activation. Macrophages derived from pannexin-1 knock-out mice present a strong compensation effect in the P2X7 receptor dye uptake pathway insensitive to carbenoxolone, probably due to the activation uncharacterized permeation pathways. The ability of P2X7 receptor to couple to a non-pannexin-1 dye uptake pathway in pannexin-1 knock out macrophages leads to a compensatory effect in the release of the pro-inflammatory cytokine IL-1β [127]. This scenario could become even more complex with the characterization of human pannexin-1 splice variants [180,189] and the recent description of pannexin-1 dominant-negative splice variants in rat pituitary cells [160].

3.2. Other P2X receptors and hemichannels

It has been described that in some conditions both P2X2 and P2X4 receptors can permeate the cell to large molecules [190–193]. Although this permeation pathway is not fully characterized and its molecular nature has not been elucidated, data so far support the idea that the P2X ion pore dilates over the time due to a conformational change [190,194], similar to P2X7 receptor pore opening properties [170,195]. The involvement of pannexin-1 channels has been questioned and pharmacological data shows that P2X2 induced dye uptake was insensitive to carbenoxolone [190]. However, it has been recently published that P2X2 receptors physically associate with pannexin-1 [160], but the function of this association was not further characterized. Also, P2X2 induced dye uptake shares some similarities with the P2X7 receptor large permeation pathways and both have been found as a separate permeation pathway sensitive to colchicine [192].

In the absence of extracellular calcium, P2X4 receptor forms a larger pore that allows permeability to big molecules including NMDG, propidium iodide or ethidium bromide [191,196], but the exact nature of this permeation pathway has not been elucidated and therefore the involvement of hemichannels in this process is mere speculation. However, P2X4 receptor homotrimmers have been found to interact with P2X7 receptor homotrimmers and form a functional association [197]. It would be interesting to evaluate if this functional association could lead to alterations in the opening of hemichannels activated by P2X7 receptors.

3.3. P2Y receptors and hemichannel activation

The generation of IP3 and the downstream intracellular calcium rise is a signal to activate the opening of plasma membrane hemichannels [64,198] (Fig. 2). While this mechanism is well characterized for different connexin hemichannels, different studies present contradictory data regarding pannexin-1 activation by intracellular calcium [173,177,179,180]. While this contradiction could be due to the use of different cell types and experimental conditions, it is clear that in some cells, such as astrocytes, pannexin-1 channels are activated due to IP3 and elevated intracellular calcium [99]. Purinergic P2Y receptors induce an increase in intracellular calcium via IP3 formation (see Section 1.3) [12]. Many studies have reported that P2Y dependent increase in intracellular calcium is a direct mechanism to open hemichannels and propagate calcium waves in a variety of cells. It has been reported that P2Y opens Cx43 hemichannels in retina epithelial cells [199] and Cx32 hemichannels in bladder cancerous epithelial cells [200]. $P2Y_2$ and $P2Y_4$ receptors mediated signaling lead to the opening of Cx26 and Cx30 in cochlear epithelial cells [88], while in astrocytes $P2Y_2$ receptor mediates the opening of pannexin-1 channels and Cx43 hemichannels [99,201]. Astrocytic calcium signaling has received considerable interest because of its role in brain function [202,203]. Originally, it was believed that inter-astrocytic signaling was mediated by diffusion of IP3 and/or calcium across gap junctions, but increasing observations suggest that an extracellular pathway exists and calcium waves can cross cell-free areas via ATP release evoking calcium increments in astrocytes physically isolated from the stimulated cell by activating P2Y receptors [204,205].

3.4. Pathophysiological implications of purinergic signaling activating hemichannels

As described in Section 2, one of the main metabolites permeating hemichannels is intracellular ATP, and therefore the opening of hemichannels after purinergic P2 receptors stimulation could be an autocrine or paracrine positive feedback loop to propagate or enhance purinergic activation by increasing the release of ATP (Fig. 2). ATP-induced ATP release is commonly inhibited by broad gap junction blockers and is strongly implicated in the propagation of calcium waves among astrocytes and epithelial/endothelia cells.

However, besides ATP, there are other metabolites that could permeate across hemichannels [63], and they could mediate some of the physiological effects of purinergic receptor stimulation. For example, it is reported that prostaglandin E2 can permeate through Cx43 hemichannels [206,207], and it has been recently found that P2X4 receptors increase the release of prostaglandin E2 from macrophages and contribute to pain sensation [208]. However as has been described, it is not known whether P2X4 receptor can activate a hemichannel (see Section 3.2). Prostaglandin E2 is also a strong mediator of fever [209], and recently a study has demonstrated that P2X7 receptor associated dye uptake pathway was important for the development of fever in a murine in vivo model induced after intraperitoneal administration of LPS and ATP [192]. Fever was reduced using colchicine, which blocked P2X7 receptor associated dye uptake without affecting the P2X7 channel itself, and although this study did not characterized the production of prostaglandin after P2X7 receptor stimulation or if colchicine was blocking an hemichannel permeation pathway [192], it is tempting to speculate that P2X7 receptor stimulation of hemichannels could mediate the release of prostaglandin and enhance fever.

The activation of hemichannel-like large pores associated with P2X7 receptors is also involved in the activation of the inflammasome/caspase-1 and the induction of cell death [73,177,210,211]. Activation of the inflammasome leads to the release of pro-inflammatory cytokines of the IL-1 family, such as IL-1β and IL-18 [212]. P2X7 receptors activating pannexin-1 channels and other permeation routes have been implicated in this process, especially in human monocytes/macrophages and the central nervous system (CNS). However, as discussed in Section 3.1, dye uptake through other functional hemichannels could also compensate the lack of pannexin-1 as observed in pannexin-1 deficient mice [73,127,177,210,213]. However, the exact signaling mechanism from cell permeabilization through

hemichannels and the activation of the inflammasome is not known. It could be that hemichannels opening causes an intracellular stress which is sensed by the inflammasome and therefore the activation of the inflammasome is a reparative response for such stress [214]. Prolonged cell permeabilization through activation of pannexin channels and/or connexin hemichannels by ATP induces cell death in macrophages, astrocytes and neurons [73,178,213,215,216] and blocking hemichannels could represent a novel and alternative strategy to reduce inflammation and neuronal loss in various pathological states.

4. Conclusions and future perspectives

The field of the purinergic signaling is tightly coupled to both the connexin and pannexin channels. There is a dual cross talk amongst hemichannels and purinergic receptors: (*i*) hemichannels represent an important pathway to control the release of ATP and other nucleotides, which are the agonists for purinergic receptors; and (*ii*) hemichannels are activated by different purinergic receptors, and mediate the release of different metabolites from the cell. This association is important to amplify purinergic signaling and to expand the downstream effects of purinergic receptors (Fig. 2).

Although the permeation of ATP through hemichannels and the activation of the purinergic signaling are well characterized, we still need to understand which other metabolites could permeate across hemichannels activated by purinergic receptors. Hemichannels are permeable to a wide range of small second messengers and molecules, and the activation of hemichannels after purine receptors activation could be an important pathway to control the release and uptake of different molecules. In that sense, they could be a pathway to permeate the cell membrane to signaling amino acids such as aspartate or glutamate, small RNA molecules or peptides, such as antigenic peptides or glutathione, or small bacterial endotoxins, such as muramyl dipeptide, that could directly enter the cytosol of the macrophage and activate intracellular pathogen sensing receptors [217–222]. In this regard it has been recently reported that P2X7 receptor and pannexin-1 mediates the release of cardioprotectants induced by ischemia in the heart [223]. The pathophysiological implication of all these permeation pathways opens a new research horizon for the purinergic signaling, but yet they bring out the importance of hemichannels as novel potential drug targets.

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