REVIEWS

P2X receptors as cell-surface ATP sensors in health and disease

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P2X receptors are membrane ion channels activated by the binding of extracellular adenosine triphosphate (ATP). For years their functional significance was consigned to distant regions of the autonomic nervous system, but recent work indicates several further key roles, such as afferent signalling, chronic pain, and in autocrine loops of endothelial and epithelial cells. P2X receptors have a molecular architecture distinct from other ion channel protein families, and have several unique functional properties.

The nucleotide ATP is the source of free energy used to maintain order within most cells, and nucleotides are building blocks for key intracellular molecules. Clever experiments some fifty years ago also indicated that some nerves released ATP[1], leading to the proposal—in 1972—of 'purinergic nerves'[2]. This was significant because it clearly explicated an extracellular role for an otherwise intracellular molecule. There is now strong molecular, cellular and systems-level evidence for extracellular ATP signalling during physiological processes, placing ATP signalling at centre stage as a widespread facet of cell sensing in diverse organisms. Apparently, evolution has assured that ATP is indispensable inside cells, and as a signalling molecule between them.

Cell-surface nucleotide receptors are called P2 purinoceptors[3]. The letter 'P' indicates that they are activated by purines (and in some cases pyrimidines), and the number '2' discriminates them from P1 (now known as A_1, A_{2A}, A_{2B} and A_3) receptors for the nucleoside adenosine. Like other transmitters, ATP activates a family of metabotropic receptors (P2Y) as well as ionotropic receptors (P2X) (Fig. 1a). Metabotropic receptors couple to intracellular second-messenger systems through heteromeric G-proteins. In contrast, P2X receptors contain intrinsic pores that switch conformation from closed to open on binding ATP, allowing ions to flow. The flow of ions is a key step in signalling, because it changes the transmembrane potential as well as local ion concentrations. Additionally, ATP is rapidly degraded in extracellular space by ecto-enzymes[4] to ADP, AMP and adenosine, which also produce physiological responses at G-protein-coupled receptors of the P2Y and adenosine type (Fig. 1a).

The focus of this review is recent discoveries concerning P2X receptors. Two general points that arise from these studies are (1) that P2X receptors are involved in pathophysiological processes throughout the body, and (2) that they differ from other ionotropic receptors in important ways. P2Y receptors are not covered here, and readers are referred to a detailed review of these molecules (ref. 3).

A new receptor architecture

Early work on ATP-gated channels in nerves and smooth muscles provided evidence for rapid activation kinetics, cation-permeable pores, and significant Ca^{2+} permeability[5]. The cloning of genes encoding P2X receptors indicated that the receptors defined a new protein family[6,7] (Figs 1b and 2). P2X genes are found in species from several phyla[5], but they seem to be absent from the genomes of *Drosophila* and *Caenorhabditis elegans*. The seven human P2X subunits range from 388 ($P2X_4$) to 595 ($P2X_7$) amino acids in length (Fig. 2). Each subunit has two hydrophobic, putative membrane-spanning segments (TM1 and TM2) separated by an ectodomain that contains ten conserved cysteine residues that form disulphide bonds[5]. Thus, the amino and carboxy termini are intracellular, and most of the molecule (about 280 amino acids) forms an extracellular loop (Figs 1b and 2); this resembles the overall form of epithelial Na^+ channels (ENaC) and acid-sensing channels (ASIC). Blue-native polyacrylamide gel electrophoresis[8] and atomic force microscopy[9] suggest that P2X channels contain three subunits. Substituted-cysteine accessibility experiments indicate that TM1 and TM2 both may contribute to the pore[5]; the resulting six α-helices—from three subunits—could form a permeation pathway.

Biophysical properties of P2X receptors

Three molecules of ATP seem to bind to the extracellular portions of P2X receptors[5,10]. Mutagenesis experiments indicate that conserved lysines near the extracellular ends of TM1 and TM2 may contribute to ATP binding[5], and that aromatic residues within the extracellular loop may coordinate the adenine group of ATP[11]; more direct structural studies are keenly awaited. Electrophysiological experiments on P2X receptors also reveal functionally important interfaces between neighbouring subunits[10]. Moreover, Zn^{2+} binds allosterically to histidine residues at subunit interfaces to modulate ATP-evoked channel opening[12]. Studies on nicotinic receptors indicate that two acetylcholine (ACh) molecules bind via electrostatic interactions within aromatic binding pockets at subunit interfaces: aspects of the binding site may undergo a conformational change to "close around the bound ACh molecule"[13]. In the case of ionotropic glutamate receptors, the transmitter binds in a clam shell formed between the S1 and S2 domains of individual subunits in the tetramer, and the clam shell closes around the bound glutamate[14].

One might expect that a narrowing pore is formed by a common subunit interface located centrally within P2X receptors, as is the case for the nicotinic superfamily and ionotropic glutamate receptors. Most P2X channels are cation-selective and—like members of the nicotinic superfamily and ionotropic glutamate receptors—they discriminate only poorly among different cations. $P2X_5$ receptors are significantly chloride permeable[15], recalling γ-aminobutyric acid receptor A ($GABA_A$), glycine, glutamate-gated chloride (GluCl) and

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MOD-1 receptors from the nicotinic superfamily. Single-channel recordings suggest that a low field-strength, cation-binding site exists in the $P2X_2$ pore[16]. Some P2X receptors also show significant permeability to divalent cations, with Ca^{2+} fluxes greater than most nicotinic family and glutamate-gated receptors, and similar to those found in *N*-methyl-D-aspartate (NMDA) receptors[17]. These data indicate a selectivity filter in P2X channels that favours the flow of selected ions over others. Polar threonine and serine residues group on one face of α-helical TM2, and their side chains from three subunits projecting into the pore may act as surrogate water molecules[17]. Thus, the $P2X_2$ selectivity filter may consist of, at least in part, a small polar stretch within TM2, extracellular to the gate.

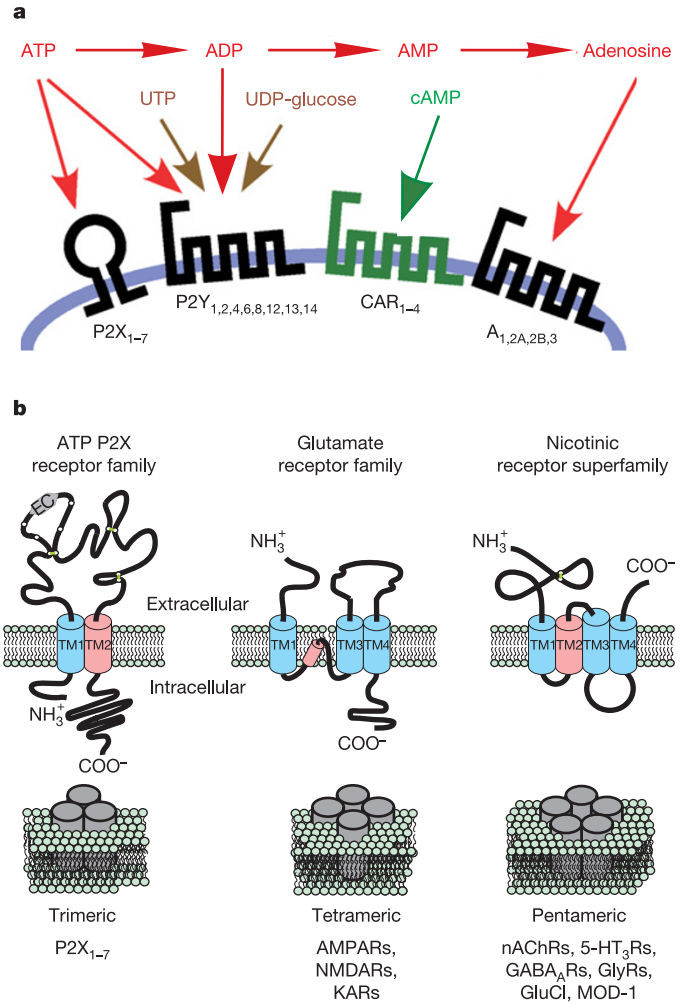

Figure 1 | ATP signalling and families of mammalian ionotropic receptors. **a**, Receptors for extracellular nucleotides. ATP is converted to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and adenosine by ecto-enzymes of several classes[4]. These are the nucleotide triphosphate diphosphohydrolases (NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP), ecto-alkaline phosphatases, and ecto-5′-nucleotidases. Nucleotide/nucleoside receptors in vertebrates (black) include P2X, P2Y and adenosine receptors. ATP acts on P2X and some P2Y receptors (G-protein-coupled, seven transmembrane domain); some P2Y receptors are activated best by ADP, uridine 5′-triphosphate (UTP), and uridine 5′-diphosphate (UDP)-glucose. Adenosine acts at four adenosine receptors. In *Dictyostelium* (green), cyclic adenosine 5′-monophosphate (cAMP) acts at a family of four cAMP receptors (CAR). **b**, Cartoon representations of subunit arrangement and proposed topology for mammalian ionotropic receptors. EC, extracellular domain; TM1–TM4, transmembrane domains 1–4; KAR, kainate receptor.

The narrowest part of the pore that moves to allow ion flow—the activation gate—in P2X channels may be near a conserved glycine residue in TM2 (Gly 342 in rat $P2X_2$), but decisive evidence is awaited. Glycine, which lacks a side chain, may favour local conformational flexibility. For example, in certain K^+ channels glycine forms a gating hinge[18], and in nicotinic channels glycines are found at the intra- and extra-cellular ends of TM2, where they may permit flexibility[19]. The conserved glycine in P2X TM2 is always followed by a hydrophobic residue, with a second hydrophobic residue positioned one helical turn away[5]. Might the activation gate be formed here? This would be consistent with the finding that mutation of the conserved glycine locks the channel into distinct permeability states[20]. Moreover, hydrophobic residues seem to be a common feature for the gates of channels with known structures. For example, the gates of nicotinic, mechanosensitive and bacterial inward-rectifier K^+ channels are all formed by hydrophobic residues[18]. By restricting the flow of water, hydrophobic residues may impede ion movement past the gate[21]. In P2X receptors, cysteine-substitution experiments indicate that TM1 and TM2 line the pore[5], but so far they fall short of pinpointing the activation gate.

Once past the gate, permeant ions enter the cytosol. Fluorescence resonance energy transfer (FRET) data indicate that the cytosolic domains of $P2X_2$ receptors may narrow[22] from their origins at the plasma membrane to their tips by ~10 Å. This implies the existence of an ordered cytosolic domain in P2X channels, which may contain additional barriers/exits for ions, as is the case for cytosolic domains of nicotinic superfamily receptors[23]. The sequences of the C termini of P2X receptors vary widely among the seven subunits[5], although a conserved YXXXK sequence (where "X" is any amino acid) in the juxtamembrane region[5,24] is involved in membrane retention (Fig. 2). Other motifs are probably involved in endocytosis[25], permeability changes[26], binding of lipopolysaccharides[27], and interactions with other proteins[28,29].

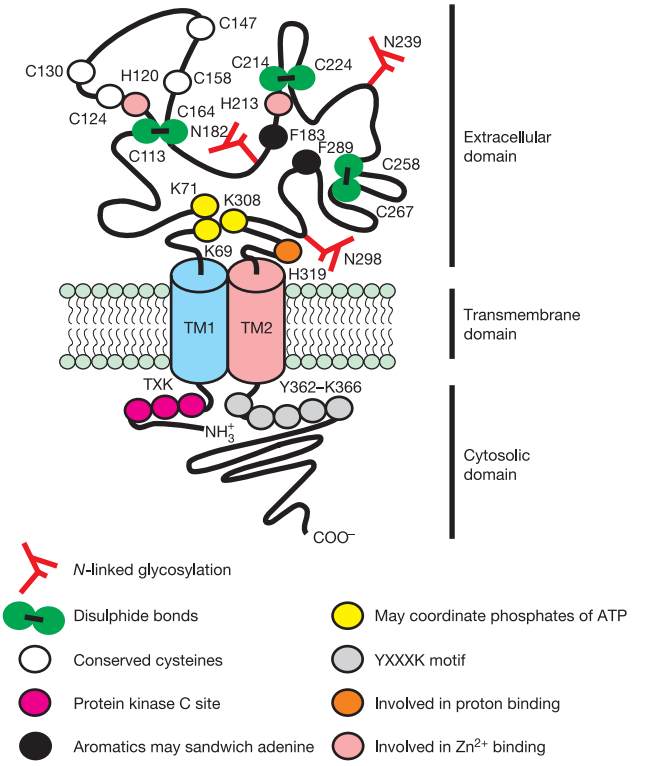

Figure 2 | Summary of P2X receptor structure–function studies. Topology and key features of P2X receptor subunits. This hypothetical view is based mainly on experiments with $P2X_1$ and $P2X_2$ receptors[5]. The numbers are for rat $P2X_2$ receptors.

Central nervous system P2X receptors and neuropathic pain

P2X receptor subunits are widely expressed in the nervous system—both in neurons and glia[5,30]—with $P2X_2$, $P2X_4$ and $P2X_6$ being the most abundant in neurons. At some central synapses, ATP is a fast neurotransmitter[5,31] where it may be released with other transmitters[32,33], as is the case in the periphery[5]. In general, recalling findings for fast nicotinic and 5-hydroxytryptamine (5-HT) brain synapses, ATP synaptic currents in the brain are small, and only occur in a subpopulation of neurons. The functional significance at central synapses might be related to the high Ca^{2+} fluxes allowed by P2X receptors, even at resting membrane potentials: Ca^{2+} entry during ATP synaptic transmission affects the frequency dependence for the induction of synaptic plasticity[34]. Whereas gene knockout experiments have confirmed a role of specific subunits at a neuroeffector junction ($P2X_1$; ref. 35) and in myenteric synapses ($P2X_2$; ref. 36), the molecular composition of synaptically activated P2X receptors in brain neurons remains poorly defined.

Antibody labelling has revealed that $P2X_2$ and $P2X_4$ subunits are found in excitatory synapses in the brain[30]. Unlike α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits, $P2X_2$ and $P2X_4$ subunits are found at the periphery of the postsynaptic density; that is, in regions where AMPA receptor subunits are of low density[29,30]. This might indicate that synaptic P2X receptors are only activated during bouts of action potential firing, perhaps when synaptically released ATP can spill out to activate P2X channels. We presume that the location of P2X receptors to the periphery of postsynaptic densities arises from specific protein interactions: one such protein that colocalizes and interacts with $P2X_2$ receptors in postsynaptic membranes is Fe65 (ref. 29), better known as a partner to β-amyloid precursor protein. Perhaps the number of synaptic P2X channels depends critically on the rates of insertion and removal by regulated trafficking.

In addition to mediating postsynaptic responses, there is now overwhelming evidence for presynaptic P2X receptors throughout the central nervous system[5,37,38]. In general, an increase in transmitter release probability is observed. The responses are triggered by calcium ions, which enter through the P2X receptor pores themselves or through voltage-dependent Ca^{2+} channels, which are activated as a consequence of the P2X-receptor-mediated nerve terminal depolarization. Presynaptic P2X channels may be activated physiologically in some synapses[38–40]. In the nucleus of the solitary tract (NTS), activation of presynaptic P2X channels evoked large-amplitude miniature synaptic currents[41]. This indicates that Ca^{2+} entry through presynaptic P2X channels may trigger multivesicular release[41], or release of glutamate from an otherwise 'silent' set of vesicles or terminals.

Presynaptic actions form one component of ATP effects in the hippocampus, specifically in the feed-forward circuit formed between CA3 pyramidal neurons, GABAergic interneurons, and output CA1 pyramidal neurons. Thus, presynaptic $P2X_2$ receptors increase glutamate release onto interneurons[40], whereas postsynaptic $P2Y_1$ receptors depolarize interneurons[42,43] and astrocyte P2Y receptors evoke glutamate release onto interneurons[42]. However, because there are few pre- or post-synaptic ATP receptors on CA1 pyramidal neurons, the net effect on output neurons is dominated by heightened GABAergic synaptic inhibition. Thus, in the stratum radiatum region of the hippocampus, although the cellular effects of ATP are excitatory, the overall result on the network is dominated by increased inhibition. ATP may act as a 'physiological brake' to runaway excitation within this feed-forward circuit[42].

Substantial ATP is released during repetitive firing of neurons[44] and from glia[45], as well as during injury[46,47] and ischaemia[48] of the brain, and the integrative actions of ATP released from these multiple sources are beginning to be understood. $P2X_4$ subunits have recently been implicated in pain sensation, but, in this case, it seems that the critical sites of expression are brain microglia rather than neurons. The tactile allodynia (whereby hitherto innocuous stimuli to the skin now elicit intense pain) that develops several days after ligation of a spinal nerve is much reduced by blocking the expression of $P2X_4$ subunits in the dorsal horn using intrathecal antisense oligonucleotides[49]. This also blocked the activation of dorsal horn microglia. Remarkably, intrathecal administration of a solution containing cultured brain microglia also produced the allodynia, but only if they had been pretreated with ATP[49]. It is suggested that ATP—by acting on $P2X_4$ receptors—causes microglia to release brain-derived neurotrophic factor (BDNF)[50]. This acts on nearby neurons to alter the expression of an anion transporter; the altered chloride gradient causes the key transmitter GABA to switch its effect from inhibition to excitation. If it occurred in the appropriate neurons, this could underlie the increased sensitivity that is a feature of allodynia. Together, these studies highlight a new systems-level mechanism involving endogenously released ATP acting on $P2X_4$ receptors, as well as a new target to treat neuropathic pain.

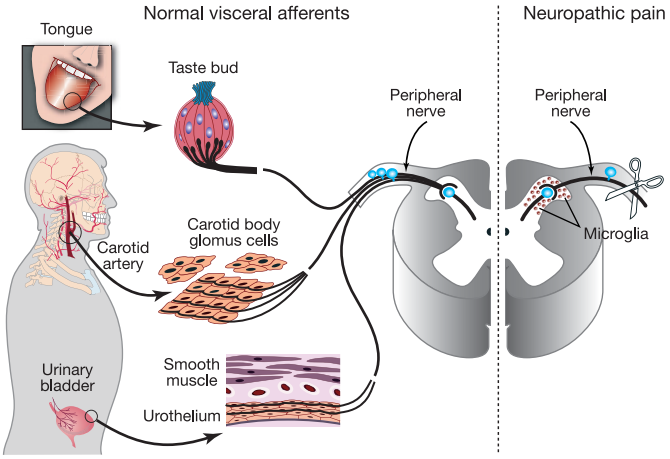

Figure 3 | Sensory transduction by P2X receptors. Left: Schematic illustration to show sensory nerve pathways from tongue taste buds, the carotid body and the bladder wall. In each case, evidence implicates ATP as the transmitter released by the sensory cell to initiate an action potential in the sensory nerve. Right: In response to injury to a peripheral nerve, microglia become activated in the dorsal horn of the spinal cord. It is proposed that ATP acts on $P2X_4$ receptors on these microglia to elicit the release of BDNF, which then acts on neighbouring neurons to change their response (and thus the perception) of incoming sensory signals. Further details are provided in the text.

Physiological and pathological sensations

ATP elicits acute pain when applied to human skin[51,52], and the cell bodies of sensory neurons were among the first sites where it was shown to activate ligand-gated channels[5]. Thus, a role for ATP in pain sensing was buttressed by the discovery that the $P2X_3$ subunit was expressed almost exclusively in a subset of primary afferents implicated in nociception[53,54], and that a predominant form of receptor in such cells is a heteromeric channel comprising $P2X_2$ and $P2X_3$ subunits[54,55]. But the behavioural response to acute painful stimuli (mechanical stimulation and heat) is unaffected in the $P2X_3$ knockout mice, arguing against ATP acting simply on $P2X_3$ as an acute pain-evoking substance[56,57]. More intriguing is the accumulating evidence that $P2X_3$ receptors are involved in the chronic or persistent nociceptive behaviour that follows nerve injury or inflammation, and may have a more widespread role in normal visceral afferent sensation.

Mechanical allodynia can be readily measured in experimental animals, and is widely used as a model of the chronic neuropathic pain in man that occurs in, for example, diabetes or AIDS. Mechanical allodynia is greatly reduced in mice with deleted $P2X_3$ receptor genes[56,57], in rats that have been treated with intrathecal antisense

oligonucleotides that reduce expression of $P2X_3$ receptors[58], and in rats receiving pharmacological antagonists selective for P2X receptors that contain $P2X_3$ subunits[58,59]. Such antagonists are effective also in other models of neuropathic and inflammatory pain, and several pharmaceutical companies are now pursuing these actions with a view to novel therapeutics. $P2X_3$-receptor knockout mice have further defects in afferent sensation (Fig. 3). Those lacking $P2X_3$ subunits have impaired ability to sense bladder filling (at least when anaesthetized)[56]. Together with earlier work showing that bladder distension releases ATP in the urothelium (the layer of the bladder wall that contains the peripheral endings of sensory nerves), this suggests that ATP forms the link between sensory stimulus (in this case mechanical) and sensory nerve activation (Fig. 3). ATP, and analogues that activate $P2X_3$-subunit-containing receptors, also excite afferent nerves in the wall of the intestine[60]; if ATP were to have a similar role in distended and inflamed intestinal mucosa, then blockade of $P2X_3$ receptors may be a valuable therapeutic approach in irritable bowel syndrome[61].

A similar link has recently been adduced for taste receptors in the tongue (Fig. 3), where the taste buds release ATP that then activates $P2X_{2/3}$ heteromeric receptors on gustatory nerves[62]. The carotid body is a collection of specialized epithelial cells in contact with arterial blood found near the bifurcation of the carotid artery (Fig. 3). These cells sense the blood oxygen and carbon dioxide concentrations. The response to hypoxia (though not hypercapnia) involves ATP release from sensory glomus cells onto terminals of the carotid sinus nerve[63]; although these sensory nerves express both $P2X_2$ and $P2X_3$ subunits, in this case studies on knockout mice indicate that the $P2X_2$ subunit has the predominant role[63]. ATP release is also involved in central chemosensory transduction[64], and other signalling roles for P2X receptors need to be tested.

P2X receptors in cell permeabilization and inflammation

One of the more remarkable actions of extracellular ATP is its ability to 'permeabilize' certain cells. This was first observed in peritoneal mast cells[65]; it is now more commonly studied in macrophages and related cells as the ATP-induced uptake of dyes (such as ethidium and YO-PRO-1)[5], which become fluorescent when they bind to intracellular nucleic acids. An important role in inflammation was inferred from the finding that ATP also elicited the processing and release of pro-inflammatory cytokines (particularly interleukin-1 and interleukin-6) from lipopolysaccharide-primed macrophages by acting on a receptor with similar properties[5]. This was initially called the P2Z receptor[66]; its distinct properties were (1) low sensitivity to ATP relative to other P2X receptors (typically 100 μM to 3 mM needed), (2) high sensitivity to the analogue 2′,3′-*O*-(4-benzoylbenzoyl)ATP (BzATP) relative to ATP, and (3) marked potentiation of the response by reducing the concentration of extracellular divalent cations. At the time of gene cloning it was recognized that these properties are closely shared by the homomeric $P2X_7$ receptor[67], which also has a widespread distribution in macrophages and other cells of the immune system. Macrophages from $P2X_7$ gene-deficient mice do not release interleukin-1β in response to ATP[68]. Such mice develop much reduced arthritis on induction by collagen injection into their joints[69], and show deficient periosteal bone formation due to absence of the receptor in osteoclasts (which behave as bone macrophages)[70].

The relationship remains enigmatic between the 'permeabilization' or 'pore formation' and the downstream actions of $P2X_7$ receptors such as interleukin release. The opening of the cation channel of the $P2X_7$ receptor occurs within milliseconds of applying the agonist ATP, and this is broadly similar to the other P2X receptors. When the agonist application is continued for several seconds, some P2X receptors[20,71], but notably $P2X_7$ receptors[67], become permeable to the large cation *N*-methyl-D-glucamine, as determined by direct biophysical measures of ion reversal potential. This time course is similar to that observed for dye uptake, but recent evidence suggests that the two phenomena may not be mechanistically related for $P2X_7$ receptors[72]. The dye uptake may reflect signal transduction from $P2X_7$ receptors to distinct membrane proteins; for instance, inhibition of a P-glycoprotein has been suggested[73]. Moreover, there is no evidence that the opening of the intrinsic cation channel, or the pathway through which dyes enter cells, are necessary for the release of interleukins evoked by ATP. It seems likely that these aspects of $P2X_7$ signalling pass through related proteins of the complex in which the receptor[28] and downstream effectors reside.

$P2X_7$-receptor-deficient mice also show reduced or absent behavioural responses to inflammation of the paw, as well as tactile allodynia[74]. A component of these effects seems to result from a reduced release of pro-inflammatory interleukin-1β in the paw itself. However, brain microglia also express abundant $P2X_7$ receptors when activated by an injurious stimulus, and a central component at some point in the nociceptive pathway also seems likely to contribute.

Towards broader physiological roles

P2X receptors are widely expressed, not only within nervous tissue. Progress on several fronts has revealed the diversity of cellular signalling. $P2X_4$ subunits, for example, are found in very many tissues, and their role in epithelial and endothelial tissues has attracted particular interest. In epithelia, they are abundant in salivary glands and bronchiolar epithelium. In the bronchioles, they seem to have a key role in maintaining the beating of cilia that moves the mucus layer, and thus helps to clear the airways of pathogens; the receptor has functional features suggesting $P2X_4$ and/or $P2X_7$ subunits[75]. It has been suggested that ATP forms part of an autocrine loop, and that the calcium entry through activated P2X receptors would be of therapeutic benefit in cystic fibrosis.

$P2X_1$ receptors are abundantly expressed in blood vessel smooth muscle and platelets, and a role in the regulation of blood pressure or haemostasis was postulated. However, knockout mice showed little deficit in blood pressure or clotting; the $P2X_1$ knockout male mice were infertile, owing to a deficit in $P2X_1$-receptor-mediated purinergic transmission in the vas deferens[35]. The first described phenotype of the $P2X_4$ knockout mice was an increase in blood pressure. In the endothelium of the vasculature, the rise in intracellular calcium resulting from shear stress is due to calcium entry through $P2X_4$ receptors[76]. In the absence of $P2X_4$, less nitric oxide is released and the normal vasodilatation evoked by acute increases in blood flow is blunted. The knockout mice have a higher blood pressure than their wild-type controls, and do not show adaptive vascular remodelling in response to a chronic decrease in blood flow[76]. The discovery of antagonist molecules effective at $P2X_4$ receptors would greatly facilitate the further elucidation of their physiological roles, and may lead to novel therapeutics. Bone metabolism is impaired in mice with a disrupted $P2X_7$ receptor gene[70]. The receptor is normally expressed on both osteoblasts and osteoclasts: its absence results in a unique skeletal phenotype, with excessive resorption of trabecular bone. Thus, the $P2X_7$ receptor is being pursued as a therapeutic target in osteoporosis.

Outlook

The widespread expression of P2X receptors in cells prepares us for the possibility that ATP may contribute generally to physiological processes in phylogenetically diverse organisms, raising several classes of question.

In the first class, what is the true extent of the family? For example, why do the genomes of slime mould and the trematode *Schistosoma mansoni* contain P2X genes[77], whereas those of the nematode *C. elegans* and the fruitfly do not? Might these organisms utilize a presently unknown family of ionotropic ATP receptors? Can the P2X channel in *S. mansoni* be targeted to treat schistosomiasis? In view of the key signalling role of extracellular cyclic AMP, what physiological functions

could P2X channels serve in slime mould? Will bioinformatics yet identify other organisms that contain P2X channels?

Questions of the second class deal with the molecular *modus operandi*. How do P2X receptors couple the energy of ATP binding to channel opening? How does the selectivity filter in P2X channels work, and select for Ca^{2+} over the more abundant Na^{+}? What is the atomic-scale structure of a P2X receptor? Are there general principles of permeation and gating that are conserved between structurally distinct ionotropic receptors? What are the molecular mechanisms of subunit-specific channel rectification and desensitization? Can the intrinsic millisecond time-scale function of P2X channels be exploited to generate new genetically encoded reporters of ATP release? Can engineered P2X channels with optimized sensitivity, kinetics and permeability be used in biosensor applications, such as remote sensing? Can the permeabilizing activity of $P2X_7$ receptors be exploited in gene therapy approaches to treat tumours?

The progressive discoveries of novel phenotypes in P2X-receptor knockout mice are driving a third class of question. How widespread is ATP as a transmitter activating primary afferent nerves? Does this reveal some biological principles in sensory processing? Is ATP released along with every other transmitter from vesicles? The study of P2X receptors has been seriously handicapped by a lack of pharmacological tools. New drugs that selectively block P2X receptors are now becoming available. Their application promises to lead us into areas of physiology hitherto not considered and, hopefully, into novel therapeutics for human disease.

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