Published in final edited form as:

Expert Opin Investig Drugs. 1995 October ; 4(10): 925–934. doi:10.1517/13543784.4.10.925.

P₂-Purinoceptors: Advances and therapeutic opportunities

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Abstract

The recent cloning of a number of distinct receptors belonging to the P₂-purinoceptor superfamily has provided conclusive evidence for a pivotal role for ATP and other nucleotides as effector molecules involved in cell-to-cell communication and the modulation of many basic aspects of tissue function. ATP itself is being clinically evaluated as a cytotoxic agent for the treatment of cancer and as an adjunct to inhalation anaesthetic use. The pyrimidine nucleotide, UTP, is in clinical trials for the treatment of cystic fibrosis. The stable ATP bioisostere, ARL 67085, is being developed as a novel antithrombotic agent, blocking with a superior safety profile and increased efficacy as compared to other agents. The diversity of P₂ receptors, with eleven having been defined using both pharmacological and molecular cloning criteria, indicates considerable additional potential and subtlety in regard to the effects of ATP on tissue function and pathophysiology.

Introduction

In the past half century, despite a considerable amount of scepticism, the role of the purine nucleotide, ATP, as a neurotransmitter/neuromodulator has been clearly delineated from its function as an intracellular building block and energy source [1,2]. Acting *via* a family of cell surface receptors, the P₂ receptor superfamily, ATP has a key role in nervous system, immune and systemic function [1,3] and acts as a trophic factor, both on its own and in combination with other pleiotropic agents to modulate cell differentiation and apoptosis [3–5].

The reason for this scepticism, so notably absent in studies related to the physiological and pharmacological roles of the various peptide neuromodulators and nitric oxide (NO), appears to reflect a conservative conceptual compartmentalisation of ATP that occurred in the 1920s; ATP was defined as the primary energy source and as an enzyme co-factor. This, then, precluded further consideration of any other role for the molecule. In contrast, studies to elaborate the function of the various peptides and NO were initiated in the absence of any preconceived role for these molecules.

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Interestingly, the first evidence for a physiological role for ATP was obtained in 1929 in the now seminal paper of Drury and Szent-Gyorgi [6]. It was not, however, until the early 1950s that further evidence demonstrated a role for ATP in transmitter function [7]. This was followed by the formulation of the purinergic nerve hypothesis by Burnstock in the early 1970s [8,9]. A considerable effort since that time has substantiated the role of ATP and related nucleotides in modulating cellular interactions. ADP functions as a humoral agent acting to stimulate platelet aggregation [10], while UTP and GTP also have effects on tissue function [11,12].

ATP as a neurotransmitter

ATP has a multidimensional role as a neurotransmitter. In addition to interacting with the various members of the P₂ receptor family, this molecule can be broken down *via* ectonucleotidase action to adenosine, a neuromodulator in its own right [13]. Thus, unlike the neuropeptides or acetylcholine, for instance, ATP has the potential to act in a cascade-like manner to produce tissue-dependent effects, some of which may be mutually antagonistic. For example, while ATP is a fast excitatory neurotransmitter at P_{2x} receptors [14,15], adenosine is a potent inhibitor of excitatory neurotransmitter release *via* its activation of presynaptic adenosine A₁ receptors [13]. The degree to which such mechanisms are physiologically relevant will be dependent on both the activity of the various ectonucleotidases in a given tissue [16] as well as the array of purinergic receptors and cell types present in the immediate extracellular space. ATP can also modulate synaptic transmission by functioning as an ecto-kinase substrate, in which role it has been found to modulate long term potentiation [17].

Conceptually, the use of ATP as a neurotransmitter has given some cause for concern in terms of an unnecessary waste of valuable cellular energy stores. For each molecule of ATP that is broken down, three high energy phosphates are cleaved at an approximate energy cost of ~ 30 kcal/mol. In contrast, if one hypothetically considers a peptide neurotransmitter consisting of ten amino acids (a 10-mer), the formation of each peptide bond involves at least one molecule of ATP being broken down to ADP at an approximate energy cost of ~ 10 kcal/mol per peptide bond for a net energy debit of ~ 100 kcal/mol when the peptide is degraded. Clearly it is somewhat short-sighted to consider the energy involved in the synthesis of a compound as the sole factor in assessing its relevance as a neurotransmitter. However, the fact that peptide transmitters, depending on their length, are more 'energy rich', albeit indirectly, than ATP should remove high energy phosphate bond utilisation from discussions related to the relevance of ATP as an extracellular messenger. Furthermore, the total amount of ATP released in the neurotransmission process represents a very small fraction of cellular stores.

ATP and GTP, as well as adenosine and guanosine, can also exert beneficial trophic effects in nervous tissue following brain trauma and/or ischaemia [4,5]. The effects of ATP are similar to those of the polypeptide growth factor, basic FGF (bFGF) in promoting astrocyte maturation. ATP can also act synergistically with bFGF to enhance mitotic processes [18] and, furthermore, can activate astrocytic MAP kinase leading to activation of the early

response gene (e.g., c-jun, c-fos, AP-1, etc.) pathways involved in cell growth, differentiation and stress responses [5].

P₂-Purinoceptors

Purinoceptors were initially divided into P₁ (adenosine) and P₂ (ATP) receptors by Burnstock in 1978 [9]. Additional data led to a further refinement to classify P₂ receptors into P_{2X} and P_{2Y} classes [19], with the subsequent addition of P_{2T} and P_{2Z} receptors [21].

Studies related to the signal transduction mechanisms for P₂ receptors led to the proposal of ligand-gated ion channel (LGIC) (P_{2X}) and G-protein-coupled receptor (GPCR) (P_{2Y}) classes of P₂ receptors [21], formalised in the seminal review by Abbracchio and Burnstock ([22]; Table 1). This has since been modified to reflect cloning and expression data (Table 2). A confounding issue in regard to the pharmacological classification is that it is dependent on agonist rank order potencies and, as such, the relative order is very tissue-dependent, reflecting the intrinsic ectonucleotidase activity of a tissue that can enhance agonist ligand lability [16].

Receptors for ATP are divided into two main groups [22]: the P_{2X} (LGIC) family [23] for which six cloned subtypes, P_{2X1-6}, currently exist; and the P_{2Y} (GPCR) family with up to seven members, four of which are designated P_{2Y1-4} [24]. Two additional members of the P₂ receptor family are the P_{2Z} receptor, an ATP-gated ion pore involved in the cytotoxic actions of ATP [3], and the UTP-sensitive, ATP-insensitive pyrimidine receptor [25]. This latter entity has been classified as a nucleotide or pyrimidine receptor raising some confusion in regard to its membership of the P₂ receptor family. Researchers in the area, including members of the IUPHAR Committee on Purinoceptor Nomenclature [26], have adopted a wait-and-see position until this receptor is cloned, with the expectation that the P₂ nomenclature may potentially be extended to encompass a 'purine/pyrimidine' receptor family.

P₂-purinoceptor characterisation has been severely limited by a paucity of potent and selective ligands for the various receptor subtypes [27]. Thus, what is known regarding potential P₂ receptor function has been dependent on localisation and *in situ* hybridisation studies, rather than the use of potent and selective compounds *in vivo* to determine functional activity. In this age of molecular biology and increasingly rational drug design, the inability to relate a defined molecular target to a given therapeutic utility can limit the prioritisation and pace of a drug discovery programme. Thus, the identification of specific ligands, agonists and antagonists, to define the physiological/pharmacological function of a particular recognition site, remains a powerful influence in the discovery of new therapeutic agents.

The first members of the P₂ receptor family were cloned in 1993, these being members of the GPCR/P_{2Y} family. The P_{2Y1} receptor was cloned from chick brain [28] and the P_{2U} receptor from the neuroblastoma/glioma cell line, NG 108–15 [29]. This P₂ receptor has been termed the P_{2Y2} receptor (Table 2).

In 1994, the first two members of the P_{2X} family were cloned, and reported as back to back articles in *Nature*. The P_{2X1} receptor was cloned from the rat vas deferens [30] and the P_{2X2} from PC12 neuroblastoma cells [31]. These LGICs had an unusual structure comprised of only two transmembrane spanning regions. The P_{2X3} receptor was cloned from rat dorsal root ganglion [32]. Other known clones of the P_{2X} family are known to exist but have yet to be published (Table 2).

The cloned P_{2X} receptors differ in their pharmacology and agonist activity profiles [23,33]. The P_{2X1} is sensitive to α,β -meATP and undergoes rapid desensitisation, while the P_{2X2} receptor is insensitive to α,β -meATP and is not readily desensitised. The P_{2X1} receptor is identical to RP-2, the product of a cDNA clone expressed in apoptotic cells.

The density of P_{2Y} receptors in brain tissue, based on binding data, is unusually high [34], with B_{max} values in the range of 38 pmole/mg protein. In comparison, receptors for the excitatory amino acid, glutamate are in the range of 7 pmole/mg protein and for adenosine in the range of 0.1 – 1 pmole/mg protein [35]. These findings suggest that P_{2Y} receptors in nervous tissue subserve an important functional role that has yet to be defined.

The P_{2Y2} receptor has been cloned from a variety of tissues and is identical to the P_{2U} receptor responsive to both ATP and UTP. This receptor is unique in that it is the only P_2 receptor containing an RGD integrin binding sequence. The P_{2Y3} receptor is a developmentally expressed receptor that is responsive to ADP. A number of other P_{2Y} receptors have been described and include clones designated as an endothelial P_{2Y4} [36], HP 212, R5 and 6HI, the latter two being cloned from rat brain and activated lymphocytes, respectively [37]. These have yet to be published. The P_{2T} , P_{2Z} and the diadenosine polyphosphate (Ap4A) receptors have not been cloned at the time of writing. Another twelve P_2 receptors are rumoured to exist as partial sequences in the various human genomic databases.

It is a major step from cloning and expression to function and, as noted, much of what is known related to cloned P_2 receptor function is either structurally inferred, as in the case of the homology between P_{2X1} and the apoptotic gene, RP-2 [31], or based on location.

Thus, the presence of the P_{2X3} receptor in the dorsal root ganglion and its localisation to small nociceptive neurones *via* anti-peripherin staining [32] suggests that this receptor is involved in some aspect of ATP-related pain perception.

The assignment of function based on structural homology and/or localisation must, however, be substantiated by more physiological data. The latter can be derived in some instances by developing transgenic knockout models or by genomic analysis of diseased cDNA libraries from human tissues. However, even when these elegant molecular biological approaches have proven successful, it is still necessary to identify selective ligands to focus drug discovery efforts.

Ligands for P₂-purinoceptors

All known agonist ligands for the P₂ receptor family are variations on the purine nucleotide pharmacophore [27,38]. While antagonist ligands like suramin and PPADS (Figure 1) show some degree of selectivity and efficacy, these parameters remain sufficiently questionable to preclude their use *in vivo* to characterise P₂ receptor function. For instance, suramin, a polysulphonated aromatic anti-trypanocidal drug that is a competitive P₂ receptor antagonist, acts at other sites including the bFGF receptor, ectonucleotidases and protein kinases. It is, thus, crucial to identify novel structural entities selective for the various P₂ receptors to advance the field effectively.

Adenine nucleotides represent an unusual challenge for the medicinal chemist. Not only is there considerable uncertainty concerning the discrete molecular site of action in terms of receptor subtype, but, as already mentioned, the identity of the active compound species can be confounded by the variety of metabolic breakdown processes occurring in the typical assay medium. The ability of a molecule to inhibit intrinsic ectonucleotidase activity and, thus, prevent or slow down nucleotide bond hydrolysis can markedly alter the rank potency order for agonists [16].

Another approach is the modification of the triphosphate moiety leading to more stable nucleotides. For example, substitution of the phosphoester bridging oxygens with methylene or dihalomethylene groups or introduction of a terminal thio group have resulted in more stable analogues [38]. While α,β -meATP is an agonist at all P_{2X} receptors except the P_{2X2}, 3'-deoxy-3'-benzylamino-ATP (Figure 2) is an even more potent P_{2X} agonist in the guinea pig vas deferens and urinary bladder.

Application of the functionalised congener concept to 2-methylthio-ATP (2-MeSATP), a potent P_{2Y} receptor agonist, by attaching substituted alkyl thio chains at the 2-thio position of adenine nucleotides can enhance activity, as in the 2-(6-cyanoheptyl) analogue (Figure 2), indicating that this is a position on the ATP molecule that has great structural tolerance, although activity at P_{2X} receptors varied to a great degree. In addition to modulating affinity and selectivity, the addition of a functionalised chain at the 2-position constituted an additional site of modification of ATP that tended to stabilise the nucleotide to enzymatic breakdown [27].

It appears likely that long chains, although at a site on the molecule distal to the triphosphate group, interfere at the nucleotidase active site. Furthermore, by serving as a secondary anchor at the receptor binding site, the functionalised chain allows for truncation of the triphosphate group with the retention of affinity, thus, circumventing the complication of triphosphate breakdown. While AMP itself is inactive at P_{2Y} receptors, 2-thioether analogues of AMP are full agonists at erythrocyte P_{2Y} receptors with greater potency than ATP, as in the case of 2-(5-hexenylthio)-AMP (Figure 2; [27]). None of the monophosphate 2-thioether analogues are active at P_{2X} receptors; thus, this may be viewed as a general approach for achieving enhanced P_{2Y} selectivity.

The N⁶-modification is also a general means of increasing P_{2Y} selectivity. N⁶-methyl-ATP is approximately equipotent to ATP at taenia coli P_{2Y} receptors and inactive at either vascular

P_{2Y}-purinoceptors or all smooth muscle P_{2X}-purinoceptors. A hybrid N⁶-methyl and 2-thioether-ATP derivative, N⁶-methyl-2-(5-hexenylthio)-ATP (Figure 2) is very potent at erythrocyte and taenia coli P_{2Y} receptors and inactive at P_{2X} receptors.

On the P₂ antagonist front, the complex structure of suramin has been simplified in XAMR 0721 (Figure 1) which is roughly equipotent to suramin but does not inhibit ectonucleotidase activity [39]. PPADS, a pyridoxal phosphate derivative is approximately ten-fold selective for P_{2X} *versus* P_{2Y} receptors. Reactive Blue 2 (RB-2; Figure 1) acts as a competitive P_{2Y} antagonist, in particular in C6 cells. At this receptor, RB2 has a K_{0.5} of 30 nM. Future challenges will be to identify potent and selective P₂ antagonists that are bioavailable and, ideally, not highly charged molecules.

An additional facet of the search for novel ligands for P₂ receptors is the potential existence of allosteric modulators. For LGICs, there is good precedent from the GABA_A/benzodiazepine receptor, the N-methyl-D-aspartate receptor and the nAChR for physiologically relevant allosteric modulation of receptor function. In this context, one of the seminal ATP antagonists, PIT (2-2'-pyridylisatogen; Figure 1), can enhance P_{2Y} receptor responses in PC 12 cells [40].

P₂ receptor binding and functional assays

Binding assays for P₂ receptors are at an early stage in their development, being exclusively dependent on agonist ligands [41]. Clearly, these are critical if high throughput screening is to be used to identify novel, non-purine, non-nucleoside pharmacophores. P_{2X} receptors have been labelled with [³H]- α,β -meATP [42–44]. P_{2Y} receptors have been labelled with [³²P]-BzATP [45] and [³⁵S]-dATP α S [34]. [³H]- α,β -meATP labels two P_{2X} receptor populations with K_d values of 0.7 – 1.5 nM and 30 – 70 nM [42,44]. The highest density of the high affinity receptor population in the brain is seen in cerebellar cortex [46]. As already mentioned, the B_{max} value for rat brain P_{2Y1} receptors labelled by [³⁵S]-dATP α S is approximately 38 pmole/mg protein [34], which is unusually high for a neurotransmitter.

P₂ receptor ligands currently under clinical evaluation

ATP is an effective and long lasting inhibitor of human tumour cell growth, an effect that is postulated to occur *via* permeabilisation of tumour cell membranes, as well as opening plasma membrane Ca²⁺ and Na⁺ channels [47]. These cytotoxic actions of ATP appear to involve the gap junction protein, connexin-43, and may, thus, be mechanistically related to the ATP-stimulated, P_{2Z} receptor-mediated apoptosis observed in macrophages [3]. The potential use of ATP as an antimetastatic agent is currently being investigated clinically by Medco.

ATP has considerable potential for use as an adjunct to inhalation anaesthetics [48]. It reduces traditional anaesthetic doses, providing an improved margin of safety and, thus, decreasing the risks associated with overdosage and toxicity as well as improving post surgical recovery time. Given *iv*, ATP induces controlled hypotension, decreasing the risk of bleeding during surgery; induces analgesia without causing respiratory depression; and blunts autonomic responses. At a perfusion rate of 103 μ g/kg/min, ATP substitutes

completely for nitrous oxide and permits a reduction of 36% in the amount of enflurane required for anaesthesia, a net 61% reduction in the amount of conventional anaesthetic agents. Similar effects have also been reported for adenosine, the active metabolite of ATP [49], which is also well characterised as an analgesic agent [50].

Purine and pyrimidine nucleotides promote airway defence by their ability to stimulate mucin secretion, activate chloride channel function and increase ciliary beat frequency [11]. Both ATP and UTP have been evaluated as treatments for cystic fibrosis, UTP being preferred to ATP to avoid the bronchoconstrictor effects of the adenosine formed from ATP. The receptor mediating the effects of both nucleotides is of the P_{2U} subtype. UTP alone, or in combination with amiloride, can restore normal function to the peripheral airways of the lung in cystic fibrosis patients.

ARL 67085 functions as a selective ADP (P_{2T} receptor) antagonist [51]. The compound has greater than 30,000-fold selectivity for the P_{2T} receptor *versus* activity at P_{2X} and P_{2Y} receptors. In a canine model of thrombosis, ARL 67085 abolished thrombosis and blocked ADP-induced platelet aggregation *ex vivo* at a mean infusion dose of 91 ng/kg/min, *iv*. This occurred in the absence of changes in blood pressure and heart rate with a good separation (twenty-eight-fold) between doses inhibiting thrombosis and increasing bleeding time. In fact, preclinical data showed that ARL 67085 had a superior margin of safety to fibrinogen receptor antagonists, which showed only a two-fold window of safety, and was superior in efficacy to aspirin. Phase I studies conducted by Fisons before their transition to Astra Charnwood substantiated the preclinical profile, and the compound is currently in Phase II trials.

Future direct ions

The challenge for the future of P₂-purinoceptor ligands as therapeutic agents will involve the application of the significant advances in basic knowledge to focus on defined therapeutic opportunities.

As knowledge in the area of P₂-purinoceptor function continues to accumulate, and as medicinal chemistry and screening efforts are focused on the various discrete receptor targets, it is anticipated that novel P₂ receptor ligands, both agonists and antagonists, will represent innovative therapeutic modalities for the treatment of disease states in a variety of tissue systems.

Abbreviations

bFGF	basic fibroblast growth factor
GPCR	G-protein-coupled receptor
LGIC	ligand-gated ion channel
NO	nitric oxide

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- 52••. BURNSTOCK G: Purinoceptor Update Newsletter 2. (9, 1995).A newsletter produced by Geoff Burnstock and colleagues that provides an on-going 'clearing house' for new information regarding receptor cloning and pharmacological characterisation. This informal newsletter is intended to aid the efforts of the IUPHAR Committee on Receptor Nomenclature by providing a framework to designate P₂ receptors in a sequential manner and avoid duplication of designators. The newsletter is available from Professor Burnstock who can be contacted via fax on +44 (0) 171 380 7349 in London, UK. (Additional information about purinoceptors is available at the World Wide Web at <http://mgddk1.niddk.nih.gov:8000/>).

Suramin

PPADS

2,2'-pyridylisatogen

XAMR 0721

Reactive Blue

Figure 1.

2-methylthio-ATP

ARL 67085

2-(6-cyanoethylthio)-ATP

N⁶-methyl-2-(5-hexenylthio)-ATP

3'-deoxy-3'-benzylamino-ATP

2-(5-hexenylthio)-AMP

Figure 2.

Table 1:

Classification of P₂-purinoceptors based on pharmacology (from Abbraccio and Burnstock, 1994).

Receptor	Agonist potency	Tissue
P _{2X1}	2-(4-nitrophenylethylthio)-ATP > 3'-amino-3'-deoxy-ATP = 2-hexylthio-ATP	Guinea-pig vas deferens
P _{2X2}	5-fluoro-UTP > 2-hexylthio-ATP > 3'-acetylaminio 3'-deoxy-ATP	Guinea-pig urinary bladder; rabbit bladder; rat urinary bladder; colon longitudinal muscle,
P _{2X3}	No selective agonists	Vascular smooth muscle
P _{2X4}	No selective agonists	Peripheral and central neurones
P _{2Y1}	2-MeSATP >>> ADP >> α,β -meATP	Chick brain; rat brain cortex
P _{2Y2}	ATP >>> ATP γ S	Rat renal mesangial cells, aortic smooth muscle cells, liver, hepatocytes, osteoblasts, myocardial epithelium, brain; rabbit neutrophils; guinea-pig brain; sheep pituitary; skeletal muscle, PC 12 cells
P _{2Y3}	2-MeSATP >>> ADP	Platelets, brain endothelium (?)
P _{2Y4}	2-MeSATP >>> ATP = ADP = α,β -meATP > β,γ -meATP	Guinea-pig taenia coli; turkey erythrocytes
P _{2Y5}	2-MeSATP >>> ATP = ADP >>> α,β -meATP (β,γ -meATP inactive)	Rabbit aorta, cortical and dorsal spinal cord astrocytes
P _{2Y6}	2-MeSATP > ATP > ADP?	Rabbit coronary, hepatic and mesenteric arteries
P _{2Y7}	Diadenosine polyphosphates - Ap ₄ A	Rat neurones, chromaffin cells; guinea-pig vas deferens

Table 2:

Classification of P₂-purinoceptors based on functional cloning (from [52]).

Receptor	Tissue of origin	Pharmacology	Transduction System	Genbank Accession #
P _{2X1}	Rat vas deferens	2-MeSATP > ATP > α , β -meATP	I _{Na} /K/Ca	X80477
P _{2X2}	Rat PC 12 cell	2-MeSATP > ATP (α , β -meATP inactive)	I _{Na} /K/Ca	U14414
P _{2X2-1}	Rat cochlea (short form)	2-MeSATP > ATP > α , β -meATP	I _{Na} /K	L43511
P _{2X3}	Rat dorsal root ganglion	ATP > 2MeSATP α , β -meATP		
P _{2X4}	Rat hippocampus	ATP active, α , β -meATP inactive		
P _{2X5}	Rat dorsal root ganglion			
P _{2X5}	Epithelium			
P _{2X6}	Myotubule			
P _{2Y1}	Chick brain	2-MeSATP > ATP > ADP (UTP inactive)	Gq/PLC β /IP ₃ /Ca ²⁺	X733268
	Turkey brain	2-MeSATP > ATP > ADP (UTP inactive)	Gq/PLC β /IP ₃ /Ca ²⁺	U09842
	Mouse insulinoma cell	2-MeSATP 2-CL-ATP ATP	Gq/PLC β /IP ₃ /Ca ²⁺	U22829
	Rat insulinoma cell	(α , β -meATP inactive)		U22830
	Human placenta Bovine endothelium	2-MeSATP > ATP >> UTP	Gq/PLC β /IP ₃ /Ca ²⁺	Z49205
P _{2Y2}	Mouse NG-108-15	ATP = UTP >> 2-MeSATP	G α /PLC β /IP ₃ /Ca ²⁺	L14751
	Human CT/43 cells	ATP = UTP >> 2-MeSATP	G α /PLC β /IP ₃ /Ca ²⁺	U07225
	Human bone			
	Rat lung	ATP = UTP	G α /PLC β /IP ₃ /Ca ²⁺	U09402
	Rat pituitary			L46865
P _{2Y3}	Chick brain	ADP > UTP > ATP = UDP	Gq/PLC β /IP ₃ /Ca ²⁺	
P _{2Y4}	Bovine endothelium			
	Rat brain			
P _{2Z}	Mouse macrophage	BzATP > ATP > UTP	Depolarisation I _{Na} /K or I _{Ca}	