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# Purinoceptors: From History to Recent Progress. A Review

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## ATP as Neuro-co-transmitter

Adenosine-5′-triphosphate (ATP) has long been known as a ubiquitous intracellular source of energy in intermediary metabolism. In 1929, potent extracellular effects both of adenine nucleotides and of adenosine were reported for the first time (Drury & Szent-Györgyi 1929). Drury and Szent-Györgyi demonstrated that the intravenous application of either adenosine or a crude extract of heart and other tissues led to cardiodepressive, antidiuretic and vasodilator effects in guinea-pigs.

Since then the list of the pharmacological actions exerted by extracellular adenine nucleotides and nucleosides and of tissues that are responsive to them has been constantly growing. Yet it was not until 1972 that Burnstock proposed that ATP was most likely to be among the active substances which had been recognized in the autonomic nervous system and responses to which were resistant to cholinergic or adrenergic blockade. This proposal formed the basis of the 'purinergic nerve hypothesis' (Burnstock 1972). The word 'purinergic' was coined in 1971 to describe nerves utilizing the purine nucleotide, ATP, as a transmitter (Burnstock 1971).

A few years later, Burnstock introduced the co-transmitter concept based on a great body of evidence that transmitter substances, e.g. noradrenaline and ATP (vas deferens, various blood vessels) or acetylcholine and ATP (urinary bladder), are co-stored, co-released and act via specific recognition sites (Burnstock 1976). Finally, ATP was identified as a neurotransmitter in its own right when Burnstock named and classified the respective receptors as 'purinergic receptors' or 'purinoceptors' (Burnstock 1978).

Today, there is no doubt about ATP being a transmitter substance at autonomic neuromuscular junctions (for a review see Hoyle 1992), autonomic ganglia (Evans et al 1992), and in the central nervous system (CNS; Benham 1992a; Edwards et al 1992; Zimmermann 1994).

## Purinoceptor Subclassification and Nomenclature

The first subdivision of purinoceptors into the $P_1$ and $P_2$ categories was based mainly on the criteria: nucleosides such as adenosine activate $P_1$ purinoceptors, whereas nucleotides like ATP stimulate $P_2$ purinoceptors; methylxanthines (caffeine, theophylline) are selective antagonists at the $P_1$ purinoceptor; and $P_1$ purinoceptors are linked to adenylate cyclase, and activation of $P_2$ purinoceptors might result in the production of prostaglandins (Burnstock 1978).

The extensive use of pharmacological, biochemical and ligand-binding studies led to the identification of a number of distinct purinoceptor subtypes. Some of these entities have been cloned (see below). The resulting framework of purinoceptor subclassification will be delineated below.

### *$P_1$ purinoceptor subtypes*

Parallel work by two groups on cultured nerve cells, adipocytes and hepatocytes demonstrated that adenosine and structural analogues either inhibited or stimulated adenylate cyclase, and the order of potency in eliciting these responses was different. Thus, the authors claimed the existence of $A_1$ and $A_2$ subtypes (Van Calker et al 1979) and $R_i$ and $R_a$ subtypes (Londos et al 1980), respectively. $A_1$ corresponded to $R_i$ (inhibition of adenylate cyclase) with the endogenous ligand adenosine having nanomolar activity and $N^6$-(2-phenylisopropyl)adenosine (*R*-PIA; Fig. 1) being a potent agonist. $A_2$ corresponded to $R_a$ (activation of adenylate cyclase) with adenosine having only micromolar activity and 5′-*N*-ethylcarboxamidoadenosine (NECA; Fig. 1) being more potent than R-PIA as agonist. 'R' was meant to indicate that the ribose moiety was essential. Today, the $A_1/A_2$ designation has gained general acceptance.

Bruns et al (1986) were the first to subdivide the $A_2$ purinoceptors into the $A_{2a}$ and $A_{2b}$ subtypes. They showed that NECA bound with high affinity to $P_1$ purinoceptors in the striatum ($A_{2a}$), but with low affinity to $P_1$ purinoceptors in fibroblasts ($A_{2b}$).

Gustafsson et al (1990) proposed the subdivision of the $A_1$ purinoceptors into $A_{1a}$ and $A_{1b}$ subtypes. This proposal was not, however, adopted by the IUPHAR Receptor Nomenclature Committee (Fredholm et al 1994).

In studies on atrial and prejunctional sites of neuronal tissues, inconsistencies of agonist potency were revealed, and it was shown that adenosine receptors might be related to a decrease in intracellular calcium levels. These observations led to the proposal of the presence of an $A_3$ purinoceptor (Ribeiro & Sebastiao 1986). The correctness of this proposal has, however, been contested and still remains questionable (Kennedy et al 1992). On the other hand, the existence of an $A_3$ subtype has been proven by cloning studies (Zhou et al 1992; see below). The cloned entity is different from that postulated by Ribeiro & Sebastiao (1986) in that it is indeed linked to adenylate cyclase, but, in contrast with the $A_1$, $A_{2a}$ and $A_{2b}$ subtypes generally does not interact with xanthine antagonists (Carruthers & Fozard 1993).

Recently, binding studies with the tritiated agonist 2-phenylaminoadenosine (CV 1808; Fig. 1) led Cornfield et al (1992) to claim the existence of an $A_4$ purinoceptor, sharing 'xanthine-insensitivity' as a general property with the $A_3$ subtype.

Even at an early stage of $P_1$ purinoceptor subclassification,

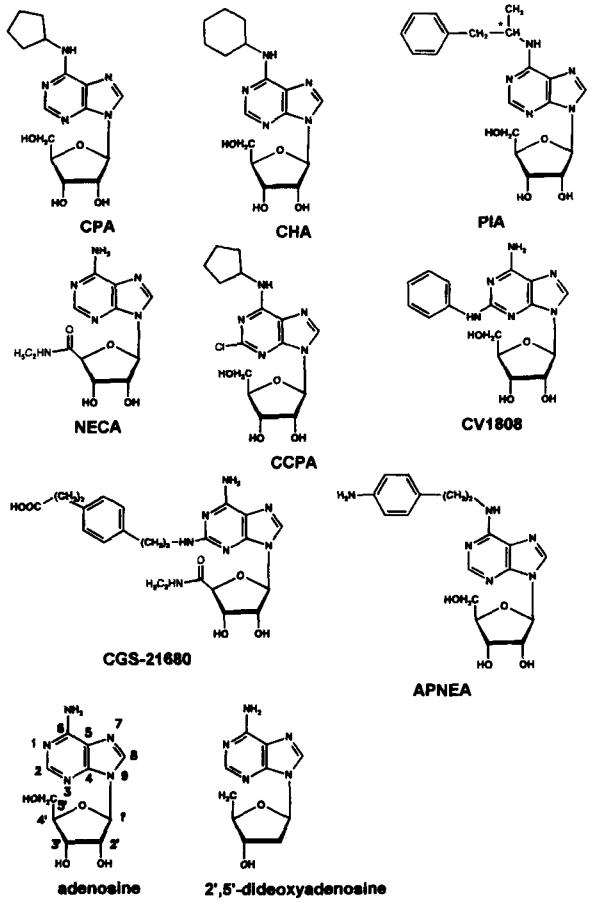



FIG. 1. The chemical structures of some key agonists at $P_1$ purinoceptors. The asterisk denotes the centre of chirality of PIA.

an intracellular nucleoside recognition site, which inhibits adenylate cyclase but is resistant to the occupation by xanthines, was recognized in homogenates of adipocytes. This site was termed a P site, because the purine moiety was crucial (Londos et al 1980). It is believed that the P site is located at the cytoplasmic face of the catalytic sub-unit of adenylate cyclase (Schwabe et al 1993). The physiological relevance of the P site remains unclear.

*Key $P_1$ receptor ligands for subclassification*

*Agonists.* Extensive structure-activity relationship (SAR) studies with adenosine analogues (Fig. 1) led to the following general rules for agonist activity at $P_1$ purinoceptors:

- the $N^6$ is essential (deamination results in the abolition of activity)
- monoalkyl or monoaryl substitution at $N^6$ generally leads to $A_1$ selectivity
- bulky residues at C-2 favour $A_2$ selectivity
- substitution at C-8 is not well tolerated
- ribose modifications are generally detrimental
- *N*-alkylcarboxamide at C-5′ (=uronamide) is tolerated
- chirality of the $N^6$ substituent is of greater significance at the $A_1$ subtype (greater difference in affinity between *R*-/*S*-PIA; cf. Fig. 1).

On the basis of this knowledge about SAR (Williams & Cusack 1990; Jacobson et al 1992), agonists have been developed, which have become useful in adenosine receptor classification (Fig. 1). Among these are the $A_1$ agonists $N_6$-cyclopentyladenosine (CPA), $N^6$-cyclohexyladenosine (CHA) and $N^6$-(2-phenylisopropyl)adenosine (*R*-/*S*-PIA), the non-selective compound 5′-*N*-ethylcarboxamidoadenosine (NECA) having nanomolar affinity, 2-chloro-$N^6$-cyclopentyladenosine (CCPA) being a potent ($K_i = 0{\cdot}4$ nM) and 10 000-fold selective $A_1$ agonist (Lohse et al 1988), 2-phenylaminoadenosine (CV 1808) being moderately selective for the $A_2$ receptor, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680) as a 140-fold selective $A_{2a}$ agonist with nanomolar affinity (Hutchison et al 1989; Fredholm et al 1994), and $N^6$-2-(4-aminophenyl)ethyladenosine (APNEA), a preferential $A_3$ agonist with nanomolar affinity which also acts at $A_1$ purinoceptors. NECA, although non-selective, has been widely used to define $A_2$ purinoceptor-mediated responses. Precautions, such as addition of CPA or *N*-ethylmaleimide (irreversibly blocks $G_i$ protein) in order to exclude interference from the $A_1$ subtype, have to be taken into account (Jacobson 1990). Whereas elimination of the ribose 2′ and 3′ hydroxyl groups abolishes activity at $P_1$ purinoceptors, the compound 2′,5′-dideoxyadenosine is a potent agonist at the so-called P site (see above).

*Antagonists.* In general, there are two major groups of $P_1$ purinoceptor antagonists, the xanthines and non-xanthines. Whereas non-xanthine antagonists are not well defined in terms of SAR, a few SARs (Jacobson 1990) for xanthine antagonists should be pointed out:

- *N*-1- or *N*-7-alkyl substituents favour moderate $A_2$ selectivity (up to 10-fold)
- homologous *N*-1,*N*-3 dialkylation results in $A_1$ selectivity (up to 16-fold)
- C-8 substitution, including alkyl, unsubstituted cyclopentyl or cyclohexyl, and phenyl residues, enhances affinity
- 8-styrylxanthines display $A_{2a}$ selectivity (up to 500-fold).

Substitution at C-8 generally leads to less water-soluble derivatives. This problem can be addressed by incorporation of sulphonate, carboxylate, or other charged groups into the C-8-phenyl residue.

Among the most relevant compounds (Fig. 2) in terms of classification of $P_1$ purinoceptor subtypes is 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; PD 116,948), a potent and 740-fold selective $A_1$ antagonist (Haleen et al 1987) with subnanomolar affinity. Another widely used xanthine is the 1,3-dipropylxanthine derivative XAC showing nanomolar affinity, 50-fold $A_1$ selectivity, and improved water-solubility. One of the most important $A_2$ antagonists is the triazoloquinoxaline 4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,3-a]quinoxaline (CP-66.713) with a 13-fold selectivity for the $A_{2a}$ over the $A_1$ subtype (IC50 = 21 nM; Sarges et al 1990). 8-(3-Chlorostyryl) caffeine (CSC), a member of the 8-styrylxanthines, is highly $A_{2a}$-selective (520-fold over $A_1$ receptors; Jacobson et al 1993a) having supra-nanomolar affinity. The essentially non-selective compounds, 8-*p*-sulphophenyltheophylline (8-SPT) and 8-phenyltheophylline, which display micromolar affinity, have been extensively used to block $A_1$ and $A_2$ purinoceptors.

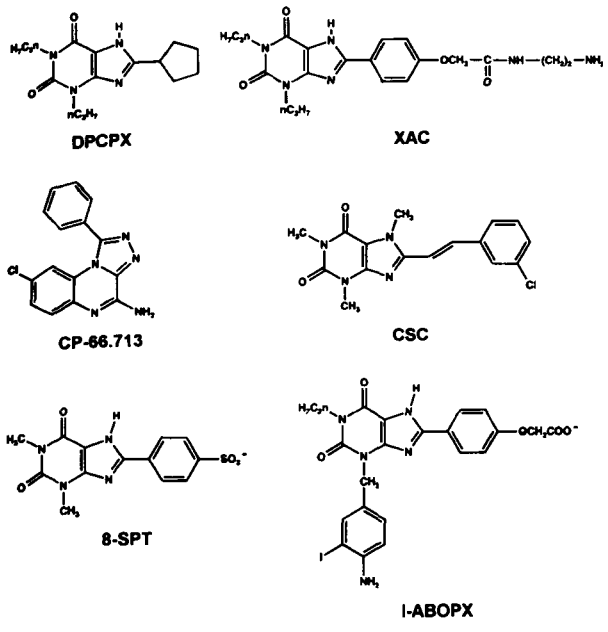



FIG. 2. The chemical structures of some key antagonists at $P_1$ purinoceptors.

Most recently, another acidic 8-phenylxanthine with nanomolar affinity for the $A_3$ receptor was shown to antagonize $A_3$ receptor-mediated hypotensive responses in the rat: 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine (I-ABOPX; BW-A522; Fozard & Hannon 1994).

The ligand-based classification of $P_1$ purinoceptors is summarized in Table 1.

*$P_2$ purinoceptor subtypes*

Growing pharmacological evidence, including type of response, rank order of agonist potency, and antagonism/desensitization by ATP and structural analogues, formed the basis for the first $P_2$ purinoceptor subdivision. Burnstock & Kennedy (1985) defined a $P_{2x}$ subtype, activation of which results in the contraction of smooth muscle organs such as urinary bladder, blood vessels and vasa deferentia, with $\alpha,\beta$-methylene ATP ($\alpha,\beta$-mATP; Fig. 3) being the most potent agonist. In addition, a $P_{2y}$ subtype was proposed, activation of which causes relaxation of taenia coli and blood vessels, with 2-methylthio ATP (2-MeSATP; Fig. 3) being the most potent agent. A further criterion typifying the $P_{2x}$ receptor has been its rapid desensitization after exposure to $\alpha,\beta$-mATP and the antagonism by arylazidoaminopropionyl ATP ($ANAPP_3$; Fig. 4) of $P_{2x}$ receptor-mediated responses. In contrast, $P_{2y}$ receptor-mediated responses are resistant to inhibition by either of these agents.

This subdivision was further extended by Gordon (1986), who has named the platelet receptor mediating aggregation $P_{2t}$ (thrombocytes) and the receptor on mast cells mediating histamine release $P_{2z}$. Apart from the responses elicited via stimulation of these receptors, the $P_{2t}$ purinoceptor is unique in that it prefers adenosine-5′-diphosphate (ADP) as agonist whereas ATP is an antagonist. The $P_{2z}$ purinoceptor is unique in that it is activated by ATP only at concentrations about a

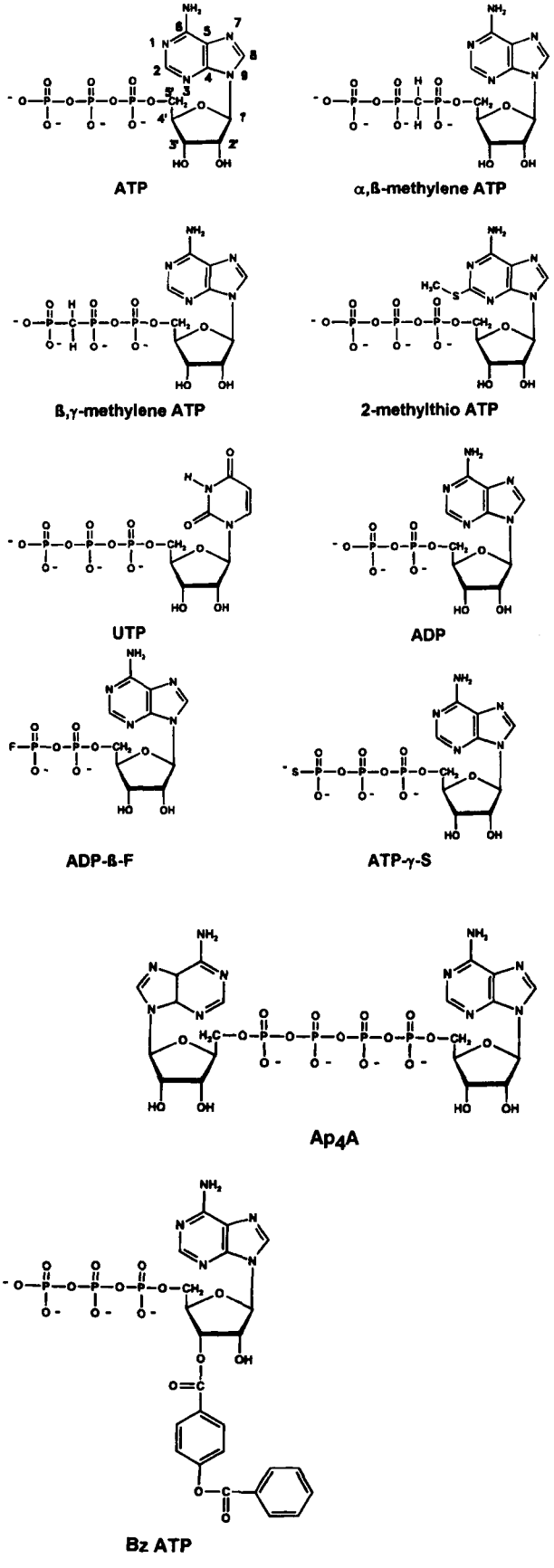



FIG. 3. The chemical structures of some key agonists at $P_2$ purinoceptors.

Table 1. Ligand-based classification of $P_1$ purinoceptors. For abbreviations see text.

| | |
|---|---|
| $A_1$ | |
| Agonist | CCPA > CPA ≥ CHA ≥ *R*-PIA > NECA ≥ adenosine > *S*-PIA > CV1808 ≥ CGS-21680 |
| Antagonist | DPCPX > XAC ≫ 8-SPT |
| $A_{2a}$ | |
| Agonist | CGS-21680 ≥ NECA ≥ adenosine > CV1808 > *R*-PIA ≥ CHA ≥ CPA > S-PIA > CCPA |
| Antagonist | CP-66·713 ≥ CSC ≥ XAC > DPCPX > 8-SPT |
| $A_{2b}$ | |
| Agonist | NECA > *R*-PIA = CHA ≥ adenosine > *S*-PIA ≥ CV1808 ≥ CGS-21680 |
| Antagonist | XAC > DPCPX > 8-SPT |
| $A_3$ | |
| Agonist | APNEA > NECA ≥ *R*-PIA > CGS-21680 ≫ adenosine |
| Antagonist | I-ABOPX |

hundred times higher than those usually required to stimulate $P_2$ purinoceptors, i.e. the tetrabasic form ($ATP^{4-}$) is the actual agonist. It should be noted, however, that there have been reports of $ATP^{4-}$ being the preferential agonist at other $P_2$ purinoceptors, also ($P_{2x}$: Fedan et al 1990; $P_{2u}$, $P_{2y}$: Motte et al 1993; Pirotton et al 1993).

A systematic analysis of observations that pyrimidine nucleotides such as uridine-5′-triphosphate (UTP; Fig. 3) and to a lesser extent cytidine-5′-triphosphate (CTP) and thymidine-5′-triphosphate (TTP) also exert various cellular responses, which could be differentiated from purine nucleotide-mediated effects with regard to the type of response, potency, desensitization and inhibition by pertussis toxin, led to the proposal of the existence of distinct 'pyrimidinoceptors' (Seifert & Schultz 1989). Soon after this concept had been introduced, O'Connor et al (1991) postulated a so-called 'nucleotide receptor' being equally sensitive to UTP and ATP, but being insensitive to 2-MeSATP.

The strongest evidence supporting their suggestion is based on relaxation experiments in rat aorta, where 2-MeSATP is more potent but significantly less efficacious than UTP and ATP. As a partial agonist, 2-MeSATP, at concentrations guaranteeing a high level of receptor occupancy, was expected to antagonize responses to ATP or UTP competitively (when activating the same receptor). Surprisingly, 2-MeSATP failed to inhibit subsequent relaxation to the other two agonists, thus indicating distinct recognition sites.

To date, the literature does not provide a clear relationship between the 'pyrimidinoceptor' and the 'nucleotide receptor' (also referred to as $P_{2u}/P_{2n}$ purinoceptor). There are reports in favour both of the pyrimidinoceptor (rabbit basilar artery, Von Kügelgen & Starke 1990; rat mesenteric bed, Ralevic & Burnstock 1991a) and the nucleotide receptor (bovine aortic endothelial cells, Pirotton et al 1993; guinea-pig trachea, Fedan et al 1994). At present, it is probably wisest to assume a common receptor for UTP and ATP, which has been differently termed in the literature (see above). The IUPHAR Receptor Nomenclature Committee adopted the $P_{2u}$ purinoceptor (Fredholm et al 1994).

There have been observations that adenine dinucleotide polyphosphates also are present and pharmacologically active in the periphery (for a review see Hoyle 1990). In addition, Hilderman et al (1991) were able to identify a unique binding site for diadenosine tetraphosphate ($Ap_4A$; Fig. 3) in mouse brain, which they refer to as 'dipurinergic receptor'. Calcium-dependent release of diadenosine polyphosphates from rat brain synaptosomes was subsequently demonstrated by Pintor et al (1992). The same group identified in rat brain synaptosomes a high affinity binding site for $Ap_4A$, which does not seem to belong to either of the $P_2$ subtypes yet postulated. This site has been tentatively designated as $P_{2d}$ (Pintor et al 1993). According to Pintor & Miras-Portugal (1993), diadenosine polyphosphates are likely to be novel neurotransmitters.

It has, furthermore, been claimed that a $P_{2s}$ subtype is present on guinea-pig ileal longitudinal smooth muscle (Wiklund & Gustafsson 1988). This proposal is mainly based on the observations that 2-MeSATP and α,β-mATP (equally potent) are more potent than ATP in eliciting contraction, and that reactive blue 2 (Fig. 4) is unable to antagonize ATP-induced contractions. These findings have been extended by Kennedy & Humphrey (1994) who were able to show that, unlike 2-MeSATP- and ATP-, α,β-mATP-induced contractile responses were antagonized by atropine and tetrodotoxin. This strongly suggests the presence of neuronal $P_{2x}$-like purinoceptors in addition to smooth muscle $P_{2y}$-like purinoceptors. Thus, the proposal of a distinct $P_{2s}$ subtype remains questionable.

Apart from the $P_1$ and $P_2$ purinoceptors, a 'hybrid' $P_3$ purinoceptor being activated both by adenine nucleosides and by adenine nucleotides and being blocked by methylxanthines has been postulated. In rat caudal artery and rat vas deferens, a $P_3$ subtype seems to mediate prejunctional inhibition of noradrenaline release (Shinozuka et al 1988; Westfall et al 1990), and-most striking-is antagonized by α,β-mATP (Forsyth et al 1991). Although Kurz et al (1993) agree that adenine nucleosides and nucleotides share a common receptor in rat and mouse vas deferens to modulate noradrenaline release, they disagree with the postulate of the existence of a novel hybrid purinoceptor by Westfall et al (1990), and rather claim that both nucleosides and nucleotides (including α,β-mATP as agonist) act via a $P_1(A_1)$ purinoceptor. Kurz et al (1993) postulate, furthermore, the presence of an additional prejunctional, release-modulating $P_{2y}$-like purinoceptor, which they admit is difficult to demonstrate in the rat. This issue clearly needs further evaluation before the IUPHAR Receptor Nomenclature Committee will adopt a $P_3$ category.

Growing information about the inadequacy of $P_2$ purinoceptor classification required a reclassification of these receptors (see below).

*Key $P_2$ receptor ligands for subclassification*

*Agonists.* Efforts to develop more potent and selective $P_2$ purinoceptor ligands have been directed towards modifications of the purine base, D-ribose sugar, and the 5′-triphosphate chain of the ATP molecule (Fig. 3). Some general rules can be derived from SAR studies in the absence of ecto-nucleotidase inhibition (see below; Williams & Cusack 1990; Cusack 1993; Bo et al 1994; Burnstock et al 1994):

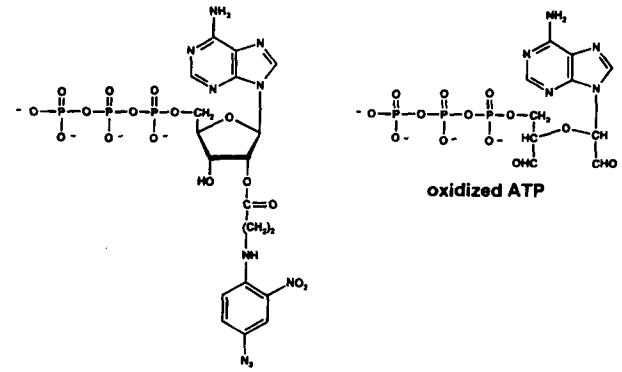
- C-8 substitution is tolerated
- $N^6$ modifications are generally not tolerated, although $N^6$-methyl contributes to $P_{2y}$ selectivity
- C-2 substitution enhances potency at the $P_{2y}$ subtype
- L-ribose markedly reduces potency at the $P_{2y}$ subtype (high degree of stereoselectivity), whereas it might significantly enhance potency at the $P_{2x}$ subtype
- removal of the 2′-hydroxyl residue in the ribose moiety is detrimental, whereas 3′ substitution might enhance potency at the $P_{2x}$ subtype
- replacement of a bridging oxygen in the triphosphate chain by a methylene group leads to metabolically more stable analogues and favours increase of potency at the $P_{2x}$ subtype
- replacement of an ionized oxygen by sulphur (= phosphorothioates) might enhance potency at the $P_{2x}$ ($P_{2y}$, $P_{2u}$) subtype
- cyclizing the triphosphate chain to the 3′ position abolishes $P_2$ activity
- exchange of adenine by other naturally occurring purine or pyrimidine bases (uracil, guanine, cytosine) is tolerated except for the $P_{2z}$ subtype (which also strictly requires a triphosphate chain and a D-ribose)
- absolute stereospecificity and absolute requirement for a diphosphate chain typifies the $P_{2t}$ subtype (where only C-2 substitution increases potency)
- diadenosine polyphosphates are agonists ($P_{2d}$, $P_{2x}$, $P_{2y}$) or antagonists ($P_{2t}$).

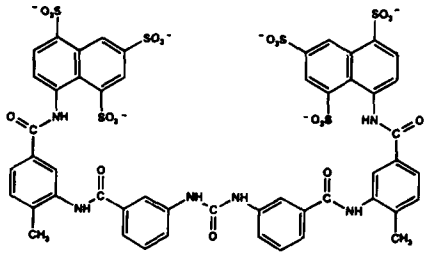
According to these SARs, a number of nucleotide agonists (Fig. 3) has proved valuable in the attempt to define $P_2$ purinoceptor subtypes. Among the most important agonists-besides ATP as endogenous, non-selective ligand-are the classical and slowly degradable $P_{2x}$ agonists, α,β-methylene ATP (α,β-mATP) and β,γ-methylene ATP (β,γ-mATP); β,γ -mATP might also stimulate $P_1$ purinoceptors (Hourani et al 1991). The potent and non-hydrolysable ligand β,γ-methylene-L-ATP (β,γ-m-L-ATP) is highly $P_{2x}$-selective being inactive at other $P_2$ subtypes (Hourani et al 1985). Notably, this compound discriminates between smooth muscle (active) and neuronal (inactive) $P_{2x}$ purinoceptors (Trezise et al 1995). The most important $P_{2y}$-ligands are 2-methylthio ATP (2-MeSATP) which-in the absence of ecto-nucleotidase inhibition-is potent and highly $P_{2y}$-selective, adenosine-5′-*O*-(2-thio)-diphosphate (ADP-β-S) as well as the slowly degradable adenosine-5′-*O*-(2-fluoro)-diphosphate (ADP-β-F; Hourani et al 1988), which is believed to be a specific $P_{2y}$ agonist. Adenosine-5′-*O*-(3-thio)-triphosphate (ATP-γ-S) acts as a preferential $P_{2u}$-ligand, whereas the pyrimidine nucleotide uridine-5′-triphosphate (UTP) is the most potent $P_{2u}$ agonist. 3′-*O*-(4-Benzoyl)benzoyl-ATP (BzATP) is a preferential $P_{2z}$ agonist. The adenine dinucleotide polyphosphate, diadenosine tetraphosphate ($Ap_4A$), is a prototypic $P_{2d}$-ligand. Both adenosine-5′-diphosphate (ADP) and the more potent 2-methylthio ADP (2-MeSADP) are $P_{2t}$ agonists.

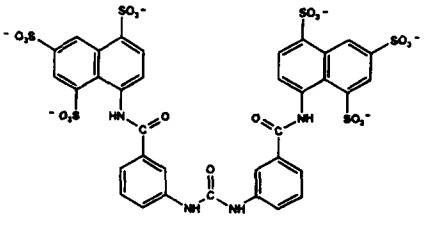
*Antagonists.* Although the lack of potent, selective and competitive antagonists has been a serious drawback for the classification of $P_2$ purinoceptors, several compounds antagonizing $P_2$ purinoceptor-mediated responses have contributed to confirm a heterogeneous $P_2$ receptor family (Fig. 4).

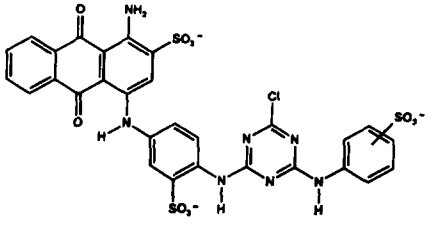
As summarized by Fedan & Lamport (1990), antagonists comprise structural analogues of ATP with an inherent initial agonist activity, such as the irreversible photoaffinity analogue arylazido aminopropionyl ATP ($ANAPP_3$). This compound has been shown to inhibit (1–100 μM) $P_{2x}$ receptor-mediated contractions, e.g. in the guinea-pig vas deferens, without affecting responses elicited by acetylcholine, histamine, noradrenaline or potassium chloride (Hogaboom et al 1980). α,β-mATP (1–30 μM) has been widely used for selective desensitization of the $P_{2x}$ purinoceptor in vascular and visceral smooth muscle preparations (Ralevic & Burnstock 1988; O'Connor et al 1990; Von Kügelgen et al 1990). The affinity label ATP-2′,3′-dialdehyde, called oxidized ATP, was shown to inhibit $P_{2z}$-mediated permeability of murine macrophages (IC50 = 30 μM; Murgia et al 1993), but it also inhibits ATP-induced contraction in the guinea-pig vas deferens (Fedan & Lamport 1990). The endogenous agonists ATP and $Ap_4A$ both represent antagonists at the platelet $P_{2t}$ purinoceptor (Cusack 1993).

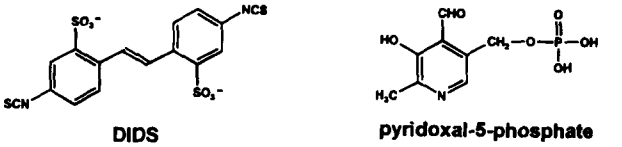
Other agents without agonist activity form a group of structurally unrelated compounds exhibiting inhibitory effects on $P_2$ purinoceptor-mediated responses. Among these the trypanocidal drug, suramin (1 μM–1 mM), has been used in many studies, including visceral smooth muscle preparations, various blood vessels, platelets, cultured coeliac ganglion neurons, and the recently cloned (see below) $P_{2x}$ purinoceptor subtypes (Dunn & Blakeley 1988; Hoyle et al 1990; Leff et al 1990; Evans et al 1992; Hourani et al 1992; Brake et al 1994; Valera et al 1994; Chen et al 1995; but see: Bo et al 1995; Buell et al 1996). Suramin is non-selective because it does not discriminate between the $P_{2x}$ and $P_{2y}$ subtypes ($pA_2 \approx 5{\cdot}0$; Hoyle et al 1990). Moreover, suramin inhibits $P_{2t}$-mediated platelet aggregation with a similar apparent $pA_2$ value (Hourani et al 1992) and might also act on $P_{2u}$ purinoceptors (Van der Zee et al 1992; but see Rubino & Burnstock 1994a, b; Wilkinson et al 1994a). Suramin is, furthermore, fairly non-specific, having various other biological effects (Voogd et al 1993) including inhibition of different enzymes. A novel suramin analogue is the symmetrical 3′-urea of 8-(benzamido)naphthalene-1,3,5-trisulphonic acid (NF023; Fig. 4). NF023 has been introduced as a specific, directly interacting, competitive $P_2$ purinoceptor antagonist (at micromolar concentrations) being highly selective for $P_{2x}$ over the $P_{2y}$ and $P_{2u}$ purinoceptors (Ziyal et al 1994, 1995; Lambrecht et al 1996 The anthraquinone dye, reactive blue 2, is thought to be a selective $P_{2y}$ antagonist over a narrow concentration range (up to 10–50 μM; Burnstock & Warland 1987; Kennedy 1990; Hoyle & Edwards 1992). Whereas this drug used to be of 60% purity only, it recently became available in a higher purity grade (Bültmann & Starke 1994b). It should, however, be noted that reactive blue 2 is able to displace radioligand binding at the rat bladder $P_{2x}$ receptor, to antagonize ATP-evoked currents via a cloned $P_{2x}$ purinoceptor and to inhibit $P_{2x}$ receptor-mediated contractions in



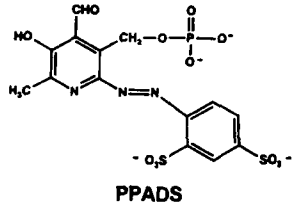




FIG. 4. The chemical structures of some ATP-antagonistic compounds.

rat vas deferens (Bo et al 1994; Brake et al 1994; Bültmann & Starke 1994b). The dye reactive red 2 is a potent (0·1–10 $\mu$M) $P_{2y}$ antagonist in guinea-pig taenia coli, being 15-fold selective over rat vas deferens $P_{2x}$ receptors (Bültmann & Starke 1995). In addition, other dyes, such as Evans blue (10–100 $\mu$M) and Trypan blue (3·2–320 $\mu$M), have been shown to antagonize $P_{2x}$ receptor-mediated contractions in the rat vas deferens with Evans blue also having non-specific effects (Bültmann & Starke 1993; Bültmann et al 1994; Khakh et al 1994). 4,4′-Diisothiocyanato-stilbene-2,2′-disulphonic acid (DIDS) is a cross-linking reagent known as an anion-transport inhibitor, and was also introduced as $P_2$ antagonist. DIDS inhibits $P_{2x}$ receptor-mediated contractions in the rat vas deferens (IC50 = 2–4 $\mu$M; Bültmann & Starke 1994a) and $P_{2z}$ receptor-mediated responses in rat parotid acinar cells (IC50 = 35 $\mu$M; Soltoff et al 1993). Pyridoxal-5-phosphate has, furthermore, been shown to inhibit $P_{2t}$ receptor-mediated ADP-induced platelet aggregation at millimolar concentrations (Kornecki & Feinberg 1980) and $P_{2x}$ receptor-mediated responses in the isolated vagus nerve, vas deferens and superior cervical ganglion of the rat (10–100 $\mu$M; Trezise et al 1994b; Connolly 1995). Interestingly, pyridoxal-5-phosphate does not antagonize UTP-evoked depolarizations in the rat superior cervical ganglion (Connolly 1995). The compound pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; Fig. 4) has been introduced as a $P_{2x}$ antagonist in rabbit vas deferens ($pK_b = 6·34$; Lambrecht et al 1992). Subsequent studies on various preparations demonstrated antagonism by and binding of PPADS at $P_{2x}$ receptors (Ziganshin et al 1993, 1994b; McLaren et al 1994; Valera et al 1994; Windscheif et al 1994a, b; Connolly 1995; Lewis et al 1995a; but see: Bo et al 1995; Lewis et al 1995b; Buell et al 1996). PPADS is specific and highly selective for $P_{2x}$ over $P_{2t}$ and $P_{2u}$ purinoceptors while having a certain selectivity for $P_{2x}$ over $P_{2y}$ receptors (Windscheif et al 1994a, b, 1995a, b; Connolly 1995; for SAR see Lambrecht et al 1996). Notably, Boyer et al (1994) described the differentiation by PPADS of phospholipase C- and adenylate cyclase-coupled $P_{2y}$ purinoceptors and Brown et al (1995) found that PPADS is able to distinguish between $P_{2y}$ and $P_{2u}$ receptors in bovine aortic endothelial cells. Also, the 2′,5′-isomer of PPADS, *iso*-PPADS, has been shown to antagonize $P_{2x}$ receptor-mediated responses; little is known about its $P_2$ purinoceptor subtype selectivity, however (Khakh et al 1994; Trezise et al 1994c; Connolly 1995). In addition, at supra-micromolar concentrations *p*-chloromercuribenzene sulphonic acid (PCMBS) has been shown to inhibit nucleotide-induced contractions in the guinea-pig ileum and vas deferens, although displaying non-specific effects (Wiklund & Gustafsson 1988; Fedan & Lamport 1990). Despite an inherent relaxant effect, 2,2′-pyridylisatogen (PIT) at supra-micromolar concentrations is able to antagonize relaxations in the guinea-pig taenia coli (Spedding et al 1975). Apamin (0·1 $\mu$M), an oligopeptide from bee venom inhibits relaxations and inhibitory junction potentials in gastrointestinal smooth muscle preparations by blocking $Ca^{2+}$-dependent increases in $K^+$ conductance, thus being relatively non-specific (Maas et al 1980; Maas 1981).

Most recently, a novel ATP analogue, 2-propylthio-D-$\beta,\gamma$-difluoromethylene ATP (FPL 66096), was shown to be a potent $P_{2t}$ antagonist ($pA_2 = 8·7$), while being a weak antagonist at vascular $P_{2y}$ purinoceptors and a very weak agonist at vascular

Table 2. Ligand-based classification of $P_2$ purinoceptors in the absence of ecto-nucleotidase inhibition. For abbreviations see text.

| | |
|---|---|
| $P_{2x}$ | |
| Agonist | $\beta,\gamma$-m-L-ATP $\geq \alpha,\beta$-mATP $\geq \beta,\gamma$-mATP $\gg$ ATP $\geq$ ADP > 2-MeSATP $\gg$ UTP |
| Antagonist | $\alpha,\beta$-mATP (desensitizing agent); $ANAPP_3$; suramin; DIDS; PPADS; NF023 |
| $P_{2y}$ | |
| Agonist | 2-MeSATP $\approx$ ADP-$\beta$-S $\gg$ ATP = ADP > $\alpha,\beta$-mATP $\geq \beta,\gamma$-mATP > ADP-$\beta$-F > $\beta,\gamma$-m-L-ATP |
| Antagonist | reactive blue 2; suramin |
| $P_{2t}$ | |
| Agonist | 2-MeSADP > ADP |
| Antagonist | ATP; $Ap_4A$; suramin; FPL 66096; FPL 67085 |
| $P_{2z}$ | |
| Agonist | BzATP > 2-MeSATP = ATP = ATP-$\gamma$-S (tetrabasic form) |
| Antagonist | DIDS |
| $P_{2u}$ | |
| Agonist | UTP $\geq$ ATP = ATP-$\gamma$-S > ADP > 2-MeSATP > $\alpha,\beta$-mATP |
| Antagonist | suramin ? |
| $P_{2d}$ | |
| Agonist | $Ap_4A$ > ADP-$\beta$-S > $Ap_5A$ > $\alpha,\beta$-mATP $\gg$ 2-MeSATP |
| Antagonist | none known |

$P_{2x}$ purinoceptors (Humphries et al 1994b, 1995). Another potent, selective $P_{2t}$ antagonist is its dichloro analogue FPL 67085 (Humphries et al 1994a, 1995).

The ligand-based classification of $P_2$ purinoceptors in the absence of ecto-nucleotidase inhibition (see below) is summarized in Table 2.

*Potential pitfalls in the subclassification of purinoceptors*

The delineation of purinoceptor subtypes has been inevitably complicated and limited by several factors which are discussed below.

*Paucity of selective antagonists.* Within the $P_1$ purinoceptor family the discovery of selective antagonists, especially for the $A_{2b}$ and $A_3$ subtypes, is highly desirable. As mentioned above, except for FPL 66096 and FPL 67085 potent, selective and competitive $P_2$ antagonists have yet to be discovered. Because the use of antagonists avoids consideration of effective receptor reserve, it is of crucial importance in defining discrete receptor subtypes.

*Ecto-nucleotidases.* One of the major problems in defining $P_2$ purinoceptor subtypes is the rapid, sequential breakdown of nucleotide agonists by widely distributed ecto-enzymes located on the outer surface of the plasma membrane of effector cells and closely associated with the sites of nucleotide release (Zimmermann 1994; Maienschein & Zimmermann 1996). The extent of degradation is difficult to assess. Predominantly, three different catabolizing enzymes are involved in the regulation of extracellular ATP concentration (Ziganshin et al 1994a). Ecto-ATPases ($Ca^{2+}$- or $Mg^{2+}$-dependent or -independent ATPases) split off the terminal phosphate group and display broad nucleotide hydrolysing activity, although ATP is the preferential substrate. In contrast, ATP-diphosphohydrolase (apyrase) equipotently cleaves ATP, ADP, and other nucleoside tri- and diphosphates. Adenylate kinase catalyses the transfer of one phosphate (from ATP) to AMP, forming two ADP molecules. In addition, ADP and AMP, thus accumulating, can be further degraded with the final step (generating adenosine) involving 5′-nucleotidase (Maienschein & Zimmermann 1996). Importantly, this enzyme was also found intracellularly, where it plays a major role in adenosine formation during conditions of increased energy demand (Meghji 1993). The activity of ecto-nucleotidases particularly complicates the interpretation of any agonist-based study, because nucleotides might act indirectly on $P_1$ purinoceptors via the formation of adenosine or adenosine analogues, and, secondly, the true order of potency might be obscured (Kennedy & Leff 1995a). It was, indeed, shown in a patch-clamp study with single cells of rat-tail artery that the rank order of potency for a putative $P_{2x}$ receptor was ATP = 2-MeSATP $\geq \alpha,\beta$-mATP, possibly reflecting reduced enzymatic breakdown (Evans & Kennedy 1994). After removal of divalent cations, a similar rank order of potency (ATP = 2-MeSATP $\gg \alpha,\beta$-mATP) for the $P_{2x}$-mediated depolarizing effects of adenine nucleotides was found in the whole rat vagus nerve (Trezise et al 1994a). Because the preferred substrate for ecto-nucleotidases in many cases is ATP complexed with $Mg^{2+}$, these authors hypothesize that removal of $Ca^{2+}$ and $Mg^{2+}$ prevents metabolic breakdown. One might speculate that in some tissues relative potencies merely mirror relative resistance to dephosphorylation. Confirmation of this hypothesis awaits the availability of compounds which selectively inhibit ecto-nucleotidases. Suramin has been described as inhibiting nucleotide breakdown (Hourani & Chown 1989; Beukers et al 1995; Ziganshin et al 1995), but is fairly nonspecific (Crack et al 1994). In addition, at 100 $\mu$M reactive red 2 and NF023 (Fig. 4) inhibit ecto-nucleotidase (ATPase) activity to a considerable extent (95 and 51%, respectively; Beukers et al 1995; Bültmann & Starke 1995). Because of the presence of ecto-nucleotidases the potency of antagonists with an inherent ecto-ATPase inhibitory activity might be underestimated when tested against agonists which are a substrate for this enzyme (Crack et al 1994; Ziyal et al 1995). The

provision of a selective ecto-ATPase inhibitor will help to circumvent the above described difficulties. A promising compound, 6-*N*,*N*-diethyl-D-$\beta,\gamma$-dibromomethylene ATP (FPL 67156), was recently shown to inhibit ATP degradation in human blood cells (pIC50 = 4·6) while being a very weak antagonist at vascular $P_{2x}$ and platelet $P_{2t}$ receptors (Beukers et al 1994; Crack et al 1995).

*Rapid desensitization.* Stimulation of $P_{2x}$ purinoceptors usually leads within seconds to receptor desensitization, the mechanism of which remains to be resolved. Precautions such as single dose techniques, minimizing agonist contact time, and allowing sufficiently long dosage intervals must, therefore, be taken into consideration. Although desensitization is a salient feature of $P_{2x}$ purinoceptors (but see Brake et al 1994; Lewis et al 1995a; Buell et al 1996), it might also be attributable to other $P_2$ purinoceptor subtypes, such as $P_{2y}$ and $P_{2u}$ receptors (Hourani et al 1993a; Wilkinson et al 1994b).

*Adenosine uptake.* Because adenosine might be removed by a specific, bidirectional uptake carrier from the extracellular space (i.e. the site of action), uptake blockade is an absolute necessity to avoid unreliable results. Dipyridamole and *p*-nitrobenzyl-6-thioguanosine (NBTG) are commonly used efficient uptake blockers (Jacobson 1990; Williams & Cusack 1990; Fig. 5).

*Adenosine deamination.* The interpretation of studies utilizing adenosine as agonist might result in an underestimation of its potency, because adenosine is efficiently deaminated by a cytosolic as well as an extracellular adenosine deaminase (ADA). The extracellular ADA is restrictively distributed, which might explain why the effect of adenosine in some cases is not potentiated by uptake blockers (Meghji 1993). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and deoxycoformycin are used as ADA blockers (Jacobson 1990; Fig. 5).

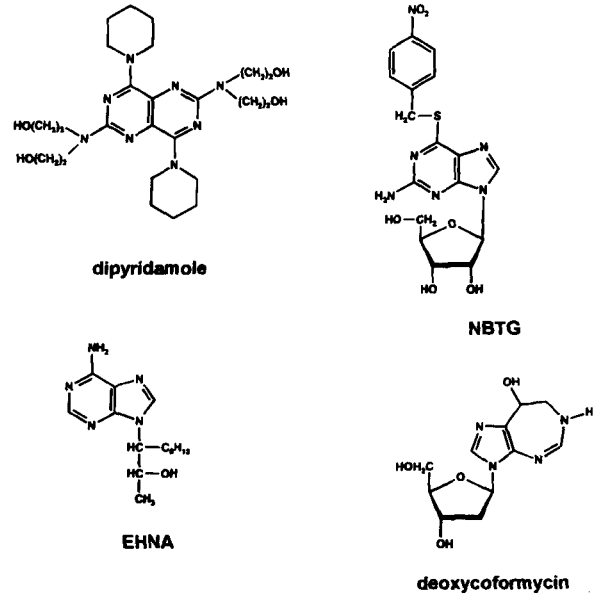



FIG. 5. The chemical structures of some nucleoside uptake blockers (above) and adenosine deaminase blockers (below).

*Heterogeneous receptor population.* An additional complicating factor is the presence of more than one type of purinoceptor, which might mediate parallel or opposing responses (Boland et al 1992; Wilkinson et al 1994b). Especially in the case of $P_2$ purinoceptors (paucity of highly selective antagonists) a mixed receptor population of already known $P_2$ receptor subtypes, which might be difficult to prove, has to be ruled out before postulating a novel purinoceptor subtype.

*Species or tissue differences.* There are many variations in the potency order of key ligands at a given purinoceptor subtype in the same tissue of different species or between different tissues believed to be endowed with the same subtype. These differences might well be because of differential expression of ecto-enzymes or purinoceptor subtypes, or both (Cusack 1993).

In an excellent review by Humphrey et al (1995) the difficulties in classifying $P_{2x}$ purinoceptors are discussed in detail.

Because of the foregoing difficulties, the classical framework of purinoceptor classification (Fig. 6) has to be regarded as provisional. In particular within the $P_2$ class, discrepancies have been increasingly reported, e.g. contraction-mediating $P_{2y}$ receptors (Bailey & Hourani 1990; Matharu & Hollingsworth 1992) or relaxation-mediating $P_{2x}$ receptors (Matharu & Hollingsworth 1992), the failure of suramin to inhibit ATP-induced contractions in guinea-pig vas deferens (Bailey & Hourani 1994), the direct stimulation by stable ATP analogues of $P_1$ receptors (Hourani et al 1991), and atypical purinoceptors with rank orders of agonists showing both $P_{2x}$ and $P_{2y}$ characteristics (Palea et al 1994). These inconsistencies seem to be indicative of subtypes of the purinoceptors delineated so far.

Indeed Abbracchio & Burnstock (1994) recently proposed reclassification of the $P_2$ receptor family. Briefly, they have categorized three superfamilies coupling to different transduction mechanisms: the $P_{2x}$ family as ligand-gated ion channels comprising four subtypes, the $P_{2y}$ family as G protein-coupled entities including seven subtypes, and the $P_{2z}$ subtype as a non-selective pore. Their proposal is based mainly on extensive analysis of literature concerned with discrepancies and on the pharmacological profile of new agonist tools (Fischer et al 1993; Bo et al 1994; Burnstock et al 1994) including the molecular biological information available (see below). These authors were able to develop agonists discriminating between endothelial and taenia coli $P_{2y}$ receptors, and vascular versus vas deferens or bladder $P_{2x}$ receptors. It should be pointed out, however, that this subtle agonist/transduction mechanism-based reclassification in itself is uncertain, thus still is tentative rather than definitive. Importantly, however, recent progress in cloning $P_2$ receptor subtypes (see below) strongly supports this proposal, which is now generally accepted and thus might eventually lead to a unifying classification scheme. As shown in Table 3 and described below, the P2X receptor family comprises at least six subclasses, whereas the P2Y receptor family consists so far of seven subclasses. This classification will be continuously updated with the availability of new information (Burnstock & King 1996).

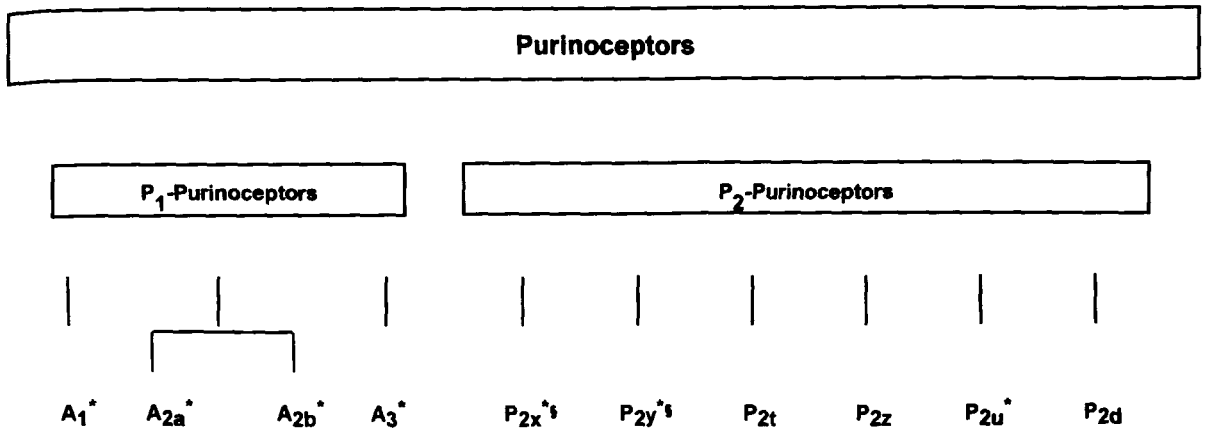



FIG. 6. Classical framework of purinoceptor subclassification. * Cloned entities. § multiple subtypes. Please note that the $P_{2u}$ purinoceptor has been identified as a $P_{2y}$ receptor subtype (for details see text).

Table 3. Current scheme of reclassification of $P_2$ purinoceptors according to Burnstock & King (1996).

| | | | | | | |
|---|---|---|---|---|---|---|
| P2X<br>ligand-gated ion channels | | | | | | |
| $P2X_1$ | $P2X_2$ | $P2X_3$ | $P2X_4$ | $P2X_5$ | $P2X_6$ | |
| P2Y<br>G protein-coupled receptors | | | | | | |
| $P2Y_1$ | $P2Y_2$ | $P2Y_3$ | $P2Y_4$ | $P2Y_5$ | $P2Y_6$ | $P2Y_7$ |
| P2Z (or P2X-like)<br>non-selective pore | | | | | | |

Due to a rapidly growing field, the given scheme of reclassification is expected to be extended.

## Signal Transduction Mechanisms of Purinoceptors

$P_1$ and $P_2$ purinoceptors both mediate their responses via multiple transduction mechanisms (for reviews see: Fredholm & Dunwiddie 1988; Seifert & Schultz 1989; Boeynaems & Pearson 1990; Linden 1991; Bean 1992; Benham 1992b; Dubyak & El-Moatassim 1993; Illes & Nörenberg 1993; Harden et al 1995). Whereas $P_1$ purinoceptors are exclusively guanine nucleotide-binding protein (G protein)-coupled entities, $P_2$ purinoceptors consist of G protein-coupled entities and intrinsic ligand-gated ion channels/pores. Although much of the transduction mechanisms has yet to be analysed, the major pathways are briefly outlined below.

### *$P_1$ purinoceptors*

*Adenylate cyclase.* This intracellular enzyme is either inhibited by $G_i$ (or as yet unidentified G proteins) or stimulated by $G_s$. As a consequence, the intracellular cyclic AMP (cAMP) level is decreased or increased, respectively. A cAMP-dependent protein kinase is activated, which in turn phosphorylates diverse protein targets. The $A_1$ ($G_i$) and $A_3$ subtypes (unknown type of G protein) are linked to this enzyme in an inhibitory manner. Conversely, the $A_2$ subtype ($G_s$) is positively linked to adenylate cyclase.

*Phospholipase C (PLC).* $P_1$ receptor stimulation might lead via $G_p$ to the activation of membrane-bound PLC, which hydrolyses phosphatidylinositol-4,5-biphosphate ($PIP_2$) to generate inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) as second messengers. $IP_3$ finally triggers $Ca^{2+}$ release from internal stores, whereas DAG activates protein kinase C (PKC), which in turn might phosphorylate diverse cell-specific proteins. The $IP_3$ pathway is one of several pathways involved in $A_1$ receptor–effector coupling, and in addition plays a role as transduction mechanism of the $A_3$ subtype. In a few tissues, however, a decrease of the $IP_3$ content might occur in response to $A_1$ receptor stimulation.

*Guanylate cyclase.* The stimulation of this enzyme via a $G_s$ leads to accumulation of the intracellular second messenger cyclic GMP (cGMP), which activates specific protein kinases to phosphorylate as yet unidentified intermediate(s). Ultimately, the intracellular $Ca^{2+}$ level is reduced. This pathway is believed to account at least partly for $A_2$ receptor-mediated vasodilations.

*Potassium channels.* After $P_1$ receptor stimulation and G protein coupling, the opening of diverse (including acetylcholine-regulated) $K^+$ channels might lead to hyperpolarization and shortening of the action potential, as has been well documented for the $A_1$ receptor in atria (Belardinelli 1993). There have also been reports of $A_2$ receptor-related opening of $K^+$ channels (Furukawa et al 1993).

*Calcium channels.* A G protein-mediated inactivation of ligand-gated $Ca^{2+}$ channels and indirect closure of voltage-dependent $Ca^{2+}$ channels is another important pathway for $A_1$ receptor-mediated responses.

### *$P_2$ purinoceptors*

*Intrinsic ion channels.* Direct activation by ATP of ligand-gated ion channels spontaneously triggers a non-specific cationic current ($Na^+$, $Ca^{2+}$, $K^+$), thus resulting in depolarization of the cell. This can promote the opening of voltage-dependent $Ca^{2+}$ channels, thereby increasing $Ca^{2+}$ influx. $P_{2x}$ receptor

subtypes have been identified as an integral part of this type of channel (Benham & Tsien 1987; see below: Brake et al 1994; Valera et al 1994, 1995; Bo et al 1995; Chen et al 1995; Lewis et al 1995a; Surprenant et al 1995; Buell et al 1996; Collo et al 1996).

*Non-selective pore.* ATP-binding to cell-surface receptors of macrophages can induce a reversible formation of a non-selective pore, permeable to small cations, anions and organic molecules with a molecular mass of less than 1 kDa. Likewise, endogenous metabolites are able to pass these pores. Sustained activation of these receptors ($P_{2z}$) will be cytotoxic. It might be speculated that this mechanism is related to programmed cell death (apoptosis).

*Phospholipase C (PLC).* This pathway (see above), which results in an increase in intracellular $Ca^{2+}$, is of prime importance in $P_{2y}$ and $P_{2u}$ receptor-coupling, the G proteins involved are, however, different (Pirotton et al 1993; Chang et al 1995). $IP_3$ accumulation is also observed after stimulation of the platelet $P_{2t}$ receptor, although it remains to be determined whether this involves a direct G protein-dependent PLC activation. The $P_{2d}$ receptor in chromaffin cells appears, moreover, to be operating by an increased intracellular $Ca^{2+}$ level via PLC and subsequent PKC stimulation (Pintor & Miras-Portugal 1993).

*Phospholipase $A_2$.* Activation of this enzyme can be direct or secondary to PLC stimulation, and is thus triggered by an increase of intracellular $Ca^{2+}$. Subsequently, arachidonic acid is metabolized, which results in the production of prostacyclin, a second messenger of endothelial $P_{2y}$ and $P_{2u}$ receptor-mediated vasodilation.

*Potassium channels.* Opening of $K^+$ channels after an increase in intracellular $Ca^{2+}$ evokes hyperpolarization. This mechanism underlies relaxations after $P_{2y}$ receptor activation.

*Other cation channels.* Influx of extracellular $Ca^{2+}$ is the initial response to stimulation of the platelet $P_{2t}$ receptor. $Na^+$ and $K^+$ also pass this type of channel. Whether this is a direct rather than G protein-coupled effect is unresolved. A G protein-linked cation channel ($Na^+$, $Ca^{2+}$) might be involved in some $P_{2y}$-elicited responses.

*Chloride channels.* The activation of airway epithelium $P_{2u}$ purinoceptors leads to $Cl^-$ secretion triggered by increased intracellular $Ca^{2+}$ levels. In addition, a $Ca^{2+}$-dependent inward current of $Cl^-$ has recently been described for the cloned $P2Y_1$ subtype (Banard et al 1994).

*Nitric oxide (NO).* The release of NO from endothelial cells requires the influx of extracellular $Ca^{2+}$, which stimulates NO-synthase to metabolize the substrate L-arginine. This pathway partly accounts for vasodilations as a response to $P_{2y}$ and probably also $P_{2u}$ receptor stimulation (Pirotton et al 1993).

*Adenylate cyclase.* Several reports have emerged demonstrating the inhibition or stimulation of adenylate cyclase (see above) as signalling pathway for the $P_{2y}$ receptor (e.g. Boyer et al 1993; Gailly et al 1993; Boyer et al 1994). Because the adenylate cyclase pathway was originally a criterion for distinguishing between the $P_1$ and $P_2$ purinoceptors, these are remarkable observations possibly supporting the heterogeneity of the $P_{2y}$ subtype. The platelet $P_{2t}$ receptor also appears to be involved in the inhibition of adenylate cyclase.

## Molecular Characterization of Purinoceptors

Receptor-cloning strategies entered the purine field in 1989, and only three years later all four adenosine receptors had been successfully cloned from different species, including man (for reviews see: Tucker & Linden 1993; Linden 1994). The recombinant receptors have been identified by ligand-binding and signal-transduction studies as the pharmacologically characterized $A_1$ (Libert et al 1991), $A_{2a}$ (Maenhaut et al 1990), $A_{2b}$ (Stehle et al 1992), and $A_3$ (Zhou et al 1992) purinoceptors. These receptors belong to the superfamily of G protein-coupled entities with seven putative transmembrane domains (α-helices), interconnecting loops, an extracellular terminal amino residue, and a cytoplasmic terminal carboxylate residue. Whereas a negatively charged aspartate in the third transmembrane region is a hallmark of cationic amine receptors (e.g. acetylcholine, catecholamines), adenosine receptors lack this amino acid (aa), and instead the corresponding residue is an uncharged valine (Jacobson et al 1993b). The $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$ purinoceptors are, moreover, fairly small having 326–328, 409–412, 328–332, and 317–320 aa, respectively. With an aa sequence homology of 49·5%, the $A_3$ subtype is more closely related to the $A_1$ than to the $A_{2a}$ (43·2%) or $A_{2b}$ (39·9%) subtypes (Linden 1994). It should be noted that the recombinant $A_1$ and $A_3$ receptors (like their native counterparts) display remarkable inter-species dissimilarities regarding their structure, tissue distribution and ligand binding characteristics.

Cloning strategies have also been introduced to the $P_2$ purinoceptor field. In 1993, the first clone (from chick brain; 362 aa) was identified as a $P_{2y}$ receptor belonging to the superfamily of G protein-coupled entities (Webb et al 1993). Because the pharmacology (near equipotency of 2-MeSATP and ATP) differed from the classical $P_{2y}$ subtype, the authors have designated this subtype as $P2Y_1$. After this report, Lustig et al (1993) described the cloning of a $P_{2u}$ receptor (373 aa), from a mouse neuroblastoma cell-line, that was equally sensitive to UTP and ATP and belonged to the same superfamily. Two other groups were successful in cloning the $P_{2u}$ purinoceptor from human airway and colonic epithelium (Parr et al 1994) and from rat heart (Gödecke & Schrader 1994). During the cloning procedure for the $P2Y_1$ receptor Webb et al (1996a) isolated a second clone from chick brain encoding a protein with the same structural motif and being preferentially sensitive to ADP. In this regard it resembled the $P_{2t}$ subtype, which, however, unlike the recombinant receptor, is not activated by ATP. The authors therefore termed it $P2Y_3$, designating the cloned $P_{2u}$ receptor $P2Y_2$ (Banard et al 1994). Communi et al (1995) and Nguyen et al (1995) cloned a $P_{2y}$ receptor from human placenta and chromosome X, respectively ($P2Y_4$; UTP-sensitive). The receptor cloned from human activated T cells by Webb et al (1996b) was designated $P2Y_5$ (ATP as the preferential agonist). Furthermore, a clone (328

aa) was isolated from rat aortic smooth muscle which encodes a receptor with a novel pharmacological profile: both UTP and ADP are more active than ATP ($P2Y_6$; Chang et al 1995). Finally, Kunapuli et al (1995) cloned a $P_{2y}$ receptor from human erythroleukaemic cells (provisionally called $P2Y_7$). Burnstock & King (1996) indicate further tissues from which the seven P2Y subclasses (Table 3) have been cloned.

These newly identified members of the G protein-coupled superfamily have small sequences and, as with adenosine receptors (see above), the aspartate residue in the third transmembrane helix is not present. Filtz et al (1994) recently established the signaling properties of the cloned turkey homologue of the $P2Y_1$, which was found to be coupled to PLC, as is the $P_{2y}$ receptor subtype cloned from rat aortic smooth muscle (see above; Chang et al 1995).

Revolutionary progress was accomplished by Valera et al (1994, 1995), who cloned the $P_{2x}$ intrinsic cation channel from rat vas deferens and human urinary bladder ($P2X_1$). The recombinant receptor, expressed in Xenopus oocytes or human embryonic kidney cells, revealed a somewhat atypical rank order of potency when measuring inward currents: 2-MeSATP $\geq$ ATP $>$ $\alpha,\beta$-mATP $\gg$ ADP. As expected, after repeated drug application responses tended to fade and were blocked by suramin or PPADS (Fig. 4). Predicting the molecular structure of this protein (Fig. 7), the $P_{2x}$ purinoceptor becomes introduced as a member of a new family of ligand-gated ion channels. The protein (399 aa), the biggest part of which is extracellularly located, contains only two membrane-spanning regions with both intracellular terminal amino and carboxylate residues and a small pore-forming motif.

An independent group cloned a $P_{2x}$ receptor (472 aa) from rat pheochromocytoma PC12 cells, which had similar electrophysiological and structural characteristics but a different pharmacological profile ($P2X_2$; Brake et al 1994) from those of the $P2X_1$ receptor.

Most recently, Chen et al (1995) and Lewis et al (1995a) described the cloning of a $P_{2x}$ receptor (397 aa), which is selectively expressed in sensory C-fibre nerves, from rat dorsal root ganglia ($P2X_3$). Importantly, this subtype forms a heteromultimer with the $P2X_2$ sub-unit, thereby constituting a novel channel phenotype ($\alpha,\beta$-mATP-sensitive but non-desensitizing; Lewis et al 1995a).

A fourth $P_{2x}$ receptor subtype ($P2X_4$; cloned from rat superior cervical ganglion and rat hippocampus) has, furthermore, been identified; this is poorly $\alpha,\beta$-mATP-sensitive or $\alpha,\beta$-mATP-insensitive and resistant to blockade by either suramin, pyridoxal-5-phosphate or PPADS (Bo et al 1995; Lewis et al 1995a, b; Buell et al 1996).

In addition, Collo et al (1996) cloned receptors from rat ganglia ($P2X_5$) and brain ($P2X_6$). For further information about the six P2X subclasses (Table 3) see Burnstock & King (1996).

Interestingly, the sequence of the cloned $P2X_1$ and $P2X_2$ receptor resembles that of a clone isolated from thymocytes which after expression was shown to induce cells to die (Brake et al 1994; Valera et al 1994). There might, therefore, be a link between $P_{2x}$ receptor subtypes and apoptosis.

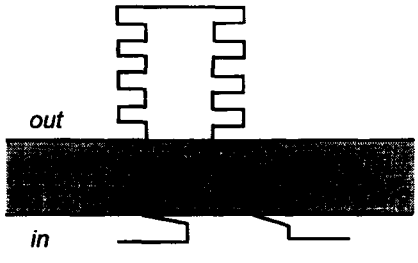



FIG. 7. Membrane topology for the $P2X_1$ purinoceptor, as suggested by Valera et al (1994). The overall pattern of hydrophobicity for the $P2X_2$, $P2X_3$ and $P2X_4$ subtypes is also consistent with this model (Buell et al 1996).

## Distribution and Biological Function of Purinoceptors

Both adenosine and ATP have been shown to participate in the regulation of a number of physiological and pathophysiological functions. Likewise, the receptors of these endogenous ligands are widely distributed in mammals. Only a few crucial examples have been selected and will be discussed below.

### *CNS*

Whereas most brain regions are rich in $A_1$ and $A_{2b}$ purinoceptors, the $A_{2a}$ subtype is specifically located in the striatum, a dopamine-rich region (Collis & Hourani 1993). The $A_3$ subtype seems to be expressed less abundantly in the brain (Linden 1994). Adenosine acts as a neuromodulator inhibiting, via prejunctional $A_1$ receptors, the release of various transmitters including excitatory amino acids (EAA; Fredholm & Dunwiddie 1988; Fredholm et al 1993). In addition, adenosine tends to limit the excitation of effector cells. Thus, adenosine reduces neuronal activity and oxygen-consumption. An increase of cerebral blood flow results, furthermore, from $A_2$ receptor activation. Collectively, adenosine might be regarded as an endogenous cerebroprotective agent, the production of which is increased, e.g., during ischaemic conditions (Rudolphi et al 1992; Fredholm et al 1993). Adenosine also exerts behavioural effects; these include the decrease of locomotor and psychomotor activity (sedative action) which is most likely to be correlated with postsynaptic $A_{2a}$ receptor stimulation (Fredholm et al 1993). Notably, a postsynaptic $A_{2a}/D_2$ interaction has been suggested in that adenosine agonists reduce dopamine binding (Fredholm et al 1993). An anticonvulsive property of adenosine has also been described. In the spinal cord, adenosine is involved in the analgesic effects of opioids (Daval et al 1991).

ATP has been identified as an excitatory neurotransmitter mediating fast synaptic transmission in the CNS (Benham 1992a; Edwards et al 1992). Receptors for ATP are present on neurones and glial cells (Zimmermann 1994). In addition to $P_{2x}$ purinoceptors which have been shown by autoradiography to be widely distributed in rat brain and spinal cord (Bo & Burnstock 1994), other purinoceptors, which do not seem to fit neatly into any of the classical $P_2$ subtypes, are localized within the CNS (Bean 1992; Illes & Nörenberg 1993). The precise function, however, is unclear. In the spinal cord, ATP participates in sensory neurotransmission (Hoyle & Burnstock 1991). Most recent data indicate that ATP is involved in the generation of pain signals via $P2X_1$ and $P2X_2$ receptors in the dorsal horn of the spinal cord (Kennedy & Leff 1995b) and via the $P2X_3$ subtype in sensory C fibre nerves (Chen et al 1995; Kennedy & Leff 1995b; Lewis et al 1995a). The $P2X_4$ subtype

is, furthermore, abundantly expressed in rat brain regions and spinal cord (Bo et al 1995; Buell et al 1996) and the $P_2X_2$ subtype has been shown by immunohistochemical data to be distributed in brain regions (Vulchanova et al 1995).

*Cardiovascular system*

$A_1$ receptors are located on atrial and ventricular myocytes, and the $A_{2a}$ and $A_{2b}$ subtypes are present on coronary smooth muscle and coronary endothelium, respectively (Tucker & Linden 1993). There is, conversely, only moderate expression of $A_3$ receptors in the heart (Linden 1994). Adenosine exerts cardiodepressive effects, i.e. a negative chronotropic, dromotropic, and antiarrhythmic action via $A_1$ receptors, and inhibition of inotropic responses to $\beta$-adrenergic agonists, thus decreasing oxygen consumption (Belardinelli 1993). Oxygen supply is, on the other hand, increased by increasing coronary blood flow after $A_2$ mediated vasodilation. Adenosine is generally a potent vasodilator ($A_2$ and $A_3$), except in the kidney (see below) and a few other blood vessels (Collis & Hourani 1993). Peripheral vasodilation in combination with reduced cardiac output results in the effective lowering of blood pressure. In addition, adenosine is an endogenous inhibitor of platelet aggregation ($A_{2a}$; Collis & Hourani 1993), owing to increased cAMP levels.

In general, ATP exerts cardiodepressant effects which might well result from its breakdown product, adenosine. In contrast, Mantelli et al (1993) were able to show a positive inotropic effect of ATP and analogues in guinea-pig left atria after blockade by DPCPX of $A_1$ receptors. They concluded that this effect might be a result of $P_{2y}$ receptor stimulation. In blood vessels, ATP mediates vasoconstriction via smooth muscle $P_{2x}$ purinoceptors and vasodilation via endothelial $P_{2y}$ purinoceptors. Endothelial-independent relaxation via smooth muscle $P_{2y}$ receptors seems to be the exception (for a review see Ralevic & Burnstock 1991b). $P_{2u}$ purinoceptors might contribute to ATP-elicited vasoconstriction and vasodilation, respectively. Notably, Chang et al (1995) described the abundant expression of a novel UTP- and ADP-sensitive $P_{2y}$ purinoceptor (see above) in rat aorta and mesentery which might be involved in UTP-induced vasoconstriction. In addition, ATP inhibits platelet aggregation via the $P_{2t}$ receptor, which might be regarded as a self-limiting regulatory process during damage of the vessel wall.

*Renal system*

Adenosine acts to reduce the glomerular filtration rate by $A_1$ receptor-mediated vasoconstriction of afferent and $A_2$ receptor-mediated vasodilation of efferent arterioles (Spielman et al 1990) resulting in an antidiuretic effect. Another important action of adenosine is the inhibition of renin release via $A_1$ receptors. The role of $A_3$ purinoceptors in the kidney remains to be elucidated.

There is, in addition, no doubt about purinergic neurotransmission by ATP in the kidney and a regulatory function by ATP of renal microvasculature (for a review see Inscho et al 1994). The most prominent response is vasoconstriction ($P_{2x}$), which appears to be restricted to preglomerular elements. There are, however, also functional vasodilation-mediating $P_{2y}$ purinoceptors on epithelial cells.

*Respiratory tract*

Whereas in healthy subjects adenosine exerts bronchodilator effects ($A_2$), in asthma patients the response is bronchoconstriction (Jacobson 1990). Although there are contraction-mediating $A_1$ receptors on respiratory smooth muscle, contraction could well be an indirect response, bearing in mind that in asthmatics adenosine is able to release histamine and leukotrienes as mast-cell mediators (Linden 1994). This effect of adenosine is weakly blocked by xanthines. Because the $A_3$ purinoceptor, which is mainly xanthine-insensitive, is abundantly expressed in the lung, it is suggested that this subtype might be involved in the aetiology of asthma (Linden 1994).

ATP has been shown to activate $Cl^-$ secretion from airway epithelium, an effect which is obviously linked to $P_{2u}$ purinoceptors (O'Connor 1992), and is involved in mucociliary clearance. Also, the recently cloned $P2X_4$ receptor is expressed in bronchial epithelium and lung (Bo et al 1995; Buell et al 1996).

*Gastrointestinal tract*

ATP has long been recognized as a non-adrenergic, non-cholinergic (NANC) inhibitory transmitter in the gut (Burnstock et al 1970). Likewise, receptors for ATP and its metabolite adenosine are widely distributed in the gut. Relaxation-mediating $P_1$ purinoceptors have been shown to be present on rat duodenum ($A_1$, $A_{2b}$; Nicholls et al 1992), rat colon longitudinal muscle ($A_2$; Bailey & Hourani 1992), and guinea-pig taenia coli ($A_2$; Burnstock et al 1984). Inhibitory $P_{2y}$ purinoceptors have been identified, e.g. in rat pyloric sphincter (Soediono & Burnstock 1994), rat duodenum (Nicholls et al 1990), and guinea-pig taenia coli being considered as archetypal $P_{2y}$ subtype (Burnstock & Kennedy 1985; Hourani et al 1991; but see: Windscheif et al 1994b, 1995a; Bültmann & Starke 1995; Dudeck et al 1995). Both excitatory $P_1$ and $P_2$ subtypes have, in addition, been found to be implicated in contractions of the gut: $A_1$ and $P_{2y}$ purinoceptors in rat colon muscularis mucosae (Bailey & Hourani 1990; Hourani et al 1993a), a probably novel $P_2$ subtype in rat duodenum (Johnson & Hourani 1994), $P_{2y}$ (together with inhibitory $P_{2x}$) receptors in rat gastric fundus (Matharu & Hollingsworth 1992), and neuronal and smooth muscle $P_2$ purinoceptors in guinea-pig ileum which have yet to be identified (Wiklund & Gustafsson 1988; Sperlagh & Vizi 1991; Kennedy & Humphrey 1994). Interestingly, moderate levels of the novel $P2Y_6$ subtype cloned by Chang et al (1995; see above) have been detected in rat stomach and intestine.

*Urogenital tract*

The neurogenic excitation of the bladder results partly from acetylcholine and partly from ATP, which is believed to participate in parasympathetic neurotransmission. Contractions to ATP of the detrusor muscle are evoked by $P_{2x}$ receptor stimulation (for a review see Hoyle & Burnstock 1991). Relaxation-mediating $P_{2y}$ purinoceptors have, on the other hand, been shown to be present on, e.g., rat urinary bladder (Bolego et al 1994). In certain disease states (e.g. interstitial cystitis), the purinergic component of bladder tone seems to be increased (Palea et al 1993). The $P2X_1$ and $P2X_4$ receptors have been shown to be distributed in urinary bladder (Bo et al 1995; Valera et al 1995).

There might, in addition, be inhibitory prejunctional $A_1$ and

postjunctional $A_2(A_{2b})$ purinoceptors, as was found in mouse and rat urinary bladder (Acevedo et al 1992; Nicholls et al 1992). In the vas deferens of various species, contractions are mediated by ATP and noradrenaline, which have been shown to act as co-transmitters (Sneddon & Westfall 1984; Burnstock 1990; Von Kügelgen & Starke 1991; Lambrecht et al 1992; Brock & Cunnane 1993; Driessen et al 1994). Briefly, prejunctional inhibitory $A_1$ (Windscheif et al 1992; Gonçalves & Queiroz 1993; Hourani et al 1993b; Driessen et al 1994; Martin & May 1994), $P_2$ (Von Kügelgen et al 1989, 1993, 1994), $P_3$ (Forsyth et al 1991; Todorov et al 1994) and facilitating $A_{2a}$ (Gonçalves & Queiroz 1993) purinoceptors and post-junctional excitatory $P_{2x}$ (Von Kügelgen et al 1989; Fedan et al 1990; Bo et al 1992; Lambrecht et al 1992; Mallard et al 1992) and additional contractile $P_2$ (Von Kügelgen et al 1989; Bailey & Hourani 1994; Bültmann & Starke 1994b) receptors and inhibitory $A_2$ (Hourani et al 1993b; Martin & May 1994) and $P_{2y}$ (Boland et al 1992) receptors might be involved in the development/modulation of the mechanical response in vasa deferentia of different species. Recent cloning and expression studies revealed the presence of the $P2X_1$ and $P2X_4$ subtype in vas deferens (Bo et al 1995; Valera et al 1995).

## Therapeutic Implications

Given the widespread distribution of purinoceptors within the body and the ample biological functions being controlled by endogenous adenosine and ATP, an immense therapeutic potential can be envisaged. Precise knowledge about receptor subtypes and the mechanistic actions of endogenous ligands is, however, the rationale for any therapy. Despite many open questions, especially in the field of $P_2$ purinoceptors, there remain several lines of evidence showing a benefit from 'purinergic' therapy with a strong emphasis on intervention with adenosine functions. Only a selection of many important examples will be considered below.

In addition to a direct ligand approach (agonists or antagonists), an indirect approach using agents that act to potentiate the actions of endogenous adenosine (see below), and might thus be regarded as 'site- and event-specific' agents, often seems more valuable because of fewer side effects.

### *CNS*

Adenosine plays an important role in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases involving EAA excitotoxicity (Daval et al 1991). Importantly, it has been shown in Alzheimer patients that the $A_1$ receptor density is reduced whereas coupling to G proteins is preserved (Ulas et al 1993). The administration of $A_1$ agonists has been found to protect against neurodegeneration (Daval et al 1991). Adenosine antagonists appear, on the other hand, to be beneficial in the treatment of these disorders (for a review see Williams 1993). $A_{2a}$ selective antagonists are promising as anti-parkinsonian agents because of their indirect enhancement of dopamine binding. $A_1$ receptor antagonists are being developed as cognition enhancers with potential use in Alzheimer's disease. To this end, a recent study must be mentioned-it has been demonstrated in mice that chronic treatment (the regimen of choice in these disorders) with an $A_1$ receptor antagonist somewhat impaired learning and memory, which is the direct opposite to the results obtained after acute treatment (Von Lubitz et al 1993). As has been shown for carbamazepine, chronic administration of $A_1$ receptor antagonists will be useful in the treatment of seizures (epilepsy) and might also serve as a prophylactic of affective diseases (Van Calker & Berger 1993). $A_1$-selective agonists in combination with hypnoanalgesics might, furthermore, render pain-therapy more effective and probably safer, because activation of adenosine receptors in the dorsal horn of the spinal cord inhibits transmission induced by pain mediators (e.g. substance P; Daval et al 1991). There is some general concern about the successful dissociation of the central effects of adenosine analogues from their cardiovascular effects.

Because ATP is the mediator of fast synaptic transmission in the CNS, and thus might play a role as excitotoxicant, one might speculate that $P_{2x}$ purinoceptor antagonists, capable of crossing the blood-brain barrier, could be useful protectors against neurodegeneration. It should be noted, however, that the widely distributed $P2X_4$ subtype is insensitive to commonly used $P_{2x}$ purinoceptor antagonists (see above). The selectively localized $P2X_3$ purinoceptor in sensory C fibre nerves represents, moreover, a likely therapeutic target for $P2X_3$ antagonists as effective analgesics (Chen et al 1995; Kennedy & Leff 1995b; Lewis et al 1995a).

### *Cardiovascular system*

Adenosine has been approved for use in man in the USA (Adenocard) as an antiarrhythmic and for controlled hypotension in aneurism surgery. Most recently, it has also been approved in Germany (Adrekar) for the treatment of paroxysmale supraventricular tachycardia. In addition, adenosine is of diagnostic use in arrhythmias and valuable in cardiac preconditioning (Bertolet & Hill 1993). $A_2$ receptor agonists and uptake blockers (e.g. dipyridamole) are potent coronary vasodilators and hypotensive agents and inhibitors of platelet aggregation. Acute treatment with $A_1$ receptor agonists, indirect agents like adenosine uptake blockers, acadesine (a purine nucleoside with antiischaemic properties being clinically evaluated) or long-term treatment with adenosine antagonists seems, moreover, to be useful in stroke therapy (Marangos et al 1990; Daval et al 1991; Williams 1993). $A_2$-selective agonists will ameliorate cerebral blood flow, thus contributing to an efficient stroke therapy. Selective $A_{2a}$ receptor agonists might also serve as antithrombotics, owing to inhibition of platelet aggregation.

$P_{2t}$ purinoceptor antagonists have been proposed as potential antithrombotic agents. A potent ATP analogue (FPL 67085) was recently shown to be effective in inhibiting ADP-induced platelet aggregation without having additional haemodynamic effects (Humphries et al 1994a, 1995). FPL 66096 is another promising compound in this regard (Humphries et al 1995).

### *Renal system*

Selective $A_1$ receptor antagonists are being developed as novel diuretics with potential protective action against acute renal failure. A recent study using a xanthine analogue (KW-3902) documented a long-lasting diuretic and natriuretic effect after oral administration in conscious dogs (Kobayashi et al 1993).

*Respiratory tract*

On the basis of insight on adenosine function as a secretagogue of histamine and leukotrienes from mast cells involving $A_3$ receptor stimulation, the development of $A_3$ selective antagonists might be an intriguing novel approach in asthma therapy (Linden 1994).

Inhaled ATP and UTP approved to be of clinical value in the treatment of cystic fibrosis, a disease which so far cannot be treated efficiently. These drugs enhance $Cl^-$ secretion independent of the defective cystic fibrosis transmembrane regulator, and thus help to improve mucociliary clearance (Boucher 1994).

*Gastrointestinal tract*

Because of the wide distribution of relaxation- and contraction-mediating $P_1$ and $P_2$ purinoceptors in the intestine, it seems likely, that selective purinergic compounds might be introduced in the therapy of gut motility dysfunctions.

*Urogenital tract*

The discovery of a purinergic component of the bladder tone (Hoyle & Burnstock 1991) is of potential importance in the development of potent, selective $P_{2x}$ purinoceptor antagonists for the therapy of stress incontinence, a disease state which is lacking efficient treatment.

*Miscellaneous targets*

Apart from the therapeutic implications described above, it should be pointed out that patients with an inherited ADA deficiency (often observed in patients with severe combined immunodeficiency) might profit from an ADA gene replacement therapy (Hirschhorn 1993).

ADA inhibitors have potential importance as anticancer or antiviral agents (Agarwal 1982). The potent lymphocytotoxic ADA inhibitor, deoxycoformycin (Fig. 5; Pentostatin; Nipent), has recently been approved for the treatment of haircell leukaemia in Germany (Hiller 1995).

ATP itself is able to arrest tumor growth (S phase of the cell cycle) and has already entered clinical phase I trials (Dubyak & El-Moatassim 1993; Rapaport 1993; Haskell et al 1994).

Finally, pancreatic B cells are endowed with $A_1$, $P_{2y}$ and $P_{2x}$ purinoceptors which, upon stimulation, mediate reduced and increased insulin secretion, respectively (Loubatières-Mariani et al 1994; Petit et al 1994). Thus, $P_{2y}$ ($P_{2x}$) agonists have the potential to be new insulin-secretory antidiabetic drugs.

Since the preparation of this review, the $P_{2z}$ purinoceptor has been identified as a member of the P2X family, $P2X_7$ (Surprenant et al 1996).

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