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Receptors for Adenine Nucleotides and Nucleosides: Subclassification, Distribution, and Molecular Characterization*

HUGH H. DALZIEL AND DAVID P. WESTFALL†

Department of Pharmacology, University of Nevada School of Medicine, Reno, Nevada

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† To whom correspondence should be addressed: Department of Pharmacology, University of Nevada, School of Medicine, Reno, NV 89557.

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I. Introduction

Adenine nucleosides and nucleotides exert a variety of effects on numerous cell types. These actions result from the activation of discrete membrane receptors, generally referred to as purinoceptors. Based on an extensive analysis of the literature, Burnstock (1978) suggested that there are two main classes of purinoceptors, called P_1 and P_2 purinoceptors. This classification was based on four criteria: (*a*) comparison of the relative potencies of ATP‡, ADP, AMP, and adenosine; (*b*) observation of the selective actions of antagonists, in particular the methylxanthines; (*c*) modulation of adenylyl cyclase by adenosine but not ATP; and (*d*) induction of prostaglandin synthesis by ATP but not adenosine.

It was indicated in the literature that P_1 purinoceptors (adenosine receptors) were more responsive to adenosine and AMP than to ADP and ATP, that methylxanthines such as theophylline and caffeine were antagonists at these receptors, and that stimulation of P_1 purinoceptors led to a modulation of adenylyl cyclase activity. P_2 purinoceptors (ATP receptors), on the other hand, were more responsive to ATP and ADP than to AMP and adenosine, were not antagonized by methylxanthines, and did not modulate the adenylyl cyclase system. Their stimulation was sometimes associated with prostaglandin synthesis (Burnstock, 1978; Burnstock and Buckley, 1985).

Since the time of this initial classification, additional subdivisions of purinoceptors have been proposed. This review summarizes the main evidence that has given rise to the current purinoceptor subclassification. Furthermore, sections of this review have been devoted to consideration of more recent work on the second-messenger systems that are thought to mediate the functional responses of purinoceptors. Also, the biochemical characterization and molecular biology of receptors for adenosine and adenine nucleotides receives consideration here. This is an exciting new field that is gaining momentum. It offers detailed insight into the structure and function of these receptors and how they interact with their ligands.

II. P_1 Purinoceptors (Receptors for Adenosine)

It has been known for 60 years that adenosine has potent actions on the heart and circulation, causing bradycardia, coronary vasodilation, and a decrease in systemic blood pressure (Drury and Szent-Gyorgi, 1929). During certain conditions such as hypoxia, adenosine is introduced into the extracellular compartment either by facilitated diffusion from the cell interior or by degradation of released ATP by ecto-ATPases. (For a review of the literature pertaining to the origin and fate of adenosine see the chapter by Meghji, 1991). Under such conditions, adenosine can exert modulatory functions on neuronal, vascular, and parenchymal cell biochemistry and physiology in many tissues. Adenosine exerts its effects through an heterogeneous population of adenosine receptors. These receptors have been subclassified by various workers and, unfortunately, the early literature is somewhat confusing, because the same receptors were referred to by different designations. This disparate nomenclature has recently been clarified (Abbracchio et al., 1993).

A. A_1/A_2 (R_i/R_a) Subclassification of P_1 Purinoceptors

1. P_1 purinoceptor subclassification based on modulation of adenylyl cyclase activity. After the discovery of the second-messenger cAMP and the associated enzymes of its formation (adenylyl cyclase) and destruction (cAMP phosphodiesterases), considerable effort was directed at evaluation of inhibitors of phosphodiesterase. These drugs inhibit the intracellular breakdown of cAMP and so potentiate the effect of adenylyl cyclase activation. It was found that the xanthine class of cAMP-phosphodiesterase inhibitors, examples of which are theophylline and caffeine, paradoxically inhibited cAMP accumulation in guinea pig brain in response to adenylyl cyclase activation by adenosine, although the expected effect was an increase in cAMP accumulation (Sattin and Rall, 1970). It was suggested that the phosphodiesterase inhibitor-induced modulation of cAMP accumulation in response to adenosine was caused by their extracellular actions as adenosine antagonists. Furthermore, it was shown that adenosine caused a concentration-dependent dual effect on adenylyl cyclase, producing an increase in tissue cAMP content at lower concentrations and a decrease at higher concentrations (Sattin and Rall, 1970). This was substantiated by the finding that discrete extracellular recognition sites for adenosine exist that are capable of either positively or negatively modulating the activity of adenylyl cyclase (Londos and Wolff, 1977; Van Calker et al., 1979; Londos et al., 1980, 1983). Further interest in phosphodiesterase inhibitors produced a series of modified xanthines, such as 8-PT, which seemed to have relatively greater effects as adenosine receptor antagonists than cAMP phosphodiesterase inhibitors (Smellie et al., 1979a).

Thus, the receptor site for adenosine could be subclassified using appropriate concentrations of adenosine to modulate adenylyl cyclase activity in fetal mouse brain cells. These receptor subtypes were termed A_1 and A_2

‡ Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; cAMP, cyclic adenosine monophosphate; $ANAPP_3$, arylazidoaminopropionyl; NECA, 5′-N-ethylcarboxamidoadenosine; 2-CA, 2-chloroadenosine; CV1808, 2-(phenylamino)adenosine; CGS21680, 2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-N-ethylcarboxamido-adenosine; 8-SPT sulfophenyltheophylline; 8-PT, 8-phenyltheophylline; SV40, simian virus 40; cDNA, complementary deoxyribonucleic acid; mRNA, messenger ribonucleic acid; PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine; EDRF, endothelium-derived relaxing factor; AP_4A, P^1,P^4-di-(adenosine-5′)tetraphosphate; AP_5A, P^1,P^5-di-(adenosine-5′)pentaphosphate; RB2, reactive blue; UTP, uridine triphosphate; ATPγS, 5′-(thio)-triphosphate.

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and could either inhibit (A_1) or stimulate (A_2) adenylyl cyclase (Van Calker et al., 1979). Independently, Londos et al. (1980) also subdivided adenosine receptors in relationship to their modulatory action over adenylyl cyclase. They termed these receptors R_i and R_a (respectively corresponding to the A_1 and A_2 receptor), because of their requirement for an intact ribose moiety (hence R) and their inhibitory (via R_i receptor) and activating (via R_a receptor) influence over adenylyl cyclase.

2. P_1 purinoceptor subclassification based on the relative potency of adenosine and related analogs. Changes in cAMP levels could not always be correlated to events modulated by adenosine, probably because adenosine also influences other second-messenger systems (Morgan, 1991). Therefore, the current subclassification of purinoceptors of the P_1 subclass into the A_1 (R_i) and A_2 (R_a) subclasses is based mainly on the relative agonist potencies of adenosine analogs in eliciting responses. The principal analogs used in these studies were N^6-*R*-1-phenyl-2-propyl adenosine, N^6-*S*-1-phenyl-2-propyladenosine, N^6-cyclohexyladenosine, NECA, 2-CA, and adenosine. The potency profile of these adenosine analogs at adenosine receptors in a variety of tissues is as follows:

A_1/R_i: N^6-*R*-1-phenyl-2-propyl adenosine = N^6-cyclohexyladenosine > NECA = 2-CA > adenosine ≥ N^6-*S*-1-phenyl-2-propyladenosine

A_2/R_a: NECA > 2-CA > N^6-*R*-1-phenyl-2-propyl adenosine, + N^6-cyclohexyladenosine > adenosine ≥ N^6-*S*-1-phenyl-2-propyladenosine

The R/S diasterioisomer potency ratio for phenyl-2-propyl adenosine is always greater than ten at A_1 receptors and generally less than ten at A_2 receptors (Smellie et al., 1979b; Murphy and Snyder, 1981; Paton and Kurahashi, 1981). The nonselective adenosine receptor agonist, [^{3}H]NECA, was used in binding studies in the presence of the A_1 selective ligand, N^6-cyclopentyladenosine, to reveal A_2 receptors. Under these conditions, the adenosine receptor ligand, CV1808, was shown to be a selective A_2 receptor agonist (Bruns et al., 1986) and has been used in studies concerned with the subdivision of A_2 receptors.

3. P_1 purinoceptor subclassification using antagonists. In addition to caffeine and theophylline, which have marked phosphodiesterase inhibitory activity, more selective and more potent adenosine receptor antagonists have been developed. For example, 8-PT is ten times more potent than theophylline at antagonizing the adenosine-induced accumulation of cAMP in guinea pig cerebral cortex slices (Smellie et al., 1979a), and three times more potent than theophylline at antagonizing 2-CA-induced inhibition of twitch responses in the rat vas deferens (Clanachan, 1981). 8-PT seems to be a P_1 purinoceptor antagonist that does not exhibit selectivity for A_1 or A_2 adenosine receptors (Collis et al., 1985).

The xanthine derivative, PACPX, is 70,000 times more potent than theophylline and 70 times more potent than 8-PT (which was 1000 times more potent than theophylline) at inhibiting the binding of [^{3}H]N^6-cyclohexyladenosine to bovine brain membranes (Bruns et al., 1983). PACPX also can discriminate between the A_1 and A_2 subclasses in that it exhibits a noncompetitive antagonism of A_1 receptor-mediated-negative-inotropic responses to adenosine in the isolated, driven left atrium of the guinea pig, and a competitive antagonism of A_2 receptor-mediated relaxant responses to adenosine in the carbachol-contracted guinea pig taenia coli (Burnstock and Hoyle, 1985). However, there are technical limitations to the use of this drug because of its low solubility. 8-Cyclopentyl-1,3-dipropylxanthine, which exhibits 700-fold selectivity for A_1 receptors (Lohse et al., 1987), also has been a widely used antagonist in the characterization of A_1 receptors. More recently, compounds such as CP66713 (Sarges et al., 1990) and KF17837 (Mayfield et al., 1993) have been developed that show antagonist selectivity for A_2 receptors. For a comprehensive consideration of adenosine receptor antagonists see paper by Williams (1991).

B. Subclassification of A_1 Receptors

A subclassification of the A_1 receptor has been proposed (Gustafsson et al., 1990). In this study, the affinities for some of the agonists and antagonists used were higher in the rat and guinea pig brain than in the rat vas deferens or guinea pig ileum, and it was suggested that the former high-affinity, centrally located receptors be called A_{1a} and the latter low-affinity, peripherally located receptors be called A_{1b} receptors (Gustafsson et al., 1990).

C. Subclassification of A_2 receptors

The A_2 receptor has been subclassified on the basis of the differences in binding affinities of a large number of compounds for the rat striatal A_2 adenosine receptor versus that of the human fibroblast; the terminology A_{2a} (striatal) and A_{2b} (fibroblast) was proposed (Bruns et al., 1986). Recently, this subclassification has been endorsed using a wide range of adenosine analogs, including the 2-substituted compounds, CV1808 and CGS21680, and NECA in an extensive study of adenosine receptors in a wide range of tissues from various species (Gurden et al., 1993). This study showed that CV1808 and CGS21680, which have previously been shown to have high affinity for the rat striatal A_{2a} receptor (Bruns et al., 1986; Jarvis et al., 1989), exhibited a high degree of selectivity for the A_2 receptors of dog coronary artery, human neutrophils, and human platelets, where CV1808 and CGS21680 were of a similar potency to NECA (A_{2a} receptors). However, in guinea pig aorta, both of these compounds were markedly less active than was NECA (A_{2b} receptors). Thus, it was proposed that CV1808 and CGS21680 could be used, with NECA as a reference, to differentiate A_{2a} from either A_{2b} or A_1 receptors (Gurden et al., 1993).

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D. Other Subclasses of P_1 Receptors

Additional subclasses of P_1 receptors have been proposed. The A_3 receptor nomenclature has been adopted by two separate groups to describe a receptor/receptors that might or might not be related (Riberio and Sebastiao, 1987; Zhou et al., 1992). The A_4 receptor has been proposed in one study that demonstrated specific, reversible, and saturable binding of adenosine analogs in a novel order of potency and correlated this binding with more functional responses (Cornfield et al., 1992). A common property of the A_3 receptor of Zhou et al. (1992) and of A_4 receptors, but not that of Riberio and Sebastiao (1987), is the lack of interaction of these receptors with xanthine antagonists. For a fuller discussion of the nomenclature of adenosine receptors, the reader is referred to a report of the Purine Receptor Nomenclature Subcommittee of the "Purine Club" meeting, which was held in Milan, Italy in 1992 to clarify the confusing array of purinoceptor names (Abbracchio et al., 1993).

1. A_3 receptor(s). It has been suggested that the presynaptic adenosine receptor at the neuromuscular junctions of the frog sartorius muscle (Riberio and Sebastiao, 1987) and the rat diaphragm (Sebastiao and Riberio, 1988), which mediates inhibition of neurotransmission, might be distinct from the A_1 or A_2 receptors. Riberio and Sebastiao (1986) suggested that such receptors be called A_3 receptors. Blakeley et al. (1988) found results with the vas deferens that were compatible with the idea of the prejunctional A_3 receptor of Riberio and Sebastiao (1987). Evidence of this proposed A_3 receptor is based on the potency profiles of adenosine analogs to inhibit nerve-mediated twitches and end plate potential magnitude. The rank order of potency of these analogs in activating this receptor would seem to be different from that at either the A_1 or A_2 receptor. There is debate, however, about this additional subclassification (Riberio and Sebastiao, 1988; Fredholm and Dunwiddie, 1988), and it does not seem that the proposed A_3 receptor has become universally accepted (Fredholm and Dunwiddie, 1988). The evidence of the existence of the A_3 receptor came largely from studies that used adenosine analogs, the effects of adenine nucleotides not being rigorously tested. It has been suggested that the potency profile of certain agonists and antagonists allows the A_3 receptor of the neuromuscular junction to be categorized as the A_{1A} receptor (Gustafsson et al., 1990). Furthermore, it is possible that the A_3 receptors of Riberio and Sebastiao (1986) might be similar to the P_3 prejunctional receptor (receptors for both adenosine and ATP) proposed by Shinozuka et al. (1988) (see second paragraph of section IV).

More recently, the cloning, expression, and molecular characterization of a novel, G-protein-coupled adenosine receptor, distinct from either the A_1 or A_2 receptor, has been described (Zhou et al., 1992). The authors of this report have also utilized the A_3 nomenclature to differentiate this receptor from the A_1 and A_2 receptors. There seems to be no relationship of this cloned receptor to the A_3 receptor of Riberio and Sebastiao (1986). The cloned A_3 receptor has been implicated in the 8-SPT-resistant hypotensive response to adenosine analogs in the pithed rat (Fozzard and Carruthers, 1993).

The characterizations described above of two, apparently separate, adenosine receptors, using very different approaches but assigning the same nomenclature to the receptors, has led to confusion in the recent literature. This confusion has been somewhat clarified by the International Union of Pharmacology Purinoceptor Subcommittee (Abbraccio et al., 1993) and a recent further comparison of the characteristics of the two A_3 receptors (Carruthers and Fozard, 1993). The concensus deemphasizes the receptor of Riberio and Sebastiao (1986) in favor of that of Zhou et al. (1992). For additional information concerning this subject, the reader's attention is directed to reports by Riberio and Sebastiao (1986); Zhou et al. (1992); Fozard and Carruthers (1993); Carruthers and Fozard (1993).

2. A_4 receptor. A novel adenosine binding site that exhibits high affinity for CV1808 has been proposed to exist in rat brain membrane preparations. Two components of CV1808 binding were exhibited, the aforementioned high-affinity site that also bound 9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-amine, and a low-affinity site that bound N^6-cyclopentyl-adenosine. The inactivity of CGS21680 and NECA at the high-affinity site suggested that it was not an A_2 receptor. Furthermore, it was not similar to the A_3 receptor of Riberio and Sebastiao (1986), inasmuch as this A_3 receptor displays high affinity for NECA. The discovery of a novel receptor, the A_4 adenosine receptor, was proposed (Cornfield et al., 1992). The inability of analogs of guanosine 5′-triphosphate to affect ligand binding at this receptor, suggesting that the receptor was not associated with G-proteins, further highlighted differences from A_1 and A_2 receptors that are known to be G-protein dependent. The known coronary vasodilator activity of CV1808, and the independence of the brain CV1808 binding site from G-proteins, prompted these workers to examine the effect of CV1808 on K^+ conductances in porcine coronary arterial smooth muscle cells. A high degree of correlation of those analogs of adenosine that inhibited binding of CV1808 in brain membranes with the ability of the same analogs to block the CV1808-induced increase in K^+ currents in the smooth muscle cells was observed, suggesting to the authors the possibility that the two receptors were the same. These data were extrapolated to tentatively suggest that the receptor site of brain membranes might also mediate its effect via K^+ channels, although the involvement of K^+ channels in the function of brain A_4 receptors was not investigated (Cornfield et al., 1992).

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E. P-site of Adenylyl Cyclase

A low-affinity recognition site for adenosine on the catalytic subunit of adenylyl cyclase, called the P-site (so named because of its requirement for an intact purine moiety), has been described in human platelets (Londos and Wolff, 1977; Haslam et al., 1978), rat liver and bovine thyroid cells (Londos and Wolff, 1977), turkey erythrocytes (Lad et al., 1980), rat brain (Johnson and Shoshani, 1990; Yeung and Johnson, 1990; Bushfield and Johnson, 1990), bovine brain (Yeung and Johnson, 1990), and human leukocytes (Marone et al., 1990). Relatively high concentrations of adenosine were required to activate this site, which mediates an inhibition of adenylyl cyclase activity (Londos and Wolff, 1977). There is evidence that suggests that this site is orientated in an intracellular direction (Londos and Wolff, 1977). Whereas the externally orientated A_1/R_i and A_2/R_a sites exhibit the characteristics of a bona fide receptor, the internal P-site does not. The physiological role of this site remains uncertain (Londos et al., 1983).

F. P_1 Purinoceptor Second-Messenger Systems

It is well known that adenosine receptor occupation is followed by alterations in the levels of cAMP in a tissue and, as described above above, the initial P_1 purinoceptor subclassification was based on this property of adenosine. These studies showed that A_1 receptor activation results in inhibition of adenylyl cyclase activity and A_2 receptor occupancy results in stimulation of adenylyl cyclase activity, leading to a fall or rise, respectively, in tissue levels of the second-messenger cAMP.

It is known that the influence of the activated adenosine receptor on adenylyl cyclase is mediated via guanine nucleotide binding proteins (G-proteins), G_s and G_i. Indeed, A_1 receptors have been copurified in association with G-proteins (Munshi and Linden, 1989), and it has been shown that the solubilized A_1 receptor retains the ability to be regulated by G-proteins (Gavish et al., 1982; Stiles, 1985). Stimulation of A_2 receptors results in the activation of G_s, which has a stimulatory effect on adenylyl cyclase. A_1 receptors activate G_i, which either inhibits adenylyl cyclase directly or reduces the effectiveness of G_s (Morgan, 1991). Cyclic adenosine monophosphate binds to and activates cAMP-dependent kinase, which has the ability to phosphorylate a large number of different cellular proteins. This ultimately results in the characteristic effect of adenosine in that particular tissue.

Interestingly, it has been demonstrated that contraction of the nonpregnant guinea pig myometrium by adenosine analogs is mediated by an A_1 receptor that is not coupled to adenylyl cyclase (Smith et al., 1989) but is coupled to adenylyl cyclase in the tissue from pregnant animals (Schiemann et al., 1991).

G. Biochemical Characterization and Molecular Biology of P_1 Purinoceptors

With the exception of the A_4 receptor, adenosine receptors belong to the G-protein-coupled family of receptors. Structurally, these receptors consist of a 320- to 420-amino acid glycoprotein with seven transmembrane domains. Two members of this family, RDC7 and RDC8, have been cloned (Libert et al., 1989) and characterized respectively as A_1 (Libert et al., 1991; Mahan et al., 1991; Reppert et al., 1991) and A_2 (Maenhaut et al., 1990) adenosine receptors. More recently, the A_{2a} (Furlong et al., 1992) and A_{2b} subtypes (Stehle et al., 1992; Pierce et al., 1992; Rivkees and Reppert, 1992) of the A_2 receptor have been cloned. To our knowledge, the A_{1a} and A_{1b} subtypes of the A_1 adenosine receptor proposed by Gustafsson et al. (1990) have not been cloned. Chronologically, the characteristics and identification of the transcript of RDC8 cDNA as an A_2 receptor were reported before the identification of RDC7 cDNA as the gene coding for an A_1 receptor, therefore the literature will be discussed in that order here.

1. A_2 adenosine receptor. Expression of RDC8 cDNA in Y1 adrenal cells, dog thyrocytes, and *Xenopus* oocytes resulted in activation of adenylyl cyclase in these cells. This effect was inhibited by exogenous adenosine deaminase or the P_1 purinoceptor antagonist 8-SPT, implying that the receptor expressed in response to introduction of RDC8 cDNA was an A_2-like adenosine receptor, responding to adenosine present in the medium. Transfection of simian virus 40 (SV40)-transformed African green monkey kidney cells (COS-7 cells) with RDC8 cDNA resulted in expression of this transmembrane protein. Binding studies using adenosine analogs on membrane preparations of these transfected cells, demonstrated that this receptor had a profile consistent with an A_2 adenosine receptor (Maenhaut et al. 1990).

A G-protein coupled receptor was isolated from a human hippocampal cDNA library which, when expressed in transfected human embryonic kidney 293 cells, showed binding properties to a range of adenosine agonists and antagonists, including CGS21680, which were characteristic of the A_{2a} (striatal subtype) adenosine receptor (Furlong et al., 1992).

A novel adenosine receptor subtype, called RFL9, has been cloned from a rat brain cDNA library. Its expression in COS-6 cells resulted in activation of adenylyl cyclase and ligand-binding characteristics resembling an A_2 receptor. Northern blot analysis revealed a distribution unlike that of either A_1 or A_2 receptors, with highest levels expressed in colon, caecum, and urinary bladder (Stehle et al., 1992). This group subsequently fully characterized this receptor in Chinese hamster ovary cells transfected with RFL9 and showed that its properties correlated well with the receptor of human fibroblasts, the cells in which the A_{2b} adenosine receptor subtype were originally characterized. Furthermore, Northern

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blot analysis of fibroblast mRNA showed a positive signal when probed for RFL9 but no hybridizing signal when probed for A_{2a} receptor mRNA, strongly suggesting that the cloned cDNA, RFL9, was the encoding sequence for the A_{2b} (fibroblast-type) adenosine receptor (Rivkees and Reppert, 1992). The A_{2b} receptor has also been cloned from a human hippocampus cDNA library that, when transfected into Chinese hamster ovary cells, resulted in the expression of adenosine receptors that caused production of cyclic guanosine monophosphate in response to a series of agonists that had a profile characteristic of the A_{2b} adenosine receptor. Binding to specific A_1 and A_{2b} agonists was not detected (Pierce et al., 1992).

2. A_1 adenosine receptor. It seems that the A_1 adenosine receptor has now been cloned for three species: dog, rat, and cow. In these studies, described in the next few paragraphs, the adenosine analog-binding characteristics and susceptibility to antagonists of the recombinant receptors corresponds well with those of the native receptor defined by more classical studies.

A_1 adenosine receptors have been solubilized from bovine brain, rat brain, and rat testis membranes using a variety of methodologies (Gavish et al., 1982; Stiles, 1985; Nakata, 1989, 1990; Olah et al., 1990). The molecular weight of the receptor glycoprotein is reported to be 34 to 36 kDa, which is reduced to 32 kDa after deglycosylation (Munshi and Linden, 1989; Olah et al., 1990). It was noted that the A_1 receptor, unlike other G-protein-coupled receptors, was still able to be regulated by G-proteins in the solubilized state. Furthermore, the solubilized receptor retained the same adenosine analog-binding characteristics as the membrane-bound receptor (Gavish et al., 1982; Stiles, 1985; Nakata, 1989; Olah et al., 1990). A_1 receptor-G-protein complexes have been copurified from bovine brain (Munshi and Linden, 1989).

RDC7, the orphan G-protein-coupled receptor retrieved from a canine thyroid cDNA library (Libert et al., 1989), has been identified as corresponding to the A_1 adenosine receptor (Libert et al., 1991). Transfection of Chinese hamster ovary cell lines with a vector containing RDC7 cDNA, constructed from dog cultured thyrocyte mRNA, resulted in expression of a receptor with the characteristics of an A_1 adenosine receptor (Libert et al., 1991). After expression of the receptor, the cells were exposed to forskolin to directly increase adenylyl cyclase activity. Subsequent exposure to the adenosine analog N^6-cyclopentyl-adenosine caused a reduction of this adenylyl cyclase activity, a result that would be expected if N^6-cyclopentyl-adenosine were acting on an A_1 receptor. Furthermore, binding studies using adenosine analogs were performed on membrane preparations from COS-7 cells transfected with RDC7 cDNA, and again the expressed receptors had the binding characteristics of the A_1 adenosine-receptor subtype (Libert et al., 1991).

Similar characteristics have been observed in another G-protein-coupled receptor constructed from rat striatal mRNA (Mahan et al., 1991). This homologue of RDC7 was shown to behave in a similar fashion with respect to its ability to mediate an adenosine analog-induced reduction of adenylyl cyclase activity in transfected cells, and its binding characteristics to a series of adenosine analogs. Furthermore, the authors demonstrated the dependence of the function of this receptor on guanosine 5′-triphosphate-binding proteins. Northern blot analysis of the receptor mRNA and in situ hybridization histochemical studies of various brain structures showed that the distribution of the message for this receptor corresponds to previous autoradiographic localization of the A_1 adenosine receptor in rat brain (Mahan et al., 1991). In another similar study, an A_1 receptor has been cloned from a rat brain cDNA library using a probe constructed from rat brain mRNA. Northern blot analysis showed that the receptor was highly expressed in brain, spinal cord, testis, and adipose tissue. In situ hybridization studies revealed an extensive distribution in the central nervous system with high levels in the cerebral cortex, hippocampus, cerebellum, thalamus, brainstem, and spinal cord (Reppert et al., 1991).

Bovine brain A_1 adenosine receptor cDNA has been cloned and expressed in COS-1 cells and the resulting receptor exhibited the expected species differences with respect to the binding characteristics of adenosine analogs and receptor antagonists (Tucker et al., 1992).

3. A_3 adenosine receptor. Another G-protein-coupled adenosine receptor that can negatively modulate adenylyl cyclase activity, R226, has been isolated from a rat brain cDNA library. When expressed in COS-7 or Chinese hamster ovary cells and subjected to binding studies, the receptor exhibits high affinity for the A_1-selective agonist [^{125}I]-N^6-2-(4-amino-3-iodophenyl)-ethyladenosine. However, its binding characteristics to a number of other A_1- and A_2-selective ligands led the authors to conclude that it was different from existing A_1 or A_2 receptors, and they proposed to call it the A_3 receptor. Examination of the distribution of this A_3 receptor showed highest predominance in the testes of the rat (Zhou et al., 1992). The authors stress that the relationship between this receptor and the putative A_3 receptor of Riberio and Sebastiao (1986) (see section II.D.1), remains unknown (Carruthers and Fozard, 1993).

III. P_2 Purinoceptors (Receptors for ATP)

Extracellular ATP elicits responses that are not antagonized by methylxanthines, and therefore not mediated by P_1 purinoceptors, in a variety of smooth muscle preparations. The receptors that mediate these ATP-induced responses were termed P_2 purinoceptors (Burnstock, 1978). Evidence has accumulated indicating heterogeneity of P_2 purinoceptors (Kennedy et al., 1985; White et al., 1985; Kennedy and Burnstock, 1985). It has been proposed that P_2 purinoceptors can be subdivided into

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P_{2X} and P_{2Y} subclasses (Burnstock and Kennedy, 1985). This proposal was based on three main observations: (*a*) comparison of the potency of ATP and its analogs, α,β-methylene ATP, β,γ-methylene ATP, and 2-methylthio-ATP; (*b*) the ability of $ANAPP_3$ to antagonize and α,β-methylene ATP to desensitize P_{2X} but not P_{2Y} purinoceptors; and (*c*) the ability of ATP to produce excitatory responses in some tissues and inhibitory responses in others, or excitatory or inhibitory responses in the same tissue under different conditions (such as contraction at resting tone and relaxation when tone is raised) was considered (Burnstock and Kennedy, 1985).

Other subtypes of the P_2 receptor have been characterized: the P_{2Z} receptors of the mast cell, the P_{2T} receptor of the platelet, and the P_{2U} purinoceptor of the human neutrophil. One other P_2 receptor has been proposed, the P_{2S} purinoceptor, in the guinea pig ileum (Wiklund and Gustafsson, 1988).

For more information on the structure-activity relationships of nucleotide analogs at the various P_2 purinoceptor subtypes, see the review by Cusack (1993).

A. P_{2X} and P_{2Y} Receptors

P_{2X} and P_{2Y} receptors are present on many tissues (see Hoyle and Burnstock, 1991). For example, P_{2X} receptors mediate the effects of ATP in smooth muscle by producing excitatory responses leading to contraction of tissues such as the vas deferens (Westfall et al., 1978; Frew and Lundy, 1982; Fedan et al., 1982; Taylor et al., 1983; Sneddon and Westfall, 1984; Sneddon et al., 1984), bladder (Ambache and Zar, 1970; Burnstock et al., 1972; Dean and Downie, 1978), and the cat nictitating membrane (Langer and Pinto, 1976). Smooth muscle P_{2Y} receptor activation, on the other hand, leads to relaxation of tissues such as the guinea pig taenia coli (Burnstock et al., 1970; Westfall et al., 1982).

In blood vessels ATP acts on P_{2X} purinoceptors on the smooth muscle cells to produce contraction and therefore vasoconstriction (Kennedy et al., 1985), and on P_{2Y} purinoceptors on the endothelial cells to produce relaxation and therefore vasodilation (De Mey and Vanhoutte, 1981). P_{2X} purinoceptors have been shown to mediate vasoconstriction, or the underlying electrical events of vascular smooth muscle contraction, in a variety of vascular preparations, including rat tail artery (Sneddon and Burnstock, 1984b), rabbit mesenteric artery (Kügelgen and Starke, 1985; Ramme et al., 1987; Muir and Wardle, 1988; Ishikawa, 1985), dog mesenteric artery (Muramatsu, 1986, 1987; Muramatsu et al., 1989; Machaly et al., 1988; Omote et al., 1989), guinea pig mesenteric artery (Ishikawa, 1985), rabbit ear artery (Suzuki, 1985; Kennedy et al., 1986; Muir and Wardle, 1988; Saville and Burnstock, 1988), rabbit saphenous artery (Burnstock and Warland, 1987a; Warland and Burnstock, 1987), guinea pig saphenous artery (Cheung and Fujioka, 1986), dog cerebral arteries (Muramatsu et al., 1980, 1981; Muramatsu and Kigoshi, 1987), rabbit portal vein (Kennedy and Burnstock, 1985), and rabbit hepatic artery (Brizzolara and Burnstock, 1990).

In most blood vessels ATP causes vasodilation via activation of the endothelial P_{2Y} purinoceptor that causes EDRF (nitric oxide)-mediated relaxation. These include dog femoral artery (De Mey and Vanhoutte, 1981), rat aorta (White et al., 1985), and rat isolated perfused mesenteric bed (Ralevic and Burnstock, 1988; see also reviews by Burnstock 1987a, 1987b). However, in some vessels ATP is thought to induce vascular smooth muscle relaxation directly by acting on P_{2Y} receptors located on the smooth muscle. These include the rat isolated femoral artery (Kennedy et al., 1985), dog isolated saphenous vein and left circumflex coronary artery (Houston et al., 1987), and guinea pig and rabbit coronary artery (Keef et al., 1992).

For comprehensive reviews of the literature pertaining to the distribution and function of P_{2X} and P_{2Y} purinoceptors see White (1988), Olsson and Pearson (1990), and Hoyle and Burnstock (1991).

1. P_{2X}/P_{2Y} purinoceptor subclassification based on the relative potency of ATP and related analogs. Burnstock and Kennedy (1985) proposed that vascular P_2 purinoceptors could be subclassified based on the relative potency of ATP and a series of ATP analogs, α,β-methylene ATP, β,γ-methylene ATP, and 2-methylthio-ATP, in inducing functional responses in various tissues. These receptors were termed P_{2X} and P_{2Y} purinoceptors. The rank order of potency of ATP analogs at inducing excitatory responses mediated by P_{2X} receptor activation, and inhibitory responses mediated by P_{2Y} receptor activation, is as follows:

P_{2X}: α,β-methylene ATP > β,γ-methylene ATP > ATP = 2-methylthio-ATP

P_{2Y}: 2-methylthio-ATP > ATP > α,β-methylene ATP, β,γ-methylene ATP

This subclassification scheme also holds true for the P_2 receptors of nonvascular smooth muscle. In the guinea pig vas deferens, α,β-methylene ATP and β,γ-methylene ATP were more potent than ATP in producing contraction (Fedan et al., 1982; Taylor et al., 1983), whereas 2-methylthio-ATP was equipotent with ATP (Burnstock et al., 1985). Furthermore, in the rat and guinea pig urinary bladder, α,β-methylene ATP and β,γ-methylene ATP are more potent than ATP in producing contraction (Kasakov and Burnstock, 1982; Brown et al., 1979), whereas 2-methylthio-ATP was equipotent with ATP (Burnstock et al., 1983). These results suggest, according to the above P_2 purinoceptor subclassification, that the contractile responses of these tissues are mediated by the P_{2X} purinoceptor. However, in the longitudinal muscle of the ergotamine precontracted rabbit portal vein, α,β-methylene ATP and β,γ-methylene ATP were less potent than ATP in producing an endothelium-independent relaxation, although 2-methylthio-ATP was more potent.

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In tissues at resting tone α,β-methylene ATP and ATP produced a contraction, α,β-methylene ATP being more potent than ATP (Kennedy and Burnstock, 1985). This suggests that in the portal vein at high tone, P_{2Y} purinoceptor-mediated relaxation responses predominate, whereas at resting tone, P_{2X} receptor-mediated contractions predominate. Subsequent studies on the rat portal vein, using simultaneous recording of electrical and mechanical responses by the sucrose gap method, have substantiated these findings (Reilly and Burnstock, 1987). In the precontracted guinea pig taenia coli, α,β-methylene ATP and β,γ-methylene ATP were less potent than ATP in producing relaxation, although 2-methylthio-ATP was more potent (Satchell and Maguire, 1975), suggesting the involvement of P_{2Y} purinoceptors in this response. Thus, in general, α,β-methylene ATP is more potent that 2-methylthio-ATP at P_{2X} receptors, whereas the converse is true at the P_{2Y} receptor.

Research into other structural analogs of adenine nucleotides revealed several compounds that have been claimed to exhibit a higher potency or selectivity as agonists at one or other of the P_2 purinoceptor subclasses. For example, adenosine 5′-(2-fluorodiphosphate) was claimed to be the first structural analog of ATP that is a specific agonist at the inhibitory P_{2Y} purinoceptors of the guinea pig taenia coli, having no effect on the P_{2X} receptors of the guinea pig bladder (Hourani et al., 1988). It is known that the diadenosine nucleotides, P^1,P^3-di-(adenosine-5′)triphosphate and AP_4A, are released from platelets during aggregation and the vasomotor activity of these substances has been examined in the rabbit mesenteric artery (Busse et al., 1988). This report provided evidence of AP_4A production of an endothelium-dependent relaxation that was blocked by RB2 (a putative P_{2Y} receptor antagonist), suggesting that it might be acting as an agonist at the endothelial cell P_{2Y} receptor. Diadenosine nucleotides have also been investigated for their ability to block P_2 purinoceptors; this will be discussed later.

Although it seems that, in the absence of specific competitive antagonists of P_{2X} and P_{2Y} purinoceptors, the examination of the relative potencies of these various ATP analogs has provided evidence of a subclassification of P_2 purinoceptors, it should be borne in mind that these are not conclusive. It is possible that differences in potency might reflect differences in efficacy of the agonists at the receptor or differences in the number of available receptors for the agonist in any given tissue, rather than solely differences in affinity of the receptors for the agonists. Also, as pointed out by Burnstock and Kennedy (1985), the influence of hydrolytic enzymes means that it might be possible that small amounts of degradation products of ATP or its analogs, however resistant to degradation they are, might contribute to the observed result. A more comprehensive review of the structure-activity relationships of a wide range of modified nucleotide compounds at P_{2X} and P_{2Y} receptors is available (Cusack, 1993).

2. P_{2X}/P_{2Y} purinoceptor subclassification using desensitizing agents and putative antagonists. A major obstacle to the definitive classification of purinoceptors has been the lack of a classical, selective, competitive antagonist of P_2 purinoceptors. Of the compounds that have been used to date, most have been stable analogs of ATP that are agonists at the P_2 receptor. These produce a desensitization and functional block of the receptor, or are photolyzed to produce a covalent bond between the receptor and ligand. One such compound was one of the first "antagonists" of the P_{2X} receptor. The photoaffinity analog of ATP, $ANAPP_3$, is a potent agonist at P_2 purinoceptors that, on exposure to light, covalently binds to these receptors producing an irreversible antagonism (Hogaboom et al., 1980). $ANAPP_3$ has been used to selectively antagonize contractions to exogenous ATP and the purinergic component of the neurogenic response in the guinea pig vas deferens, leading to the first pharmacological evidence suggesting that ATP was the non-adrenergic, noncholinergic cotransmitter in sympathetic nerves in this tissue (Fedan et al., 1981).

The stable analog of ATP, α,β-methylene ATP, which is more resistant than ATP to hydrolysis by ecto-ATPases present on the cell membrane, was first shown to produce a selective desensitization of P_2 purinoceptors in the guinea pig bladder (Kasakov and Burnstock, 1982) and vas deferens (Meldrum and Burnstock, 1983). Since then, α,β-methylene ATP has been the most widely used compound in the investigation of excitatory (P_{2X} receptor-mediated) responses to exogenous or endogenous ATP, providing evidence of purinergic involvement in the neurogenic responses of the rodent vas deferens (Fedan et al., 1981; Meldrum and Burnstock, 1983; Sneddon and Burnstock, 1984a; Allcorn et al., 1986), rat tail artery (Sneddon and Burnstock, 1984b), cat nictitating membrane (Duval et al., 1985), rabbit portal vein (Kennedy and Burnstock, 1985), rabbit mesenteric artery (Kügelgen and Starke, 1985; Ramme et al., 1987), rabbit saphenous artery (Burnstock and Warland, 1987a), guinea pig, rabbit and pig urinary bladder (Fujii, 1988), rabbit central ear artery (Kennedy et al., 1986), and dog mesenteric artery (Muramatsu et al., 1984; Muramatsu, 1986, 1987; Machaly et al., 1988; Muramatsu et al., 1989). There have been reports indicating a lack of specificity of action of α,β-methylene ATP (Byrne and Large, 1986), but the majority of reports indicate that α,β-methylene ATP and $ANAPP_3$ behave selectively. They seem to be the best pharmacological tools presently available for studying P_2 purinoceptor-mediated excitatory responses.

The anthraquinone-sulfonic acid derivative, RB2, might be an ATP antagonist (Kerr and Krantis, 1979; Choo, 1981). Studies on the rat caecum have revealed that the pharmacology of this compound is complicated, however. RB2 was able to selectively antagonize inhibi-

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tory junction potentials and ATP analog-induced hyperpolarizations, suggesting that it might have some antagonistic effect on the P_{2Y} purinoceptors in this tissue (Manzini et al., 1986). In the isolated, Langendorff-perfused rat heart (Hopwood and Burnstock, 1987), dog coronary artery (Houston et al., 1987), and rabbit mesenteric artery (Burnstock and Warland, 1987b), RB2 seems to be more effective in antagonizing P_{2Y} purinoceptor-mediated vasodilation than P_{2X} receptor-mediated vasoconstriction. Furthermore, in the isolated, Krebs-perfused rabbit ear, RB2 dose dependently inhibited vasodilator responses to ATP and reversed these to a vasoconstrictor response, providing evidence of two subpopulations of P_2 purinoceptors. However, RB2 also inhibited vasodilator responses to carbachol and, to a lesser extent, papaverine, indicating limited selectivity (Taylor et al., 1989). Although it seems that RB2 has potential as a P_{2Y} purinoceptor antagonist, or at least as a starting point for the development of such a drug, RB2 itself seems to have nonspecific actions that should be taken into account when this drug is used.

Investigators have proposed that AMP is a selective antagonist of P_{2X} purinoceptors in the rat vas deferens (Satchell, 1986), but a more recent study has provided direct evidence against the proposal that AMP acts as an antagonist of P_{2X} purinoceptors (Dalziel and Sneddon, 1988).

The trypanocidal drug suramin might be an effective, selective antagonist of P_2 purinoceptors. This was first shown in the mouse vas deferens (Dunn and Blakeley, 1988), and subsequent studies have investigated the P_2 receptor antagonist effect of suramin and related compounds in a variety of tissues, such as mouse vas deferens (Kügelgen et al., 1989a), rat vas deferens (Mallard et al., 1989), pithed rat (Schlicker et al., 1989; Urbanek et al., 1990), guinea pig urinary bladder and taenia coli (Hoyle et al., 1990), and rabbit ear artery (Leff et al., 1990). Suramin also selectively inhibits ATP-induced membrane currents in cultured vas deferens smooth muscle (Hoiting et al., 1990) and P_{2X} receptor-mediated excitatory junction potentials in the guinea pig isolated vas deferens (Sneddon, 1992). Although suramin can block P_{2X} receptors, it seems that it might have some antagonist activity at P_{2Y} receptors also (Den Hertog et al., 1989; Hoyle et al., 1990; Voogd et al., 1993). Although suramin might be a useful tool in discriminating between P_1 and P_2 receptor-mediated responses, the ability of suramin to discriminate between P_{2X} and P_{2Y} receptors might be limited. Furthermore, suramin has been shown previously to be a potent inhibitor of various hydrolytic and oxidative enzymes (Wills and Wormall, 1950) as well as Na^+/K^+ ATPase (Fortes et al., 1973), indicating that suramin possesses a spectrum of biological activities that might further limit its usefulness as a specific receptor antagonist.

Diadenosine nucleotides, which have been discussed above with respect to their properties as P_2 purinoceptor agonists, have also been investigated as potential P_2 purinoceptor desensitizing agents. AP_5A is approximately equipotent with α,β-methylene ATP in producing contraction and desensitization of the P_{2X} receptors of the guinea pig vas deferens (MacKenzie et al., 1988). The inability of AP_4A to emulate the actions of AP_5A in the guinea pig vas deferens might be attributable to the ability of AP_5A, but not AP_4A, to act at P_{2X} purinoceptors. Busse et al. (1988) showed that AP_4A could stimulate EDRF release, probably by acting on endothelial cell P_{2Y} purinoceptors. When this is considered with the results of MacKenzie et al. (1988), it may be speculated that AP_4A and AP_5A are acting as selective agonists (and therefore desensitizing agents) at P_{2Y} purinoceptors and P_{2X} purinoceptors, respectively. The discovery of selective agonists/desensitizing agents for the P_2 purinoceptor subtypes, which are resistant to hydrolysis, as diadenosine nucleotides seem to be (Busse et al., 1988), would be extremely useful in substantiating existing evidence of P_2 purinoceptor subclassification.

B. Other P_2 Purinoceptors

1. P_{2Z} receptors. Mast cells possess nucleotide receptors that, when stimulated by ATP^{4-} (but not by most other forms of ATP analogs) cause an activation of degranulation and histamine secretion that becomes an inhibition as the nucleotide concentration is increased further. Additional characterization showed the ATP^{4-} receptor of mast cells to be distinct from P_{2X} and P_{2Y} receptors (Tatham et al., 1988; Jaffar and Pearce, 1990), and tissue- and species-specific for the rat serosal mast cell (Jaffar and Pearce, 1990). ATP originating within the secretory granule of the mast cell acts on neighboring mast cells via ATP receptors to amplify the intracellular Ca^{2+} concentrations and degranulation process initiated by antigen stimulation of these cells (Osipchuk and Cahalan, 1992). The pharmacology of the P_{2Z} receptor is discussed in more detail elsewhere (Cusack, 1993).

2. P_{2T} receptors. The adhesiveness of human platelets is increased by exposure to ADP (Gaarder et al., 1961). The human platelet nucleotide receptor mediates platelet activation and responds only to ADP, exhibiting stereoselectivity for the naturally occurring enantiomer of ADP: ATP has no agonist effect per se (Macfarlane and Mills, 1975; Cusack et al., 1979). Activation of this receptor by ADP derived from ATP from damaged cells in the vessel wall results in the pathophysiological changes associated with platelet aggregation and with the platelet role in hemostasis (Born and Kratzer, 1984; McClure et al., 1988). The activation of the platelet ADP receptor results in inhibition of adenylyl cyclase activity and platelet aggregation (Cusack and Hourani, 1982).

A comprehensive review of the literature concerning the P_{2T} receptor can be found in the articles by Cusack and Hourani (1990, 1991). More detailed structure-

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function relationships of the behavior of nucleoside and nucleotide analogs at this receptor are reviewed in the article by Cusack (1993).

3. P_{2U} receptors. A nucleotide receptor has been shown to exist in human neutrophils; the receptor is activated by ATP and coupled to phospholipase C via a pertussis toxin-sensitive G-protein (Cockcroft and Stutchfield, 1989a, 1989b). This results in an elevation of intracellular Ca^{2+} from intracellular and extracellular origin (Merritt and Moores, 1991). When stimulated by ATP, this receptor mediates a five-fold increase in the rate of degranulation (as monitored by β-glucuronidase secretion), however UTP was also able to activate this receptor to increase degranulation three-fold (Cockcroft and Stutchfield, 1989a). ATP and UTP were able to potentiate the formyl-methionine-leucine-phenylalanine-induced superoxide formation in human neutrophils and were able, per se, to induce aggregation of neutrophils with similar effectiveness (Seifert et al., 1989). Therefore, the P_{2U} purinoceptor of the human neutrophil is able to respond to purine and pyrimidine nucleotides to induce neutrophil activation and potentiation of the effects of other cytokines on neutrophil function. A detailed review of the effects of various purine and pyrimidine nucleotides on this receptor is available (Cusack, 1993).

A similar receptor exists on HL60 cells at which UTP is a more potent agonist than ATP (Cockcroft and Stutchfield, 1989b).

4. P_{2S} receptors. One other P_2 purinoceptor has been proposed, the P_{2S} receptor in the guinea pig ileum. This receptor-mediated contractile responses to ATP analogs and could be blocked by *p*-chloromercuribenzene sulfonic acid (which also blocked vas deferens P_{2X} receptors) but not by RB2 or α,β-methylene ATP desensitization (Wiklund and Gustafsson, 1988).

C. P_2 Purinoceptor Second-Messenger Systems

Of the six suggested subclasses of P_2 purinoceptors, the P_{2Y}, P_{2T}, and P_{2U} receptors are thought to be coupled to G-proteins. The P_{2X} receptor modulates a nonselective cation channel and thus can be viewed as a ligand-gating site on an ion channel. There is less known about the P_{2Z} receptor, and this aspect of the P_{2S} receptor has not been investigated.

1. P_{2X} receptors. ATP can activate an excitatory P_2 receptor-operated cation channel in vascular smooth muscle that is independent of diffusible second- messenger production and that carries Ca^{2+} ions with a selectivity of 3:1 over Na^+ ions (Benham and Tsien, 1987). The function of this channel seems to be two-fold. It allows direct Ca^{2+} entry and also causes depolarization of the cell leading to additional Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Benham, 1990) and subsequent contraction. ATP-activated channels have been demonstrated in other tissues where P_{2X} receptors have been demonstrated, such as vas deferens smooth muscle (Friel, 1988) and bladder (Schneider et al., 1991). Although this ATP-activated channel is of the P_2 receptor subclass, its pharmacological profile obtained using these electrophysiological techniques has so far precluded its unequivocal allocation into either the P_{2X} or the P_{2Y} subclass (Bean, 1992).

2. P_{2Y} receptors. The P_{2Y} receptor is coupled to phospholipase C via a G-protein (Boyer et al., 1989; Cooper et al., 1989; Jeffs et al., 1991). This results in increased levels of inositol 1,4,5-trisphosphate and, as a consequence, an elevated cytoplasmic Ca^{2+} ion concentration (Hallam and Pearson, 1986; Pirotton et al., 1987; Okajima et al., 1987; Pearce et al., 1989). The increased cytoplasmic Ca^{2+} concentration results in the phosphorylation of proteins by calmodulin-dependent kinases. In the vascular endothelial cell, activation of nitric oxide synthase occurs, leading to the increased synthesis and release of nitric oxide EDRF (Busse and Mulsch, 1990). Also in the endothelial cell, activation of Ca^{2+}-sensitive phospholipase A_2 results in the increased production of prostacyclin (Forsberg et al., 1987). Studies of the P_{2Y} receptor and its transduction mechanisms in the endothelial cell have been recently reviewed (Boeynaems and Pearson, 1990).

3. Other P_2 receptors. The G-protein-linked P_{2T} receptor of human platelets, which is activated by ADP, is known to inhibit the formation of cAMP (Cooper and Rodbell, 1979). Furthermore, platelet activation is also associated with inositol 1,4,5-trisphosphate generation and platelet inhibition is associated with cAMP generation (Cusack and Hourani, 1991).

The P_{2U} receptor of human neutrophils is another G-protein-associated receptor and its activation results in the stimulation of inositol 1,4,5-trisphosphate generation by phospholipase C, with a resulting elevation of cytosolic Ca^{2+} ion concentrations (Cockcroft and Stutchfield, 1989a, 1989b).

Work on P_{2Z} receptors of mast cells show that their activation by ATP causes transient elevations of Ca^{2+} concentration (Osipchuk and Cahalan, 1992). Elevated cytosolic Ca^{2+} concentration in the mast cell promoted degranulation but was not necessary or sufficient, on its own, for initiation of this process (Neher, 1988). This secretion was dependent on mobilization of Ca^{2+} but independent of increased phosphatidyl-inositol turnover (Cockcroft and Gomperts, 1979, 1980).

The transduction mechanisms of the P_{2S} receptor of Wiklund and Gustafsson (1988) have not been investigated.

D. Biochemical Characterization and Molecular Biology of P_2 Purinoceptors

Labeled versions of relatively stable ligands selective for P_{2X} and P_{2Y} receptors have been used to study both binding characteristics from isolated membrane preparations of these receptors and autoradiographic localiza-

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tion of the receptors in situ (Bo and Burnstock, 1989, 1990, 1992; Bo et al., 1992). The receptors expressed in *Xenopus* oocytes in response to injection of mRNA isolated from tissues have also been studied to a limited extent (Russell et al., 1992). As described above, the encoding cDNAs have been cloned for the A_1 and A_2 receptors (P_1 purinoceptors) and even for subtypes of the A_2 receptor, however, progress into the molecular characterization of P_2 purinoceptors has been hindered by the lack of specific, stable ligands. Despite this, two recent studies report the cloning of two P_2 purinoceptors. Expression cloning in *Xenopus* oocytes of a P_2 receptor from mouse neuroblastoma cells, which has the pharmacological profile of a P_{2U} receptor, has recently been reported (Lustig et al., 1993). In another report that appeared the same month, hybridization screening of a chick brain cDNA library using guinea pig partial, RDC1-like, cDNA sequences, has resulted in cloning of a P_{2Y}-like receptor (Webb et al., 1993).

1. P_{2X} receptors. The distribution of P_{2X} purinoceptors has been studied autoradiographically, using $[^3H]\alpha,\beta$-methylene ATP (Bo and Burnstock, 1989). This tritiated derivative of the P_{2X} receptor agonist/desensitizing agent, α,β-methylene ATP, was used to compare the distribution of P_{2X} receptors in the rat urinary bladder, rat vas deferens, and rabbit central ear artery. The specific binding was localized to the smooth muscle structures of these three tissues, in which P_{2X} receptors are known to mediate contraction in response to ATP.

Studies on rat bladder smooth muscle membrane preparations indicated that $[^3H]\alpha,\beta$-methylene ATP had a binding that was saturable, rapid, and able to be competitively displaced by other ATP analogs (Bo and Burnstock, 1989). These studies were technically refined and extended using the rat urinary bladder smooth muscle membrane preparation, and the existence of a high- and a low-affinity binding site for $[^3H]\alpha,\beta$-methylene ATP was revealed in this preparation (Bo and Burnstock, 1990). Competition studies using unlabeled purinoceptor ligands confirmed the performance of $[^3H]\alpha,\beta$-methylene ATP as a P_{2X}-selective ligand (Bo and Burnstock, 1990). Subsequent comparative studies on the urinary bladder of the rat, guinea pig, and rabbit have demonstrated species differences in the binding of this labeled analog, with the highest binding occurring in those species with the greatest purinergic component to their neurogenic contraction, i.e., the rat (Bo and Burnstock, 1992).

Solubilization of the high-affinity $[^3H]\alpha,\beta$-methylene ATP binding site from the rat vas deferens has been performed, demonstrating a $[^3H]\alpha,\beta$-methylene ATP-binding polypeptide with a molecular weight of 62 kDa (Bo et al., 1992). Characterization of this solubilized receptor by competition for the $[^3H]\alpha,\beta$-methylene ATP-binding site with purinoceptor ligands revealed a rank order of potency comparable to that for a P_{2X} purinoceptor (Bo et al., 1992).

Exogenous ATP has been shown to produce a P_2 purinoceptor-mediated, Ca^{2+}-activated Cl^- current in the *Xenopus* oocyte (Lotan et al., 1986). Poly A mRNA extracted from guinea pig vas deferens and injected into *Xenopus laevis* oocytes resulted in a significant increase in the ATP-induced Ca^{2+}-activated Cl^- current (Russell et al., 1992). This presumably reflects expression of guinea pig vas deferens P_{2X} receptor mRNA. However, there is no report to date in the literature indicating that the gene for this receptor has been cloned.

2. P_{2Y} receptors. The ^{32}P-labeled P_{2Y} receptor agonist, $[^{32}P]3'$-O-(4-benzoyl)-benzoyl ATP, has been used as a label for the P_{2Y} receptor of turkey erythrocyte membranes (Boyer et al., 1990). In the absence of light, this compound bound to membrane preparations with high affinity ; it bound in a saturable, reversible manner that could be competitively inhibited by ATP and ADP analogs with a potency profile similar to that of a P_{2Y} purinoceptor. When exposed to ultraviolet light, photolysis of the ^{32}P occurred, and it became incorporated into a 53 kDa protein, suggesting that this was the P_{2Y} purinoceptor. This photoaffinity labeling procedure was applied to other tissues known to express the P_{2Y} receptor, resulting in the recovery of a ^{32}P-labeled 53 kDa protein from primary cultures of newborn rat astrocytes, rat hepatocytes, rat brain, 1321N1 human astrocytoma cells, and bovine pulmonary aortic endothelial cells. However, the labeling of this protein was not observed in membranes of human platelets or erythrocytes (Boyer et al., 1990). The P_{2Y} receptor has also been isolated as a complex from turkey erythrocyte plasma membranes (Jeffs et al., 1991).

Expression of P_2 purinoceptors has been reported in *Xenopus* oocytes injected with embryonic guinea pig brain mRNA (Fournier et al., 1990). These receptors were subsequently partially characterized and seemed to be most like a P_{2Y} purinoceptor (Honore et al., 1991).

Cloning of a P_{2Y} receptor obtained from a chick whole brain cDNA library has been reported (Webb et al., 1993). In this study, hybridization screening of the chick brain cDNA library was performed using guinea pig partial cDNA sequences. Analysis of one of the isolated clones showed homology with the G-protein-coupled family of receptors. Injection of cRNA of this clone into *Xenopus* oocytes resulted in functional expression of a P_{2Y}-like receptor. After evaluating preliminary pharmacological data using nucleotide agonists and antagonists, the authors concluded that this receptor was different from known P_{2Y} receptors and named it P_{2Y1} (Webb et al., 1993).

3. Other P_2 purinoceptors. *Xenopus* oocytes acquire responsiveness to ATPγS when injected with purified mRNA from the leukemia cell line HL60. In this study, measurement of $^{45}Ca^{2+}$ efflux showed that the injected oocytes demonstrated an increased efflux in response to ATPγS, UTP, and ionosine triphosphate with similar

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potency, but the receptor involved was not activated by ADP or AMP. This profile deviates from that of any previously described P_2 receptor (Murphy and Tiffany, 1990).

Expression cloning in *Xenopus* oocytes of total RNA from a mouse neuroblastoma cell line, NG108–15, has resulted in the isolation of a cDNA clone, P2R, which encodes a P_2 receptor. In vitro transcription and injection of RNA into *Xenopus* oocytes, resulted in expression of a receptor that exhibited the pharmacological profile of a P_{2U} receptor, as ascertained using a single electrode voltage clamp and $^{45}Ca^{2+}$ release assay (Lustig et al., 1993).

IV. P_3 Purinoceptors (Receptors for Adenosine and ATP)

Many studies have demonstrated the ability of adenosine to modulate the release of norepinephrine from postganglionic sympathetic nerves. This phenomenon has been observed in a variety of tissues, including the vas deferens, salivary gland, heart, and various blood vessels (Hedqvist and Fredholm, 1976; Clanachan et al., 1977; Enero and Saidman, 1977; Verhaeghe et al., 1977; Wakade and Wakade, 1978; Paton, 1979, 1981; Fredholm and Hedqvist, 1980; Fredholm et al., 1982; Su, 1978, 1983; Khan and Malik, 1980; Moylan and Westfall, 1979; Husted and Nedergaard, 1981). This effect of adenosine has been attributed to its action on prejunctional P_1 purinoceptors. ATP has also been shown to have an inhibitory effect on the release of norepinephrine from several tissues (Paton 1981; Su, 1983), an effect that was largely attributed to the action of adenosine, derived from ATP degraded by ecto-ATPases, acting on P_1 purinoceptors, rather than ATP acting directly on P_2 purinoceptors. Although some of the effects of ATP could be attributed to the formation of adenosine, there is currently strong evidence showing nucleotides can act directly to inhibit transmitter release without conversion to adenosine (Lukacsko and Blumberg, 1982; Husted and Nedergaard, 1981; Wiklund et al., 1985; Shinozuka et al., 1988; Kügelgen et al., 1989b, 1992; Forsyth et al., 1991; Fuder et al., 1992).

Recently, a third subclass of purinoceptors has been proposed, distinct from P_1 or P_2 receptors, and located on the sympathetic nerves of the rat tail artery (Shinozuka et al., 1988) and the rat vas deferens (Forsyth et al., 1991). This proposal is based partially upon the findings that ATP and other nucleotides act per se to limit norepinephrine release from these tissues. This unique purinoceptor was termed the P_3 receptor (Shinozuka et al., 1988). The characteristics of the P_3 purinoceptor that distinguish it from P_1 or P_2 receptors include the following: (*a*) whereas the P_1 purinoceptor can be considered primarily as a nucleoside (e.g., adenosine) receptor and the P_2 receptor as a nucleotide (e.g., ATP) receptor, the P_3 receptor is activated by both nucleotides and nucleosides; (*b*) alkylxanthines such as 8-SPT, which antagonizes responses mediated by P_1 but not P_2 receptors, also antagonize P_3 receptor-mediated responses. This effect is apparently independent of the formation of adenosine from ATP; (*c*) α,β-methylene ATP, which is a potent agonist and desensitizing agent at P_2 but not at P_1 receptors, seems to be purely an antagonist at P_3 purinoceptors.

Fundamental to the concept of P_3 receptors is the idea that adenine nucleotides do not need to be converted to adenosine to be active. Several pieces of evidence indicate that this is indeed the case in some tissues. These are as follows:

1. It has been shown in the rat tail artery (Shinozuka et al., 1988) that the relative order of potency of adenine nucleosides and nucleotides at P_3 receptors for the inhibition of nerve stimulation-induced overflow of norepinephrine is distinct from that at P_1 or P_2 purinoceptors:

2-CA $>$ β,γ-methylene ATP $>$ ATP $=$ adenosine

Similar results have been observed in the rat vas deferens (Forsyth et al., 1991). This rank order of potency is incompatible with the idea that nucleotides need to be metabolized to adenosine in order to decrease the overflow of norepinephrine in that ATP and adenosine were equipotent. Furthermore, β,γ-methylene ATP, a compound that is less rapidly hydrolysed to adenosine than is ATP, was more potent than ATP or adenosine.

2. Studies using the adenosine uptake inhibitor, nitrobenzyl thioguanosine, have shown that this compound can potentiate the inhibitory effect of exogenous adenosine on norepinephrine overflow but was unable to potentiate the effects of nucleotides, indicating that the actions of the nucleotides were independent of the formation of adenosine (Shinozuka et al., 1988; Forsyth et al., 1991).

3. The observation that the inhibitory effects of exogenous adenosine could be blocked by inclusion of adenosine deaminase in the Krebs solution (but the inhibitory effects of ATP could not) provides additional evidence against the requirement of nucleotides to be degraded to adenosine (Forsyth et al., 1991).

4. The pyrimidine nucleotide UTP, which does not liberate adenosine, was also able to reduce the overflow of norepinephrine (Forsyth et al., 1991). In contrast to the effects of UTP, uridine itself does not produce an inhibition of norepinephrine overflow (Kügelgen et al., 1989b), again indicating that nucleotides can act directly without being broken down to the nucleoside.

Although none of the above findings from our laboratory provides any more than circumstantial evidence of the definition of an additional subclass of purinoceptor, when considered together, this information provides compelling evidence of the existence of an atypical purinoceptor. However, not all studies indicate that a P_3 receptor mediates this prejunctional effect of adenine nucleotides and nucleosides. For example, Kügelgen et

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TABLE 1
Characteristics of purinoceptors

P_1 Purinoceptors						
Nomenclature	A_{1A}	A_{1B}	A_{2A}	A_{2B}	A_3	A_4
Agonists	High affinity	Low affinity	CGS21680 = NECA	NECA > CGS21680	Two disparate receptors have been named by separate groups as the A_3 receptor. One of these has been cloned. See text for details.	CV1808
	R-PIA ≥ NECA > S-PIA		NECA > R-PIA ≥ S-PIA			
Antagonists	DPCPX, 8-CPT, 8-SPT		KF17837, CP66713			CGS15943A
Transduction mechanism	G-protein coupled, ↓ cAMP		G-protein coupled, ↑ cAMP			
Gene	A1 (RDC7, canine)		A2A (RDC8, canine)	A2B (RFL9, rat)		
Location/function	Widely distributed in the CNS and periphery of many species/diverse functions					K^+ channel Rat brain
P_2-Purinoceptors						
Nomenclature	P_{2X}	P_{2Y}	P_{2Z}	P_{2T}	P_{2U}	P_{2S}
Agonists	$\alpha\beta$>ATP = 2-SATP	2-SATP ≫ ATP > $\alpha\beta$	ATP^{4-}	ADP	UTP ≥ ATP > ADP > AMP	2-SATP > ATP = ADP ≫ 8BrATP
Antagonists	$ANAPP_3$, $\alpha\beta$, suramin	RB2, suramin	2-SATP	ATP		PCMBS
Transduction mechanism	Cation channel	G-protein coupled IP_3/Ca^{2+}/DAG	Nonselective pore ↑ Ca^{2+}	G-protein coupled IP_3/Ca^{2+}/DAG/↓ cAMP	G-protein coupled IP_3/CA^{2+}/DAG	
Gene		P2Y1 (chick brain)			P2R (murine)	
Location/function	Smooth muscle/ contraction	EDRF release relaxation	Mast cell/ degranulation	Platelets/activation	Neutrophils/degranulation	Guinea pig ileum/ contraction
P_3-Purinoceptors						
Nomenclature	P_3 (inhibitory)		P_3 (facilitatory)			
Agonists	2–CA > $\beta\gamma$ > ATP = ADO					
Antagonists	8–SPT, $\alpha\beta$					
Transduction mechanism						
Gene						
Location/function	Vascular and non-vascular postganglionic sympathetic nerves/ ↓ neurotransmitter release		Vascular postganglionic sympathetic nerves/ ↑ neurotransmitter release			

Abbreviations: CV1808, 2-(phenylamino)adenosine; CGS21680, [[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-N-ethylcarboxamido-adenosine, UTP, uridine triphosphate; DAG, sn-1, 21-diacylglycerol; ADO, adenosine; $\alpha\beta$, α,β-methylene ATP; $\beta\gamma$, β,γ-methylene ATP; 8-BrATP, 8-bromo-ATP; $ANAPP_3$, arylazidoaminopropionyl ATP; 2-CA, 2-chloroadenosine; 8-CPT, 8-cyclopentyltheophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5′-N-ethylcarboxamidoadenosine; R-PIA, N^6-R-1-phenyl-2-propyladenosine; S-PIA, N^6-S-1-phenyl-2-propyladenosine; 8-SPT, 8-sulfophenyltheophylline; 2-SATP, 2-methylthio-ATP; PCMBS, *p*-chloromercuribenzene sulfonic acid.

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al. (1989a, 1989b) report that, although both nucleotides and nucleosides reduce evoked release of norepinephrine from the mouse vas deferens, they apparently do so by acting at separate P_1 and P_2 receptors. Their conclusion was based primarily on the finding that 8-SPT, although antagonizing the effect of adenosine, did not antagonize the effect of ATP. This is unlike the situation in the rat and rabbit vas deferens (Forsyth et al., 1991; Todorov et al., 1994). Available data seem to indicate that both nucleosides and nucleotides can attenuate evoked transmitter release either by acting on separate P_1 and P_2 receptors or by activating a "hybrid" P_3 receptor that possesses some of the characteristics of P_1 and P_2 receptors.

The bulk of studies aimed at understanding the modulation of transmitter release by adenine nucleotides and nucleosides indicate that these substances inhibit evoked-transmitter release. However, it is of interest to note that there are some reports that indicate that these substances can facilitate the release of norepinephrine from sympathetic nerves. Miyahara and Suzuki (1987) and Zhang et al. (1989) reported that ATP and adenosine increased the release of norepinephrine and its metabolite 3,4-dihydroxyphenylglycol, induced by perivascular nerve stimulation of the rabbit ear artery. α,β-Methylene ATP was an antagonist, not an agonist, of this effect. Recently, preliminary studies by Ishii et al. (1993) confirmed previous results (Suzuki, 1985; Zhang et al., 1989) and also demonstrated that 2-CA enhanced the evoked overflow of norepinephrine from the rabbit ear artery. Furthermore, the facilitatory effect of both nucleosides and nucleotides was antagonized by 8-SPT and α,β-methylene ATP. Similar results have been reported for the rabbit saphenous artery (Todorov et al., 1994). Collectively, these results indicate that there might be a facilitatory prejunctional purinoceptor that exhibits a number of structure-activity relationships similar to that of the prejunctional inhibitory P_3 purinoceptor. Thus, subtypes of the P_3 receptor might exist, one of which mediates an inhibition, and one a facilitation, of neurotransmitter release.

V. Concluding Remarks

As expressed in this review article, it is becoming clear, from both classical pharmacological studies and modern molecular biological techniques, that there is great diversity in purinoceptors. There are distinct receptors for adenosine and distinct receptors for adenine nucleotides. Furthermore, evidence is accumulating that raises the possibility that there are also receptors that interact with both nucleotides and nucleosides. Among the receptors for adenosine, there might be up to six subtypes and perhaps as many subtypes of receptors for ATP and related nucleotides. A summary representation of these established and putative receptors is shown in table 1 along with information, if available, about their pharmacological properties, distribution, function, second-messenger system, and genetics. For additional information about specific points, the reader is referred to the appropriate section of this article and the references therein.

Although we have discovered much about purinoceptors during the past few years, it is clear that much remains to be learned. This article is intended to serve as a summary of the major pieces of information known about purinoceptors and as a starting point for the newcomer to this diverse research field.

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