Annu. Rev. Pharmacol. Toxicol. 1995. 35:541–79




# $P_2$-PURINERGIC RECEPTORS: Subtype-Associated Signaling Responses and Structure

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KEY WORDS: adenine nucleotides, adenylyl cyclase, phospholipase C, G protein–linked receptors



## ABSTRACT

Extracellular adenine nucleotides interact with $P_2$-purinergic receptors to regulate a broad range of physiological processes. These receptors include the $P_{2Y}$-, $P_{2U}$-, $P_{2T}$-, $P_{2X}$-, and $P_{2Z}$-purinergic receptor subtypes. This review focuses on the first three of these receptor subtypes, which couple to G proteins and regulate the inositol lipid, cyclic AMP, and other second-messenger signaling cascades. Both pharmacological data and the occurrence of selectivity of coupling to second-messenger pathways indicate the existence of multiple members in several of the classes of $P_2$-purinergic receptor subtypes. Complementary DNAs cloned for $P_{2Y}$- and $P_{2U}$-purinergic receptors predict proteins with seven transmembrane-spanning motifs, typical of that of other G protein–linked receptors.

## INTRODUCTION

Extracellular adenine nucleotides and nucleosides interact with cell surface receptors in the central nervous system and peripheral tissues to produce a broad range of physiological responses. Although pharmacological effects of ATP were acknowledged as early as 1929 (1), the important and diverse signaling roles played by ATP and its metabolic products have been realized only in the last 25 years (2, 3). Adenosine achieved its status as a major extracellular signaling molecule first; however, during the last decade the roles



0362-1642/95/0415-0541$05.00

of ATP and ADP as receptor agonists have been unambiguously established, and cell surface receptors for adenine nucleotides are now known to be ubiquitously distributed in mammals. This review focuses on recent advances made in (*a*) subclassification of receptors for extracellular nucleotides, (*b*) G protein–linked second-messenger pathways associated with nucleotide receptors, (*c*) structure-activity relationships for nucleotide analogues, and (*d*) molecular cloning of G protein–linked nucleotide receptors. Burnstock (2) was the first to propose that ATP and its metabolic products were released as cotransmitters from a variety of nerve types or as neurotransmitters from putative purinergic nerves. A broad range of target-tissue responses to extracellular adenosine and ATP had been reported by 1978, and Burnstock (4) envisioned that these effects were mediated by two major receptor types: $P_1$-purinergic receptors, which are physiologically activated by adenosine and exhibit a potency order of adenosine > AMP > ADP > ATP, and $P_2$-purinergic receptors, which are activated by ATP or ADP and exhibit a potency order of ATP > ADP > AMP > adenosine. This original classification has been retained, although subclassification of each of the two major classes into multiple receptor subtypes has been necessary (5–9).

The physiological effects of extracellular ATP and other nucleotides have been broadly described and include contractile responses of cardiac, vascular, and visceral smooth muscle; excitatory and inhibitory effects on neurons; stimulatory and inhibitory effects on ion, hormone, exocrine gland, platelet, mast cell, and inflammatory cell secretions; and many other effects. The reader is referred to Table 1 for a partial and general listing of these physiological responses, to an excellent recent review by Dubyak & El-Moatassim (9), and to a number of earlier comprehensive reviews (2–4, 8, 10).

Although the existence of such a diverse set of physiological responses to the same molecule suggests involvement of multiple receptor subtypes, the subclassification of $P_2$-purinergic receptors has only been accomplished recently and remains incomplete. Classification of $P_2$-purinergic receptors has proven difficult because there are no selective $P_2$-receptor antagonists, and very few receptor-selective analogues of ATP or ADP have been available. Further, extracellular ATP is rapidly metabolized by nucleotidases and ATPases. These so-called ectonucleotidases are differentially distributed (11, 12), which has made the comparison of relative orders of potency of $P_2$-purinergic receptor agonists across various preparations difficult.

The idea that multiple subtypes of $P_2$-purinergic receptors exist was first proposed by Burnstock & Kennedy (7) on the basis of differential pharmacological effects of analogues of ATP and ADP on smooth muscle responses (Table 2). $P_{2X}$-purinergic receptors were proposed to mediate contractile effects of ATP on smooth muscle; they exhibit the potency order of α,β-methylene ATP > β,γ-methylene ATP > ADP > 2-methylthioATP. $P_{2Y}$-purinergic

**Table 1** Some of the physiological effects of extracellular adenine nucleotides

| |
|---|
| Contraction and relaxation of vascular smooth muscle |
| Release of vasorelaxing substances from endothelial cells |
| Contraction of urinary bladder smooth muscle |
| Contraction of vas deferens smooth muscle |
| Contraction of myometrial smooth muscle |
| Ionotropic and chronotropic effects on cardiac muscle |
| Rapid depolarization of CNS and sensory neurons |
| Modulation of neurotransmitter release from peripheral and CNS neurons |
| Regulation of glial cell second-messenger production |
| Regulation of $Cl^-$ secretion by epithelial cells in airway, renal, gastrointestinal, and other tissues |
| Stimulation of the acrosome reaction in human spermatozoa |
| Stimulation of hepatic glycogenolysis |
| Stimulation of mucin secretion and ciliary beat frequency in airway cells |
| Stimulation of inflammatory responses in neutrophils, monocytes, and macrophages |
| Modulation of immune responses of lymphocytes |
| Promotion of secretion from endocrine and neuroendocrine tissues, including the pancreas and thyroid, parotid, and adrenal glands |
| Promotion of secretions from mast cells and platelets |
| Regulation of mitogenic responses of fibroblasts and other cells |



receptors were proposed to mediate the relaxant effects of ATP on smooth muscle; they exhibit the potency order of 2-methylthioATP > ATP > α,β-methylene ATP = β,γ-methylene ATP. $P_2$-purinergic receptors in addition to $P_{2X}$- and $P_{2Y}$-purinergic receptors exist. The $P_{2T}$-purinergic receptor responds to ADP but not to ATP and is expressed on platelets (8, 13, 14). The $P_{2U}$-purinergic receptor is a widely distributed phospholipase C–activating receptor that is stimulated by both ATP and UTP (9). Very high concentrations of ATP permeabilize cells through the so-called $P_{2Z}$-purinergic receptor (15, 16), but the general physiological significance of this entity is unknown. Several other potential members of the $P_2$-purinergic receptor family have been proposed (Table 3). Each of the subclasses of $P_2$-purinergic receptors is considered individually in later sections of this review. The classification of receptors presented in Table 2 is essentially the one suggested by the International Union of Pharmacology subcommittee on the classification of purinoceptors (17).

## SECOND-MESSENGER SIGNALING

The $P_2$-purinergic receptors signal either through G protein–linked second-messenger signaling pathways or as ion-gating proteins. This review focuses on the first of these two classes of proteins, although we discuss general properties of the ion-gating, i.e. $P_{2X}$- and $P_{2Z}$-purinergic, receptors.

A broad range of extracellular hormones, neurotransmitters, chemoattract-

**Table 2** Classification of the major nucleotide receptors

| Subtype | Agonist | Pharmacological selectivity | Effector | G protein |
|---|---|---|---|---|
| $P_{2X}$ | ATP and ADP | $\alpha,\beta$-methyleneATP = $\beta,\gamma$-methyleneATP > ATP >> ADP$\beta$S or 2-MeSATP | Ion channel (Primarily $Na^+$ and $Ca^{2+}$) | None |
| $P_{2Y}$ | ATP and ADP | 2-MeSATP > ADP$\beta$S > ATP >> $\alpha,\beta$-methylene-ATP = $\beta,\gamma$-methyleneATP | ↑ Phospholipase C<br>↓ Adenylyl cyclase<br>Ion channel<br>[↑ Phospholipase $A_2$<br>↑ Phospholipase D<br>↑ Adenylyl cyclase][a] | $G_q$<br>$G_i$ |
| $P_{2T}$ | ADP | 2-MeSADP > ADP<br>ATP, AMP = Antagonists | ↓ Adenylyl Cyclase<br>↑ $Ca^{2+}$ (Ion channel?)<br>↑ Phospholipase C | $G_i$<br><br>? |
| $P_{2Z}$ | $ATP^{4-}$ | $ATP^{4-}$ = BzATP >> ATP | Membrane Pore | None |
| $P_{2U}$ | ATP and UTP | ATP = UTP > ATP$\gamma$S >> ADP; Inactive: 2-MeSATP, ADP$\beta$S, and $\alpha,\beta$-methyleneATP | ↑ Phospholipase C<br>[↑ Phospholipase $A_2$<br>↑ Phospholipase D][a] | $G_q$ and $G_i$ |

[a] These effector responses may be secondary to another signaling pathway, e.g. inositol lipid hydrolysis.

ants, and other extracellular stimuli produce their physiological effects by stimulating the second-messenger signaling cascades that involve the action of an intermediate guanine nucleotide–regulating protein (G protein). Deduced amino acid sequences based on cloned nucleotide sequences are available for over 150 of these G protein–linked receptors (18). All of these proteins have a predicted seven transmembrane–spanning structure and share notable conservation of sequence in their transmembrane-spanning domains. Agonist-occupied receptors in this superfamily of proteins specifically couple to one of at least 20 different heterotrimeric G proteins (19). Exchange of GTP for GDP is promoted by agonist-occupied receptors, which leads to activation as a result of dissociation of the inactive heterotrimeric protein into a GTP-liganded $\alpha$-subunit and a tightly associated dimer of $\beta$- and $\gamma$-subunits. In most cases the GTP-liganded $\alpha$-subunit then interacts with and activates an effector enzyme or channel. However, compelling evidence also exists for the direct regulation of many effector proteins by $\beta\gamma$-subunits (20). G protein–regulated effector proteins range from adenylyl cyclase—the classical effector of the cyclic AMP (cAMP) signaling cascade—to inward-rectifying $K^+$ channels. Phospholipase C is perhaps the most common effector protein associated with

**Table 3** Other potential nucleotide receptors

| Subtype | Agonist | Pharmacological selectivity | Effector | G protein |
|---|---|---|---|---|
| Uridine nucleotide | UDP and UTP | UDP > 5BrUTP > UTP | Phospholipase C [↑ Phospholipase $A_2$][a] | $G_i$ |
| $P_{2D}$ | $Ap_4A$ | $Ap_4A$ > ADPβS > App(NH)p > $Ap_5A$ > α,β-methyleneATP > 2-MeSATP | ↑ $Ca^{2+}$ | ? |
| $P_{2(PC12)}$ | ATP | ATPαS > ATP > ATPγS >■> BzATP, ADP, UTP, α,β-methyleneATP, β,γ-methyleneATP | $Ca^{2+}$ Channel | ? |

[a] This effector response may be secondary to another signaling pathway, e.g. inositol lipid hydrolysis.



$P_2$-purinergic receptors. This enzyme catalyzes the hydrolysis of PtdIns(4,5)$P_2$ to the $Ca^{2+}$-mobilizing second messenger Ins(1,4,5)$P_3$ and to *sn*-1,2-diacylglycerol, which activates protein kinase C (21). Extracellular stimuli that regulate this pathway predominately do so by activating seven transmembrane–spanning receptors that activate one of the four members of the $G_q$ class of G proteins (19, 22). An activated $G_q$ α-subunit then stimulates one of the multiple members of the phospholipase C–β class of isoenzymes. In addition, a number of receptors activate phospholipase C through a pertussis toxin–sensitive mechanism, implying that the $G_i$ class of G proteins, which are substrates for this bacterial toxin, is involved (23). However, $G_i$ or $G_o$ α-subunits do not activate any of the known phospholipase C isoenzymes (19). The fact that several phospholipase C–β isoenzymes are markedly stimulated by G protein βγ-subunits has led to the suggestion that the release of βγ-subunits from $G_i$ heterotrimers accounts for pertussis toxin–sensitive inositol lipid signaling (22).

# $P_2$-PURINERGIC RECEPTOR SUBTYPES

## *$P_{2Y}$-Purinergic Receptors*

INTRODUCTION $P_{2Y}$-purinergic receptors were originally identified as receptors that mediate ATP-induced relaxation in the guinea pig *taenia coli* and the longitudinal layer of the rabbit portal vein, and release of relaxing substances from rat and pig arterial endothelial cells (7). These receptors were proposed to be potently activated by 2-MeSATP and insensitive to α,β-methyleneATP and β,γ-methyleneATP. Until recently very few additional drugs have been available to delineate potential multiple subtypes of receptors within the $P_{2Y}$-

purinergic receptor class, and investigators have tacitly assumed that the various physiological responses occurring with a pharmacological specificity of a $P_{2Y}$-purinergic receptor are mediated through a single receptor type. We now know from both second-messenger signaling and pharmacological studies with new analogues of ATP and ADP that multiple subtypes of receptors exist within the $P_{2Y}$-purinergic receptor class.



## SECOND-MESSENGER RESPONSES REGULATED BY $P_{2Y}$-PURINERGIC RECEPTORS

*Inositol lipid hydrolysis* The inositol lipid signaling pathway was the first second-messenger cascade shown to be associated with $P_{2Y}$-purinergic receptors (Figure 1). Inositol lipid hydrolysis and/or $Ca^{2+}$ mobilization occurred in response to ATP and ADP (and in some cases to analogues of these nucleotides) in hepatocytes (24, 25), in vascular (26) and adrenal medullary endothelial cells (27), in Erlich ascites tumor cells (28), and in turkey erythrocytes (29, 30). Most of these responses have subsequently been confirmed to be mediated by $P_{2Y}$-purinergic receptors, although lack of appreciation of the widespread existence of $P_{2U}$-purinergic receptors (see below) led to the erroneous assumption in a number of studies in the late 1980s that any ATP-promoted inositol lipid signaling response or mobilization of $Ca^{2+}$ was mediated through a $P_{2Y}$-purinergic receptor.

One of the most extensively studied $P_{2Y}$-purinergic receptor–regulated signaling responses is the one that exists on turkey erythrocytes (29–34). Regulation of the inositol lipid signaling cascade occurs in intact erythrocytes

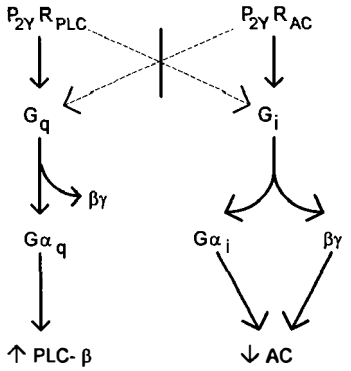



*Figure 1* $P_{2Y}$-purinergic receptor–associated signaling pathways. At least two different $P_{2Y}$-purinergic receptors apparently exist, based on second-messenger signaling properties. One receptor type activates phospholipase C but has no effect on adenylyl cyclase, whereas the second receptor type inhibits adenylyl cyclase but has no effect on phospholipase C. Activation of phospholipase C–β is pertussis toxin insensitive and is mediated by GTP–liganded $G\alpha_q$. Inhibition of adenylyl cyclase is pertussis toxin sensitive and is mediated by either α- or βγ-subunits, depending on the adenylyl cyclase isoform.

through a receptor that clearly expresses the properties of a $P_{2Y}$-purinergic receptor (30). Moreover, membranes prepared from turkey erythrocytes have provided the only reliable test system for studying $P_{2Y}$-purinergic receptor–mediated signaling in a cell-free preparation. Boyer et al (29) illustrated that the stimulatory effects of $P_{2Y}$-purinergic receptor agonists on phospholipase C are absolutely dependent on the presence of guanine nucleotides and that the first-order rate of activation of phospholipase C by guanine nucleotides is markedly enhanced by $P_{2Y}$-purinergic receptor agonists. The involved phospholipase C (35, 36) and G protein (37) have subsequently been purified and identified as members of the phospholipase C–β class of isoenzymes (38) and the $G_q$ family protein, $G_{11}$ (39), respectively. Although the $P_{2Y}$-purinergic receptor of turkey erythrocytes markedly activates phospholipase C, it does not couple in a stimulatory or inhibitory way to adenylyl cyclase. The turkey erythrocyte remains the only cell in which the signaling proteins to which a $P_2$-purinergic receptor couples have been directly identified. However, coupling to members of the $G_q$ family of G proteins and to a phospholipase C–β isoenzyme may be responsible for $P_{2Y}$-purinergic receptor–promoted inositol lipid signaling and $Ca^{2+}$ mobilization in most tissues (Figure 1). Whether this response occurs through a single $P_{2Y}$-purinergic receptor subtype is unknown but seems unlikely. For example, at least four different $\alpha_1$-adrenergic and three different muscarinic cholinergic receptors stimulate the inositol lipid signaling pathway (40, 41).

*Inhibition of adenylyl cyclase* Inhibition of cAMP accumulation in response to extracellular ATP has been observed in several tissues (Figure 1). Okajima and colleagues (25) reported that a $P_2$-purinergic receptor on rat hepatocytes inhibits cAMP accumulation. Differentiation of this receptor from another receptor that activated phospholipase C in the same preparation of cells proved difficult, and the concentration of nucleotides necessary to observe effects on cAMP accumulation was relatively high. Furthermore, the pharmacological selectivity of the putative receptor mediating the effects on cAMP levels did not clearly match that of any of the known $P_2$-purinergic receptor subtypes. Sato et al (42) reported that activation of a $P_2$-purinergic receptor on FRTL-5 thyroid cells inhibits cAMP accumulation. Although the activity of 2-MeSATP was not reported, the lack of effect of α,β-methyleneATP and the relatively potent effects of ATP, ATPγS, and ADPβS were consistent with the involvement of a $P_{2Y}$-purinergic receptor. Yamada et al (43) reported that incubation of cultured mouse ventricular myocytes with ATP resulted in both an increase in inositol lipid hydrolysis and a decrease in cAMP accumulation. It was unclear whether a single or multiple $P_2$-purinergic receptors were responsible for these effects. The agonists tested did not permit a clear definition of the receptor subtype that was involved, although observation of effects of both

ATP and ADP suggested the involvement of a $P_{2Y}$-purinergic receptor. The fact that the inhibition of cAMP accumulation was sensitive to inactivation by pertussis toxin, whereas the inositol lipid response was not, also suggested that the effects on cAMP accumulation occurred as a consequence of inhibition of adenylyl cyclase rather than as a secondary effect of elevation of $Ca^{2+}$ and activation of a $Ca^{2+}$-activated cAMP phosphodiesterase.

Pianet et al (44) demonstrated that extracellular ADP and ATP produced a concentration-dependent decrease in cAMP levels in rat C6 glioma cells. Sensitivity of this response to inactivation by pertussis toxin suggested that the effect of a $P_2$-purinergic receptor was probably mediated through $G_i$ and inhibition of adenylyl cyclase (Figure 1). Subsequent kinetic studies with C6 cell membranes conclusively showed that the effects of the ADP analogue ADPβS were mediated by $P_2$-purinergic receptor–promoted inhibition of adenylyl cyclase (45). Lin & Chuang (46) also confirmed that ATP reduced cAMP levels in C6 glioma cells by a mechanism that primarily involved $G_i$. However, a small contribution of $Ca^{2+}$-dependent activation of cAMP phosphodiesterase also was identified. Boyer et al (47) have extended these observations to demonstrate that the order of potency of a series of 11 analogues of ATP and ADP for inhibition of cAMP accumulation in C6 glioma cells followed that expected of a $P_{2Y}$-purinergic receptor. This work has been expanded to a new series of $P_{2Y}$-purinergic receptor–selective agonists, several of which inhibited cAMP levels in C6 cells with $K_{0.5}$ values of less than 100 pM (JW O'Tuel, JL Boyer, B Fisher, KA Jacobson & TK Harden, unpublished data).

Several reports have suggested that $P_2$-purinergic receptors also can lead to increases in intracellular cAMP accumulation (49–52). In several of these studies the contribution of the breakdown of ATP to adenosine has been incompletely considered, and the possibility that the ATP-induced release of substances that activate adenylyl cyclase through another receptor has not always been ruled out. Nonetheless, in the case of ATP-induced relaxation of mouse vas deferens, involvement of a $P_{2Y}$-purinergic receptor linked in an undefined way to activation of adenylyl cyclase has been proposed (51). In contrast, the receptor promoting cAMP formation in mouse C2C12 myotubes displayed a pharmacological specificity that was more similar to a $P_{2X}$-purinergic receptor than to a $P_{2Y}$- or $P_{2U}$-purinergic receptor (52).

*Other responses regulated by $P_{2Y}$-purinergic receptors* Phospholipase $A_2$ catalyzes the hydrolysis of a fatty acid, i.e. arachadonic acid, from the C2 position of the glycerol backbone of phospholipids. Activation of phospholipase $A_2$ occurs in essentially all target tissues in which inositol lipid hydrolysis is promoted by receptor activation. Although the molecular details of activation of phospholipase $A_2$ have not been fully delineated, activation

clearly occurs secondarily to mobilization of intracellular $Ca^{2+}$ and may well involve one or more phosphorylation reactions (53, 54). Phosphatidylcholine is a primary substrate for receptor-activated phospholipase $A_2$, and liberation of arachidonic acid and increases in lysophosphatidylcholine have been observed in many target tissues. Thus, arachidonic acid release is a predictable response to $P_{2Y}$-purinergic receptor activation and enhancement of inositol lipid hydrolysis (27).

$P_{2Y}$-purinergic receptors have also been reported to stimulate a phosphatidylcholine-specific phospholipase C, which liberates phosphocholine and diacylglycerol, and/or a phospholipase D, which increases cellular phosphatidic acid and choline (55–58). Although neither the molecular mechanism nor the physiological significance of these phenomena have been established, they may account for a large percentage of the diglyceride that is formed in response to $P_{2Y}$-purinergic receptor activation.

Although $P_{2Y}$-purinergic receptors principally act through the generation of second messengers, they also regulate ion channels. Whereas such regulation could potentially occur through production of a second messenger (e.g. diacylglycerol), activation of a protein kinase (e.g. protein kinase C), and phosphorylation of an ion channel (e.g. a $Ca^{2+}$ channel), this is not likely to be the full story. For example, $P_{2Y}$-purinergic receptor–promoted relaxation of smooth muscle (7) likely involves regulation of a $K^+$ channel. Although there are no direct data for the $P_{2Y}$-purinergic receptor, a number of $G_i$-linked receptors, e.g. m2-muscarinic and somatostatin receptors, have been shown to couple through $G_i$ to activate a $K^+$ channel (59). Moreover, the $P_{2Y}$-purinergic receptor on C6 glioma cells (45–47) is $G_i$ linked, and therefore, $G_i$-mediated coupling of $P_{2Y}$-purinergic receptors to $K^+$ channels probably occurs. Several tissues have been shown to express receptors that loosely fit a $P_{2Y}$-purinergic receptor specificity and that regulate an ion channel. Bjornsson et al (60) reported that activation in rat ventricular myocytes of a receptor with a $P_{2Y}$-like pharmacological selectivity resulted in depolarization, activation of voltage-activated cation channels, and influx of extracellular $Ca^{2+}$. Scarpa and coworkers (61, 62) also have studied a similar receptor in rat myocardial cells that is activated by 2-MeSATP but not by α,β-methyleneATP or β,γ-methyleneATP, leading to depolarization and activation of an L-type $Ca^{2+}$ channel. Illes & Nörenberg (63) discuss the potential existence of two neuronal $P_{2Y}$-purinergic receptors: one that directly regulates a cation channel and a second that regulates a $K^+$ channel through a G protein.

EVIDENCE FOR MULTIPLE $P_{2Y}$-PURINERGIC RECEPTOR SUBTYPES *Subtypes differentiated on the basis of specificity of second-messenger signaling* G protein–linked receptors generally couple selectively to a given G protein and second-messenger signaling pathway. For example, hormones that produce

their physiological effects through elevation of intracellular cAMP do so through activation of the G protein $G_s$, which in turn activates adenylyl cyclase. Although multiple signaling responses may be observed at high receptor concentrations (64), there is only limited evidence that the $G_s$-linked subclass of receptors will couple under physiological conditions to other G proteins to produce other second-messenger responses. Consequently, receptor subtypes that exhibit similar pharmacological selectivities may be distinguished by their specificity of G protein and effector coupling. For example, although they have very similar pharmacological selectivities, m1-muscarinic cholinergic receptors couple to $G_q$ and activate phospholipase C, whereas m2-muscarinic cholinergic receptors couple to $G_i$ and inhibit adenylyl cyclase (40). A similar difference exists in signaling properties of $\alpha_1$- versus $\alpha_2$-adrenergic receptors (41), as well as in receptor subtypes activated by serotonin or angiotensin II, to name a few.

Because the most prominently described second-messenger response to $P_{2Y}$-purinergic receptor activation is promotion of inositol lipid hydrolysis, it was important to establish whether a phospholipase C–activating receptor could also couple through $G_i$ to inhibit adenylyl cyclase. This would not be expected, based on the fidelity of coupling to $G_q$ family G proteins and phospholipase C exhibited by other nonpurinergic receptor classes. Thus the observation of $P_{2Y}$-purinergic receptor-mediated coupling to adenylyl cyclase suggested the existence of an additional member(s) of the $P_{2Y}$-purinergic receptor family. This idea has been supported by recent studies on the signaling responses of C6 glioma cells (47). Although $P_{2Y}$-purinergic receptor agonists markedly inhibited cAMP accumulation in C6 cells, they had no effect on inositol lipid hydrolysis or on $Ca^{2+}$ mobilization (Figure 1). The failure of ATP analogues to activate phospholipase C cannot be explained by lack of the appropriate signaling components in C6 cells because muscarinic cholinergic receptor agonists markedly enhanced inositol lipid hydrolysis and $Ca^{2+}$ mobilization. The most parsimonious interpretation of these results is that a $P_{2Y}$-purinergic receptor is expressed on C6 cells that couples to $G_i$ and adenylyl cyclase but does not couple to $G_q$ family G proteins and phospholipase C (Figure 1). Whether the $P_{2Y}$-purinergic receptor expressed by C6 glioma cells is the same receptor subtype that mediates ATP- and ADP-promoted inhibition of cAMP accumulation in other tissues has not been established. What is apparent, however, is that the $G_i$- and adenylyl cyclase–linked $P_{2Y}$-purinergic receptor is different from the $P_{2Y}$-purinergic receptor in other tissues, e.g. turkey erythrocytes, that stimulates phospholipase C but has no effect on adenylyl cyclase activity.

*Pharmacological differentiation of multiple $P_{2Y}$-purinergic receptor subtypes*

The idea that different $P_{2Y}$-purinergic receptor subtypes can be identified based

on their second-messenger coupling specificities has also been supported by pharmacological data. For example, Boyer et al found that the putative $P_2$-purinergic receptor antagonist pyridoxal phosphate 6-azophenyl-2′,4′-disulphonic acid (PPADS) competitively blocked the $P_{2Y}$-purinergic receptor that activates phospholipase C on turkey erythrocytes but had no effect on the adenylyl cyclase–coupled $P_{2Y}$-purinergic receptor of C6 glioma cells (34). Suramin and reactive blue 2 were antagonists at both $P_{2Y}$-purinergic receptors, but the $pK_B$ of reactive blue 2 differed by two orders of magnitude between the two systems. Although remarkable differences in agonist potencies between the turkey erythrocyte and C6 cell receptor were not observed in an initial comparison with 11 analogues of ADP and ATP (47), expansion of this work into a comparison of agonist affinities of newly synthesized $P_{2Y}$-purinergic receptor–selective analogues of ATP and ADP revealed differences in agonist potencies of up to 300-fold (JW O'Tuel, JL Boyer, B Fisher, KA Jacobson & TK Harden, unpublished data). Because some agonists were more potent for the adenylyl cyclase–coupled receptor and other agonists were more potent for the phospholipase C–coupled receptor, this work adds additional evidence for the existence of at least two different $P_{2Y}$-purinergic receptor subtypes that couple to two different second-messenger signaling pathways.

Jacobson and colleagues recently synthesized a series of drugs that exhibit high potency and selectivity for $P_{2Y}$-purinergic receptors (65). Examination of the structure-activity relationships of these adenine nucleotide analogues in guinea pig *t. coli,* endothelial cells of the rabbit aorta, and smooth muscle of the rabbit mesenteric artery has led to the conclusion that multiple $P_{2Y}$-purinergic receptors exist in these tissues (65, 66). For example, although 2-cyclohexylthio-ATP was a potent full agonist at the $P_{2Y}$-purinergic receptors of *t. coli,* this agonist was two orders of magnitude less potent and produced maximal effects that were smaller than those of 2-MeSATP in aorta and mesenteric artery. In contrast, 8-(6-aminohexylamino)ATP was selective for $P_{2Y}$-purinergic receptors of the endothelial cells of the rabbit aorta over the $P_{2Y}$-purinergic receptors of *t. coli* and rabbit aorta smooth muscle; $N^6$-methyl-ATP was selective for the $P_{2Y}$-purinergic receptor in the *t. coli* and was inactive at the receptors on rabbit aorta and mesenteric artery. Assuming that these differences in effects are not produced by differential rates of degradation of nucleotides in the preparations used, these data strongly suggest heterogeneity in $P_{2Y}$-purinergic receptors. Based on differential orders of potencies of agonists in various tissues, Abbracchio & Burnstock (67) have proposed the existence of at least four subtypes of the classical $P_{2Y}$-purinergic receptor.

SIGNALING RESPONSES OF CLONED $P_{2Y}$-PURINERGIC RECEPTORS There are few model systems available to test the hypothesis that multiple $P_{2Y}$-purinergic receptors couple to multiple second-messenger responses. Similarly, although

new pharmacological agents are becoming available that may permit delineation of multiple pharmacological specificities for $P_{2Y}$-purinergic receptors in various tissues, the availability and selectivity of these agents is not yet high. This nascent stage of differentiation of multiple $P_{2Y}$-purinergic receptor subtypes is also accompanied by problems inherent in studying only agonist molecules, and these agonists may be differentially metabolized in various tissues and converted to undefined products that have undefined or even opposite effects on the $P_2$-purinergic receptors that are under investigation.

Unambiguous delineation of multiple members in a receptor class requires molecular cloning of the cDNA that encodes each subtype, expression of the receptor in appropriate null cells, and definition of the pharmacological and second-messenger coupling specificities of this protein of defined structure. Webb et al (68) cloned a cDNA from a chick brain library that, when expressed in *Xenopus* oocytes, produced a slowly developing ATP-stimulated, $Ca^{2+}$-activated, inward current. The order of potency of a series of adenine nucleotides for activation of the inward current was predictive of that of a $P_{2Y}$-purinergic receptor. Because this was the first receptor of this class of signaling proteins to be cloned, it was referred to as the $P_{2Y1}$-purinergic receptor.

Filtz et al (69) have cloned the turkey homologue of the chick brain $P_{2Y1}$-purinergic receptor and have examined its second-messenger signaling properties after stable transfection in 1321N1 human astrocytoma cells. Activation of the expressed receptor with adenine nucleotides resulted in marked elevation of intracellular inositol phosphate levels. Pharmacological studies with a series of 11 adenine nucleotides confirmed that a $P_{2Y}$-purinergic receptor had been cloned and that its overall agonist selectivities were similar to those of the turkey erythrocyte $P_{2Y}$-purinergic receptor that had been previously studied. The inositol phosphate response to $P_{2Y}$-purinergic receptor agonists was unaffected by pretreatment of cells with pertussis toxin, and no evidence for inhibitory coupling to adenylyl cyclase of the $P_{2Y1}$-purinergic receptor was observed after its expression in either 1321N1 human astrocytoma cells or Chinese hamster ovary (CHO) cells. Simon et al (J Simon, TE Webb, BF King, G Burnstock & EA Barnard, unpublished data) also have recently observed that expression of the chick $P_{2Y1}$-purinergic receptor in COS-7 cells results in conferrence of adenine nucleotide–stimulated inositol lipid hydrolysis to these cells. Thus the cloned $P_{2Y1}$-purinergic receptor apparently couples selectively to the $G_q$–phospholipase C signaling pathway, with no interaction with $G_i$ and adenylyl cyclase. As is discussed above, these results suggest that a yet-to-be-cloned gene for a second (probably more) $P_{2Y}$-purinergic receptor subtype exists that couples to $G_i$ and adenylyl cyclase but not to $G_q$ and phospholipase C (Figure 1). Direct association of the cloned $P_{2Y1}$-purinergic receptor with any one of the myriad physiological responses that occur to $P_{2Y}$-purinergic receptor agonists has not been accomplished.

## $P_{2U}$-Purinergic Receptors

INTRODUCTION In the late 1980s it became apparent that activation of phospholipase C by ATP in many target tissues occurs through a receptor that is pharmacologically distinct from the $P_{2Y}$-purinergic receptor, which had been previously considered the principal inositol lipid hydrolysis–promoting purinergic receptor. This so-called $P_{2U}$-purinergic receptor is activated by ATP, UTP, and ATPγS (9). Other analogues of ATP, including those that are selective for $P_{2X}$- or $P_{2Y}$-purinergic receptors, only weakly activate or have no effect on this receptor. The presence of this receptor has been recognized on many different types of target cells, ranging from glial cells to airway epithelial cells to circulating monocytes. Physiological responses to $P_{2U}$-purinergic receptor activation include promotion of inflammatory responses and stimulation of transepithelial $Cl^-$ secretion in human airways (9, 71).

$P_{2U}$-PURINERGIC RECEPTOR–ASSOCIATED SIGNALING RESPONSES The overall signaling response promoted by $P_{2U}$-purinergic receptors is that of a phospholipase C–linked receptor. Therefore, mobilization of $Ca^{2+}$ and activation of protein kinase C are prominent effects of $P_{2U}$-purinergic receptor activation (72–76). Activation of phospholipase $A_2$, which apparently occurs secondarily to activation of the inositol lipid signaling pathway, is also a prominent sequelae of $P_{2U}$-purinergic receptor activation (74, 77, 78). As with $P_{2Y}$-purinergic receptors, stimulation of phosphatidylcholine breakdown and activation of phospholipase D occur in response to $P_{2U}$-purinergic receptor agonists (42, 58, 79). No convincing evidence of either stimulatory or inhibitory coupling of a $P_{2U}$-purinergic receptor to adenylyl cyclase has been reported. The prominent effect of $P_{2U}$-purinergic receptors on $Cl^-$ conductance in airway epithelial cells appears to be secondary to activation of phospholipase C. However, it would not be surprising if these receptors were shown to regulate ion conductance more directly through G proteins or potentially by other mechanisms. For example, Connolly et al (80) have reported that activation of a receptor with the general pharmacological specificity of a $P_{2U}$-purinergic receptor leads to depolarization of rat superior cervical ganglion neurons.

PROPERTIES OF CLONED $P_{2U}$-PURINERGIC RECEPTORS Lustig et al (81) used an expression-cloning strategy to isolate a cDNA that conferred an ATP-stimulated inward current and $Ca^{2+}$ mobilization to *Xenopus* oocytes. The amino acid sequence predicted by this cDNA was homologous to that of the superfamily of G protein–linked receptors. The fact that responses to ATP, UTP, and ATPγS occured in oocytes injected with this cDNA, whereas 2-MeSATP, α,β-methyleneATP, ADP, and adenosine had little or no effect in these cells

was consistent with the idea that a $P_{2U}$-purinergic receptor had been cloned. Erb et al (82) obtained similar results after stable expression of this mouse cDNA in K-562 human leukemia cells. The human homologue of this receptor has been cloned and stably expressed in 1321N1 human astrocytoma cells, where it was shown to activate phospholipase C and mobilize intracellular $Ca^{2+}$ in response to extracellular ATP and UTP (83).

One observation that has been made from studies with cloned $P_2$-purinergic receptors is that ATP and/or ADP is released from cultured cells in what may in some cases be very high concentrations. Parr et al (83) observed that overnight labeling with [$^3$H]inositol resulted in very high levels of [$^3$H]inositol phosphates in 1321N1 human astrocytoma cells that had been stably transfected with $P_{2U}$-purinergic receptor cDNA. The inclusion of apyrase, an adenosine di- and triphosphatase, in the labeling medium greatly reduced this background of inositol phosphates. The most likely explanation of these results was that ATP was released from cells during the labeling period and stimulated the expressed $P_{2U}$-purinergic receptor prior to any addition of exogenous agonist. By virtue of metabolizing released ATP, apyrase reduced this background response. Filtz et al (69) obtained a similar set of results after stable transfection of $P_{2Y}$-purinergic receptors in either 1321N1 human astrocytoma cells or CHO cells or transient expression in COS cells. Presently, the physiological significance of these results is unclear, but the possible release of endogenous ATP and ADP from cultured cells adds a level of complexity to the study of $P_2$-purinergic receptors that should be taken into account in all experimental designs regardless of whether the study involves endogenously or heterologously expressed $P_2$-purinergic receptors. The release of cellular adenine nucleotides can be predicted to cause an apparent increase in basal second-messenger levels and coincidentally down-regulate $P_2$-purinergic receptors (69).

POSSIBLE SUBMEMBERS OF THE $P_{2U}$-PURINERGIC RECEPTOR FAMILY Whether the cloned $P_{2U}$-purinergic receptor is the receptor that accounts for $P_{2U}$-purinergic receptor–mediated signaling in the myriad of target tissues studied thus far has not been proven. Very little pharmacological or second-messenger response data exist that suggest the existence of multiple $P_{2U}$-purinergic receptors. However, the lack of a broad range of agonist nucleotides, particularly analogues of UTP, make pharmacological distinction of any potential $P_{2U}$-purinergic receptor subtypes problematic. As has been reviewed by Seifert & Schultz (84), many tissue responses to extracellular uridine nucleotides have been reported, and although the underlying reasons for differences have not been identified and the pharmacological analyses have been incomplete, these tissue responses occurred with differences in relative effects of various pyrimidine nucleotides.

A novel receptor has been identified on C6-2B rat glioma cells that could prove to be a submember of a $P_{2U}$-purinergic receptor class of receptors (85). Activation of this receptor resulted in marked activation of phospholipase C, but in contrast to the classical $P_{2U}$-purinergic receptor, the receptor on C6-2B cells is activated by uridine nucleotides but not by adenine nucleotides. UDP is the most potent agonist ($K_{0.5} < 1$ μM) at this so-called uridine nucleotide receptor; UTP is approximately 50-fold less potent than UDP. ATP, ADP, and analogues of these nucleotides are not agonists at this receptor, and other nucleotides—e.g. CTP, XTP, and GTP—have no effect. Neither the physiological significance nor the tissue distribution of this uridine nucleotide receptor is known.

## *$P_{2T}$-Purinergic Receptors*

Platelets express $P_{2T}$-purinergic receptors, which play a role in the development and extension of arterial thromboses. These receptors exhibit a unique specificity in that ADP is an agonist, and ATP is an antagonist. Although the $P_{2T}$-purinergic receptor was first recognized in the 1970s, its signaling mechanisms have not been fully delineated, and its protein structure has not been elucidated. Excellent reviews have been published covering this class of $P_2$-purinergic receptor (14, 86, 87); thus only general aspects of this signaling molecule(s) are presented here.

The integrated response observed in ADP-stimulated platelets is a rapid shape change followed by aggregation and secretion of dense granules. The initial change in shape has been correlated with a rapid increase in intracellular $Ca^{2+}$ and with the phosphorylation of myosin light chain (14, 86). A rapid increase in $Ca^{2+}$ influx has been shown to precede a second wave of $Ca^{2+}$ influx that apparently is induced by the release of intracellular stores of $Ca^{2+}$ (88). Although these results suggest that the earliest response triggered by ADP is activation of a cation channel, data from other aggregating agents and from $Ca^{2+}$ ionophores indicate that the increase in $Ca^{2+}$ alone cannot explain the platelet response to ADP, suggesting the involvement of another second-messenger pathway (89). ADP stimulates the mobilization of $Ca^{2+}$ from intracellular stores in the absence of extracellular $Ca^{2+}$ (89), which is consistent with the participation of $Ins(1,4,5)P_3$. ADP-promoted increases in inositol phosphate levels have been reported (90, 91), but other investigators have not observed these effects (92–94). The reasons for these discrepancies are unclear, but they could be explained by a variable contribution of indirect effects introduced by shear forces or by other agents such as prostaglandins that are released by ADP (95).

Cooper & Rodbell (96) and Mellwig & Jakobs (97) initially reported that activation of an ADP receptor on platelets resulted in inhibition of adenylyl cyclase activity. These were among the first direct demonstrations of recep-

tor-mediated inhibition of adenylyl cyclase and were followed by subsequent studies confirming that a GTP-hydrolyzing G protein is involved (98). The absolute identity of this protein, which is likely a member of the $G_i$ family of G proteins, has not been established.

Although a number of aggregating agents, including ADP, inhibit adenylyl cyclase and reduce cAMP levels in platelets, other aggregating agents, such as vasopressin and platelet-activating factor (PAF), do not inhibit adenylyl cyclase. Also, $\alpha_2$-adrenergic receptor activation inhibits adenylyl cyclase, but this is not the mechanism by which epinephrine induces and potentiates platelet aggregation (86). These data and the fact that some analogues of ADP are more potent as inhibitors of adenylyl cyclase than as aggregating agents indicate that there is not a causal relationship between the two responses. Alternatively, these two effects could be mediated by two different $P_{2T}$-purinergic receptors. Support for two receptors has been suggested from studies using the affinity analogue 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA). This compound inhibits ADP-induced aggregation and has no effect on ADP-mediated inhibition of adenylyl cyclase or on the increase in intracellular $Ca^{2+}$. FSBA is covalently incorporated into a 100-kDa membrane protein termed aggregin (99), which has been proposed to be a platelet ADP receptor. However, this conclusion seems unlikely, due to the lack of specificity of FSBA (100), its inability to inhibit ADP-induced $Ca^{2+}$ increases (101), and the fact that 1 mM of ADP inhibited incorporation of FSBA by only 50% (102).

Very little data are available that address the existence of multiple $P_{2T}$-purinergic receptors. Most pharmacological analyses of these receptors have been carried out with platelets, but the recent demonstration of $P_{2T}$-purinergic receptors on cultured hematopoietic cell lines (103–106) and osteoblasts (107, 108) provides new possibilities for the study of these receptors. The $Ca^{2+}$ stimulatory and adenylyl cyclase inhibitory effects of ADP on platelets are suggestive of the existence of two different receptors that couple to two different second-messenger pathways. This idea is supported by the results of Boyer & Kim (JL Boyer & W-L Kim, unpublished data) with MEG-01 cells, in which activation of a $P_{2T}$-purinergic receptor results in an inositol phosphate response but has no effect on cAMP levels. Although Barnard et al (109) state that cDNA for a $P_{2T}$-purinergic receptor has been cloned, no primary data have been reported.

## *$P_{2X}$-Purinergic Receptors*

$P_{2X}$-purinergic receptors were originally delineated by Burnstock & Kennedy (7) as receptors that mediate the contractile responses to ATP in vas deferens and urinary bladder of guinea pig and rat, in frog and rat ventricle, and in smooth muscle of the rat femoral artery and rabbit central ear artery. These receptors are now known to be widely distributed in the central nervous system

as well as on many different types of smooth muscle. The order of potency of agonists initially described for $P_{2X}$-purinergic receptors, i.e. α,β-methyleneATP, β,γ-methyleneATP > ATP = 2-MeSATP, is still the basis on which these receptors are distinguished. Excellent comprehensive reviews of this class of receptors have been published by Bean and colleauges (110, 111) and by Dubyak & El-Moatassim (9); thus these receptors are considered in only very general terms here.

$P_{2X}$-purinergic receptors regulate cell function by gating ion permeability. This does not exclude the possibile existence of a receptor with the general pharmacological specificity of a $P_{2X}$-purinergic receptor that functions by a mechanism not involving direct regulation of ion movement; however, such a species has not yet been reported. Interaction of ATP and several of its analogues with receptors on neurons and on muscle cells results in a rapid depolarizing current (9, 110). The kinetics of these responses in intact cells, as well as their capacity to be measured by patch-clamp technology as unitary channel currents, have led to the conclusion that these receptors for extracellular ATP exist as ligand-gated ion channels that are analogous to the glutamate or nicotinic cholinergic type of receptor/ion channels. Activation of ATP-regulated channels typically occurs within milliseconds, is in general followed by a rapid inactivation of the depolarizing current, and accounts for fast excitatory neurotransmission at a number of defined synapses (112, 113). Neither G proteins nor second-messenger molecules appear to be directly involved in the function of these ATP receptors, although in most cases direct data do not exist to unambiguously rule out this possibility. These channels exhibit little selectivity for monovalent cations, but under physiological conditions $Na^+$ is the predominant depolarizing ion (113–115). Activation of $P_{2X}$-purinergic receptors in many target tissues also results in increases in cytosolic $Ca^{2+}$ levels (114–117), apparently occurring as a consequence of permeation of the ATP-regulated channel by $Ca^{2+}$ (111, 114). Activation of voltage-dependent $Ca^{2+}$ channels may also account for secondary changes in intracellular $Ca^{2+}$ levels following activation of $P_{2X}$-purinergic receptors (9).

Although the general pharmacological specificity of the ATP-gated ion channels fits that of a $P_{2X}$-purinergic receptor, detailed pharmacological data do not exist. For example, although the pharmacological specificity of the receptor that mediates contraction of bladder smooth muscle is well established, direct pharmacological equivalence of this tissue response with pharmacological data from a single channel current is not available. The data for pharmacological selectivity of the receptors on neurons is even less well developed but, nonetheless, points to involvement of a receptor with a $P_{2X}$-purinergic receptor selectivity. Pharmacological data in three tissues with a broad series of newly synthesized analogues of ATP has lead to the suggestion that multiple $P_{2X}$-purinergic receptors exist (66). Differences in agonist spec-

ificity also have been observed in depolarizing responses in various tissues. For example, α,β-methyleneATP is not a potent agonist for the ATP-gated ion channel on rat ventricular myocytes (118), frog arterial cells (119), or PC-12 cells (120, 121). Suprenant (122) has reported multiple neuronal depolarizing responses to adenine nucleotide analogues that suggest the existence of more than one $P_{2X}$-purinergic receptor.

Complimentary DNAs that encode two different ATP-gated ion channels have recently been cloned (122a,b). The sequences for both receptors predict protein structures that include two transmembrane-spanning domains and a pore-forming domain similar to that of potassium channels. Although these cDNAs apparently provide the first identification of $P_{2X}$-purinergic receptor structure, the pharmacological specificities of the recombinant receptors expressed in oocytes are not those originally used to classify $P_{2X}$-purinergic receptors. That is, Valera et al (122a) found the 2MeSATP was more potent than α,β-methyleneATP for activation of the receptor cloned from rat deferens. Brake et al (122b) reported that although 2MeSATP was a potent activator of the receptor cloned from rat pheochromocytoma PC12 cells, neither α,β-methyleneATP nor β,γ-methyleneATP were agonists. These results illustrate the uncertainty inherent in delineating receptor subtypes on the basis of agonist selectivities alone and support the view that association of protein structure with pharmacological selectivity will be necessary for confident $P_2$-purinergic receptor subclassification.

## *$P_{2Z}$-Purinergic Receptors*

ATP produces a nonselective increase in permeability of a number of cell types, including macrophages (123–125), mast cells (16), fibroblasts (15, 126, 127), and parotid cells (128). This effect can be distinguished from that mediated by $P_{2X}$-purinergic receptors in that the increase in permeability occurs to both anions and cations and has been shown in several cases to occur as a bidirectional increase in plasma membrane permeability to molecules up to 900 Daltons in size (9). This so-called receptor for ATP has been termed the $P_{2Z}$-purinergic receptor. ATP is the only nucleotide triphosphate activator, and only a limited number of analogues of ATP, e.g. BzATP (127), have been shown to mimic the effect of ATP. $ATP^{4-}$ has been shown to be the form of ATP that activates these receptors (9, 129, 130). The physiological significance of $P_{2Z}$-purinergic receptors has not yet been fully established; the concentration of ATP, i.e. 0.1–1 mM, necessary for pore-forming activity far exceeds that necessary for activation of $P_{2X}$- or $P_{2Y}$-purinergic receptors. Nuttle et al (125) have illustrated that injection of *Xenopus* oocytes with partially purified poly(A)$^+$ RNA from macrophages results in conferrence of a response to ATP or BzATP that is consistent with that observed for $P_{2Z}$-purinergic receptors in native tissues. The cDNA encoding this signaling protein has not yet been

isolated. The reader is referred to the review by Dubyak & El-Moatassim (9) for more details on $P_{2Z}$-purinergic receptors.

## *Other $P_2$-Purinergic Receptors*

Data exist suggesting that the classification scheme presented in Table 2 does not completely account for all of the receptors through which extracellular adenine nucleotides regulate cellular function, and thus additional $P_2$-purinergic receptors likely exist (Table 3). Some of these are probably signaling proteins that should not be considered $P_2$-purinergic receptors. For example, the uridine nucleotide–selective receptor on C6–2B glioma cells (85) is likely related to $P_{2U}$-purinergic receptors because UTP activates both the $P_{2U}$-purinergic receptor and the novel uridine nucleotide receptor. However, since the latter receptor is not activated by adenine nucleotides, it obviously is not a purinergic receptor. On the other hand, a $P_2$-purinergic receptor on PC12 cells apparently activates a $Ca^{2+}$ channel with a pharmacological specificity that distinguishes it from other purinergic receptors (120). Although the principle effect of the PC12 cell receptor appears to be through an ion channel, this receptor is not activated by α,β-methyleneATP or β,γ-methyleneATP, which are classical $P_{2X}$-purinergic receptor agonists. Thus, in a classification scheme grouping ion channel–coupled receptors with a $P_{2X}$-purinergic receptor superfamily (67), this receptor would stand out as a signaling protein with a very different pharmacological specificity. Moreover, regulation of a $Ca^{2+}$ channel might well be through a G protein (59), and the PC12 receptor might structurally resemble the G protein-linked $P_{2Y}$- and $P_{2U}$-purinergic receptors. The relationship of the receptor studied by Kim & Rabin (120) to the receptor shown to regulate [$^3$H]dopamine release and $Ca^{2+}$ influx in PC12 cells (131) is unclear.

Perhaps the most novel class of signaling molecules that may reveal a new class of purinergic receptors derives from the work of Miras-Portugal and coworkers (132–135). Diadenine polyphosphates are naturally occurring components of secretory granules and of neural tissues (132–135). Adenine dinucleotides modify a number of physiological responses, including inhibition of platelet aggregation and inhibition of catecholamine secretion. Although unambiguous association of a physiological effect of adenine dinucleotides with a distinct class of receptors has not been accomplished, a binding site for [$^3$H]-Ap4A has been identified in neural tissues that follows the general order of specificity of Ap4A > ADPβS > AppNHp > Ap5A > α,β-methyleneATP > 2-MeSATP. This putative receptor has been tentatively designated the $P_{2D}$-purinergic receptor by Pintor et al (135). Observation of potent stimulatory effects of Ap4A on the stably expressed human $P_{2U}$-purinergic receptors indicates that effects of adenine dinucleotide are not limited to the putative $P_{2D}$-purinergic receptor (ER Lazarowski & TK Harden, unpublished results).

# STRUCTURE-ACTIVITY RELATIONSHIPS FOR $P_2$-PURINERGIC RECEPTOR AGONISTS

## *Introduction*

Study of $P_2$-purinergic receptors has been difficult owing to the lack of potent and selective agonists and antagonists. The analysis of structure-activity relationships for the available nucleotide analogue agonists also has been problematic because these analogues are hydrolyzed by ectonucleotidases and kinases, which are present at variable levels in different cell preparations (11, 12). Until recently, essentially all of the structure-activity analyses of $P_2$-purinergic receptor agonists had been carried out by Cusack in collaboration with several laboratories (136, 137). However, in the last year Jacobson and coworkers (65, 66; JW O'Tuel, JL Boyer, B Fisher, KA Jacobson & TK Harden, unpublished data) have introduced several analogues of ATP and ADP that have substantially advanced our understanding of the determinants of $P_2$-purinergic receptor selectivity. These agonists have also been applied to studies of $P_2$-purinergic receptor–regulated second-messenger response pathways.

## *Structure-Activity of $P_{2X}$- and $P_{2Y}$-Purinergic Receptor Agonists*

Substitutions at the C8 position in the adenine ring ($B_2$ in Figure 2), such as 2-chloro-ATP and 2-MeSATP, resulted in increases in potency at $P_{2Y}$-purinergic receptors, with a small increase or no increase in the potency for $P_{2X}$-purinergic receptors (65). Substitutions at the C2 position in the adenine ring ($B_1$ in Figure 2)—such as 8-BrATP, 8-(6-aminohexylamino)-ATP (66), and 8-azido-ATP—resulted in complete abolishment of $P_{2X}$-purinergic responses and decreased activity at $P_{2Y}$-purinergic receptors. Bridging the positions $N^1$ and $N^6$ with an ethylene group, as in $N^1$,$N^6$-etheno-ATP, resulted in loss of activity at both $P_{2X}$- and $P_{2Y}$-purinergic receptors. Substitutions at the $N^6$ position, as in $N^6$-methyl-ATP or $N^6$-dimethyl-ATP, resulted in no change in agonist activity at $P_{2Y}$-purinergic receptors, but the activity of these compounds at $P_{2X}$-purinergic receptors was lost completely. Further, the double substitution of $N^6$-methyl and 2-thioether in the same molecule, i.e. $N^6$-methyl-2-[(5-hexenyl)thio]-ATP, resulted in a compound that was inactive at $P_{2X}$-purinergic receptors but exhibited a potency at $P_{2Y}$-purinergic receptors that increased by more than two orders of magnitude relative to $N^6$-methyl-ATP (65).

Several nucleotide analogues substituted in the ribose moiety have been studied (Figure 2). All of these compounds exhibited a decrease in potency for $P_{2Y}$-purinergic receptors, with the exception of 3′-amino-3′-deoxy-ATP, which was significantly more potent than ATP at $P_{2Y}$-purinergic receptors in turkey erythrocytes and in the endothelial cell–dependent response of rabbit aorta (66). Activities at $P_{2X}$-purinergic receptors decreased or remained unchanged by

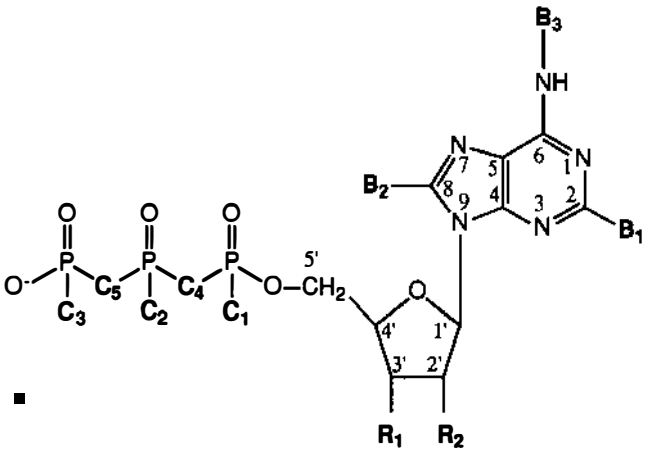
ribose substitutions (Figure 2). Enantiomeric forms of ATP analogues have been prepared by replacing the naturally occurring D-ribose sugar of adenine nucleotides by L-ribose (138). $P_{2Y}$-purinergic receptor–mediated responses in the guinea pig *t. coli* showed very little stereoselectivity between these enantiomers (three- to sixfold). The naturally occurring enantiomeric forms of 2-substituted ATP analogues showed a higher stereoselectivity (about 125-fold), probably due to enhancement of potency of the 2-sub- stituted ATP versus the poor enhancement in potency of 2-substituted L-ATP (139, 140). In contrast, no stereoselectivity towards the enantiomeric forms of ATP, ADP, AMP, or their 2-substituted analogues was observed for the $P_{2X}$-purinergic receptor of the urinary bladder. In fact, it has been shown that the unnatural L-enantiomeric form of ATP is more potent than ATP (140, 141), although this effect is probably due to resistance of L-ATP to dephosphorylation by ectonucleotidases.

Phosphate chain substitutions of sulfur for oxygen at the α, β, or γ positions results in ATPαS, ATPβS, and ATPγS, respectively (Figure 2). The substitutions at nonterminal phosphates result in chirality so that the analogues exist as pairs of Rp and Sp diastereoisomers. Pharmacological activities of these compounds indicated very little stereoselectivity (142). Substitution of methylene or imido groups for the phosphorous-bridging oxygens results in compounds that exhibit selectivity among the various $P_2$-purinergic receptor subtypes. ATPγS is a potent agonist at both $P_{2Y}$- and $P_{2U}$-purinergic receptors but has no activity at $P_{2X}$-purinergic receptors. In contrast, α,β-methyleneATP and β,γ-methyleneATP are potent $P_{2X}$-purinergic receptor agonists with very weak or no effects at $P_{2Y}$- and $P_{2U}$-purinergic receptors, respectively. Cyclization of the phosphate of β,γ-methyleneATP at the 3′ position as in β,γ-Me-adenosine-3′,5′-cyclic triphosphate resulted in abolishment of activity at $P_{2X}$-purinergic receptors (66). The ADP analogue ADPβF is more potent than ADP or ATP at $P_{2Y}$-purinergic receptors and has no effect on $P_{2X}$-purinergic receptors (143), as is the case for ADPβS.

Adenine nucleotide diphosphate analogues are equipotent to triphosphates as agonists at $P_{2Y}$-purinergic receptors. The marked increase in potency of 2-thioether analogues of adenine nucleotides at $P_{2Y}$-purinergic receptors also has allowed preparation of 2-thioether adenine nucleotide monophosphates that are full agonists at these receptors. Moreover, some of these monophosphate derivatives are more potent than ATP (65). This observation indicates that one phosphate is sufficient for activity at $P_{2Y}$-purinergic receptors, which could potentially overcome difficulties produced by nucleotide degradation by nucleotidases.

## *Structure-Activity at other $P_2$-Purinergic Receptors*

Analyses of structure-activity relationships at $P_{2U}$-purinergic receptors have been very limited. $P_{2U}$-purinergic receptors are fully activated by three known




*Purine base modifications*

| | $B_1$ | $B_2$ | $B_3$ | $P_{2X}$ | $P_{2Y}$ | $P_{2U}$ |
|---|---|---|---|---|---|---|
| ATP | H | H | H | | | |
| 2-MeS-ATP | $CH_3$-S | H | H | [a] | ↑[b] | ↓[c] |
| 2-(hexylthio)-ATP | $CH_3$-$(CH_2)_5$-S | H | H | [a] | ↑[d] | |
| 2-[(2-(*p*-nitrophenyl)ethyl)thio]-ATP | *p*-$NO_2C_6H_4(CH_2)_2$-S | H | H | [e] | ↑ | |
| 2-[(6-cyanohexyl)thio]-ATP | $NC(CH_2)_6$-S | H | H | ↑[f] | ↑ | |
| $N^6$-methyl-ATP | H | H | $CH_3$ | ↓ | | |
| $N^6$-methyl-2-[(5-hexenyl)thio)]-ATP | $CH_2$ = CH-$(CH_2)_4$-S | H | $CH_3$ | ↓ | ↑ | |
| 8-Br-ATP | H | Br | H | ↓ | ↓ | ↓ |
| 8-(6-aminohexyl)amino-ATP | H | $NH_2$-$(CH_2)_6$-NH | H | ↓ | [g] | |

*Ribose modifications*

| | $R_1$ | $R_2$ | $P_{2X}$ | $P_{2Y}$ | $P_{2U}$ |
|---|---|---|---|---|---|
| ATP | OH | OH | | | |
| 2',3'-deoxy-ATP | H | H | | ↓[h] | ↓ |
| 3'-amino 3'-deoxy-ATP | $NH_2$ | OH | | ↑[i] | |
| 3'-acetylamino 3'-deoxy-ATP | $CH_3$-CO-NH- | OH | | ↓[j] | |
| 3'deoxy 3'-(4-hydroxyphenylpropionyl)amino-ATP | OH-$C_6H_4$-$(CH_2)_2$-CO-NH- | OH | | ↓[k] | |
| 3'-benzylamino 3'-deoxy-ATP | $C_6H_5$-$CH_2$-NH- | OH | ↑[l] | ↓ | |

*Phosphate chain modifications*

| | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $P_{2X}$ | $P_{2Y}$ | $P_{2U}$ |
|---|---|---|---|---|---|---|---|---|
| ATP | OH | OH | OH | O | O | | | |
| ATP$\alpha$S | S | OH | OH | O | O | | ↑ | ↓ |
| ATP$\beta$S | OH | S | OH | O | O | | ↑ | |
| ATP$\gamma$S | OH | OH | S | O | O | | ↑ | ↓ |
| $\alpha,\beta$-methyleneATP | OH | OH | OH | $CH_2$ | O | ↑ | ↓ | ↓ |
| $\beta,\gamma$-methyleneATP | OH | OH | OH | O | $CH_2$ | ↑ | ↓ | ↓ |
| App(NH)p | OH | OH | OH | O | NH | | | ↓ |

nucleotides—ATP, UTP, and ATPγS; other nucleotide analogues that are selective for $P_{2X}$-, $P_{2Y}$-, and $P_{2T}$-purinergic receptors are inactive or exhibit low potencies. A systematic evaluation of the effect of substitutions of UTP on $P_{2U}$-purinergic receptor activity is needed because such derivatives are not yet available, and they offer promise for identification of high potency, stable, selective $P_{2U}$-purinergic receptor agonists. Structure-activity relationships of nucleotide analogues acting at $P_{2T}$-purinergic receptors have been broadly studied, and the reader is referred to an extensive and excellent review by Hourani & Cusack (87).

## RADIOLIGAND-BINDING ASSAYS FOR $P_2$-PURINERGIC RECEPTORS

Successful radiolabeling of cell surface receptors for extracellular stimuli has usually depended on the availability of ligands of high affinity, stability, and protein-binding specificity. Although many peptide hormones have possessed the requisite affinities and specificity for receptor radioligands, success in receptor labeling has usually required the availability of high-affinity receptor antagonists. Such requirements cannot yet be met with molecules that interact with $P_2$-purinergic receptors. There are no selective antagonists, and the several compounds that can be shown to competitively inhibit $P_2$-purinergic receptors (i.e. suramin, reactive blue 2) do so with only micromolar affinity and interact with many other proteins. Agonists of $P_2$-purinergic receptors also present problems. Their binding affinities are only slightly higher than their affinities for other ATP binding proteins, and they are subject to hydrolysis by nucleotide hydrolyzing enzymes.



*Figure 2* Some examples of structural analogues of ATP and their pharmacological selectivity

[a] Inactive at saphenous artery and as potent as ATP in guinea pig vas deferens and urinary bladder.
[b] ↑, increased potency relative to ATP.
[c] ↓, decreased potency relative to ATP.
[d] $K_{0.5}$ for inhibition of adenylyl cyclase in C6 rat glioma cells is 23 pM.
[e] More potent than ATP in guinea pig vas deferens; inactive in saphenous artery.
[f] More potent than ATP in guinea pig vas deferens and urinary bladder; inactive in saphenous artery.
[g] Less potent than ATP in all $P_{2Y}$-purinergic responses, except in rat aorta, in which it is 10-fold more potent than 2-MeSATP.
[h] These analogues were inactive in rabbit aorta and mesenteric artery, less potent than ATP in turkey erythrocytes, and as potent as ATP in the *taenia coli.*
[i] Increased potency in turkey erythrocytes and aorta; inactive in mesenteric artery and in C6 rat glioma cells.
[j] Inactive in turkey erythrocytes, *taenia coli* and aorta; more potent than ATP in mesenteric artery.
[k] Inactive in turkey erythrocytes, *taenia coli* and mesenteric artery; more potent than ATP in rabbit aorta.
[l] Inactive in the saphenous artery $P_{2X}$-purinergic receptor.

With these concerns in mind, radiolabeling of $P_2$-purinergic receptors has proven difficult. Although a number of reports have suggested successful labeling of $P_{2Y}$-, $P_{2X}$-, and $P_{2T}$-purinergic receptors, in none of these studies have data been provided that unambiguously prove that a physiologically relevant receptor was specifically quantitated. A consistent problem is that binding assays have been carried out under conditions, e.g. with membranes, that are very different than the conditions in which biological responses to the receptor can be measured, e.g. with intact cells or tissues. As such, direct correlations of binding constants with receptor activity constants usually have not been possible. Attempts to radiolabel the $P_{2Y}$-purinergic receptor on turkey erythrocyte membranes were made under conditions that were somewhat similar to those in which receptor stimulatory activity of nucleotide analogues could be tested (144). Thus, radioligand-binding assays were carried out in purified plasma membranes and the capacity of $P_{2Y}$-purinergic receptor agonists to stimulate phospholipase C was determined in membrane ghosts prepared from erythrocytes. $K_i$ values of a series of ten agonists for inhibition of [$^{35}$S]ADPβS binding to plasma membranes closely matched the $K_{0.5}$ values determined for the same agonists for stimulation of phospholipase C activity in the ghost membrane preparation. GTP and hydrolysis-resistant analogues of GTP inhibited radiolabeled agonist binding noncompetitively and with potencies that were consistent with the potencies of the same guanine nucleotides for supporting $P_{2Y}$-purinergic receptor–stimulated phospholipase C activity. These pharmacological and biochemical data strongly suggested that the site on turkey erythrocyte membranes that was labeled by [$^{35}$S]ADPβS was the same site utilized by $P_{2Y}$-purinergic receptor agonists to activate phospholipase C. Subsequent work has cast doubt on this initial assertion. The availability of newly synthesized, highly selective $P_{2Y}$-purinergic receptor agonists (65) has permitted more detailed pharmacological analyses of the [$^{35}$S]ADPβS-binding site on turkey erythrocyte membranes. A number of these agonists activated phospholipase C and inhibited [$^{35}$S]ADPβS binding with very different apparent affinities (TK Harden, unpublished data). Such results could potentially be explained by differential hydrolysis of nucleotides or important differences in conditions necessary for assays of binding versus activity. However, major differences in binding versus biological activity seriously call into question the validity of the [$^{35}$S]ADPβS-binding assay of $P_{2Y}$-purinergic receptors on turkey erythrocyte membranes. van Galen et al (145) have found that, although [$^{35}$S]ADPβS labels a binding site(s) in bovine brain membranes with high affinity, the pharmacological selectivity of this site does not fit that of any known $P_2$-purinergic receptor.

Boyer and coworkers (31, 32) have utilized [$^{32}$P]benzoylylbenzoylylATP ([$^{32}$P]BzATP) to competitively label and supposedly covalently incorporate radiolabel into $P_{2Y}$-purinergic receptors on turkey erythrocyte plasma mem-

branes. The reversible binding of [$^{32}$P]BzATP closely correlated with that of [$^{35}$S]ADPβS binding in the same membranes (31). Moreover, following exposure of prelabeled turkey erythrocyte plasma membranes to UV light, [$^{32}$P]BzATP was covalently incorporated into a 53-kDa protein. Analogues of ATP and ADP and guanine nucleotides inhibited [$^{32}$P]BzATP labeling of the 53-kDa protein with potencies and properties that would be expected of that of a $P_{2Y}$-purinergic receptor. However, this suggestion was based on results with a limited number of drugs that would be expected to show $P_{2Y}$-purinergic receptor selectivity. Based on the questions of the validity of the [$^{35}$S]ADPβS-binding assay for $P_{2Y}$-purinergic receptors in turkey erythrocyte membranes, a similar concern holds for [$^{32}$P]BzATP labeling. Moreover, the selectivity of [$^{32}$P]BzATP is seriously questioned by results from other studies. For example, Erb et al (127) illustrated that BzATP was an effective agonist at $P_{2Z}$-purinergic receptors and could apparently be covalently incorporated into this protein upon exposure of BzATP-prelabeled fibroblast membranes to UV light. Subsequent work by Erb and coworkers has demonstrated that [$^{32}$P]BzATP labels $P_{2U}$-purinergic receptors after their overexpression in K562 human leukemia cells (82). However, [$^{32}$P]BzATP apparently does not label the $P_{2U}$-purinergic receptor agonist–binding site because BzATP is only a weak agonist at this receptor, and radiolabeling was not inhibited by $P_{2U}$-purinergic receptor agonists. [$^{32}$P]BzATP also has been shown to label an approximately 53-kDa protein in membranes derived from PC12 cells (146, 147), although insufficient pharmacological data were provided to unambiguously delineate the identity of this protein(s). [$^{3}$H]α,β-methyleneATP has been used as a radioligand for $P_{2X}$-purinergic receptors in membranes isolated from bladder and vas deferens smooth muscle (148–150). Although agonist-binding affinities generally followed those observed in intact tissues for $P_{2X}$-purinergic receptors, these correlations were far from absolute. For example, the differences in apparent binding affinity of α,β-methyleneATP and 2-MeSATP for competition at the vas deferens–binding site were only about 30-fold, and many nucleotides that apparently are not $P_{2X}$-purinergic receptor agonists also fully inhibited radioligand binding (150). The density of binding sites labeled with [$^{3}$H]α,β-methyleneATP also far exceeded that observed with essentially all other neurotransmitter receptors. Bo et al (151) have covalently incorporated [$^{3}$H]α,β-methyleneATP into a 62-kDa protein in vas deferens membranes, but the equivalence of this protein with the $P_{2X}$-purinergic receptor has not been firmly established. [$^{3}$H]arylazidoaminoproprionyl-ATP also has been used in an attempt to covalently label $P_{2X}$-purinergic receptors (152), but the equivalence of $P_{2X}$-purinergic receptors with the 57- and 62-kDa proteins that were labeled was not demonstrated.

Other radioligands have been used to supposedly label $P_{2T}$-purinergic receptors (153–155) and the putative $P_{2D}$-purinergic receptor (135, 156).

[$^{35}$S]ATPγS also has been used to label binding sites on intact TEA3A1 cells that supposedly represented a cell surface $P_2$-purinergic receptor (157). Similarly, Giannattasio et al (61) have used 8-azido-ATP to label a binding site that was tentatively suggested to be a cardiac ATP receptor. As with $P_{2Y}$- and $P_{2X}$-purinergic receptor labeling, questions still remain in all of these studies.

Two encouraging radioligand-binding studies recently have been carried out with [$^{35}$S]ATPαS. [$^{35}$S]ATPαS labeled a binding site on intact P12 cells with a pharmacological specificity that was very similar to the potencies of a series of drugs for $P_2$-purinergic receptor–mediated activation of a $Ca^{2+}$ channel on the same cells. Moreover, Simon et al (J Simon, TE Webb, BF King, G Burnstock & EA Barnard, unpublished data) have shown that overexpression of the cloned chick $P_{2Y1}$-purinergic receptor in COS-7 cells results in conferrence of a [$^{35}$S]ATPαS-binding site that exhibits the general pharmacological specificity of a $P_{2Y}$-purinergic receptor. The latter study is the first to take advantage of the powerful attribute of controlled elevation of receptor-binding sites to levels that should considerably exceed those of other nonreceptor nucleotide-binding proteins that exist on cells. However, a major uncertainty arises from such studies with agonist radioligands: High-affinity agonist binding to G protein–linked receptors commonly is thought to require a stoichiometric presence of the G protein to which the receptor couples. As such, there will be a limitation to which elevation in expression of receptors can be detected by an agonist radioligand. For example, the percentage of receptors existing in a high-affinity guanine nucleotide–sensitive state after overexpression of muscarinic cholinergic (158), dopamine (159), or many other G protein–linked receptors is very low. The density of expressed $P_{2Y1}$-purinergic receptors detected by [$^{35}$S]ATPαS was 8 pmol $mg^{-1}$ protein (J Simon, TE Webb, BF King, G Burnstock & EA Barnard, unpublished data), which is a level that exceeds what might be expected for the presence of $G_q$ or $G_{11}$ in COS-7 cells. The meaning of these observations will require more quantitative studies and the availability of radiolabeled $P_2$-purinergic receptor antagonists of sufficient affinity and specificity.

## STRUCTURE OF $P_2$-PURINERGIC RECEPTORS

A mouse and a human $P_{2U}$-purinergic receptor (81, 83) and a chicken and a turkey $P_{2Y}$-purinergic receptor (68, 69) represent the only G protein–linked $P_2$-purinergic receptors whose cDNA and amino acid sequences have been reported. These sequences confirm that the $P_2$-purinergic receptors are members of the superfamily of G protein–coupled receptors, which contain seven putative α-helical transmembrane segments, with their amino termini located on the extracellular side and their carboxy termini located on the intracellular side of the membrane (Figure 3). $P_{2U}$- and $P_{2Y}$-purinergic receptors have

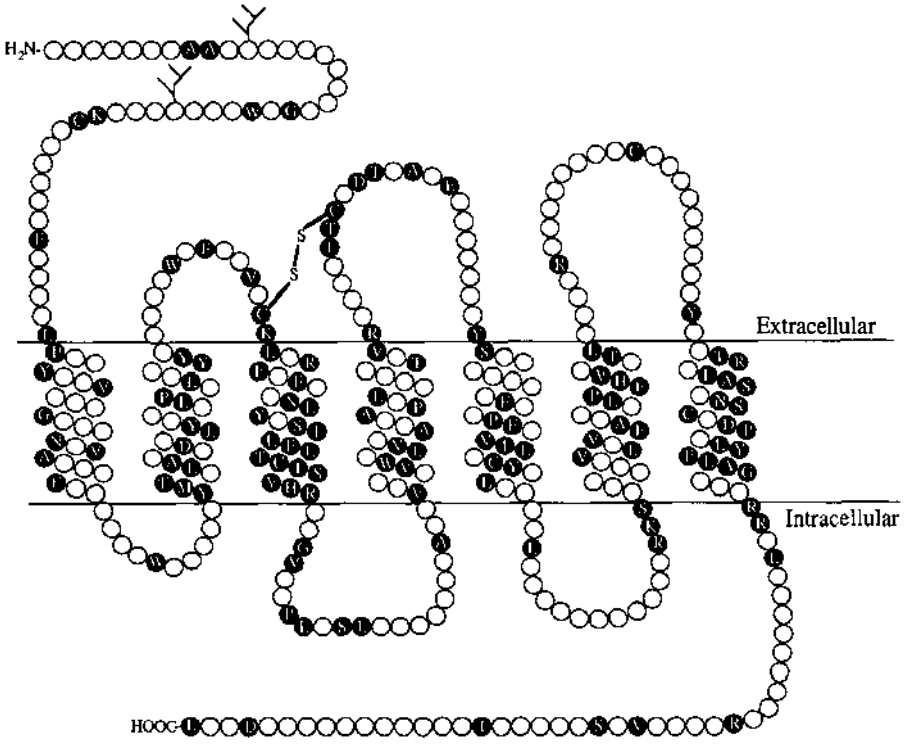



*Figure 3* Schematic representation of the $P_{2Y}$-purinergic receptor and its homology with $P_{2U}$-purinergic receptors. The hypothetical structure of the avian $P_{2Y}$-purinergic receptor is shown. The black-filled circles represent amino acids (indicated by their one letter code) conserved between the avian $P_{2Y}$- and mouse $P_{2U}$-purinergic receptors. The overall amino acid identity between these two receptors is about 35%. Note the span of twelve consecutive identical amino acids at the intracellular side of the third transmembrane segment. The two branched structures in the amino terminus indicate possible glycosylation sites. Two other possible sites in the $P_{2Y}$-purinergic receptor are not shown, but their location is indicated in Figure 4. The disulfide bond connecting the first and second extracellular loops is surmised from data obtained with other more well-studied G protein–coupled receptors.

relatively low amino acid homology (36% identity) (Figure 4). Because partial clones of the rat and human homologues of avian $P_{2Y}$-purinergic receptors are about 85% homologous at the amino acid level with the avian receptors (Q Li, JB Schachter, TK Harden & RA Nicholas, unpublished data), the low homology of $P_{2U}$- and $P_{2Y}$-purinergic receptors is not a result of species differences. Barnard et al (109) described a third purinergic receptor cloned from chick brain that is activated by ADP with a potency equal to that of ATP. This receptor was reported to possess 39% homology with the chick brain $P_{2Y}$-purinergic receptor. Because no primary data have been published on this

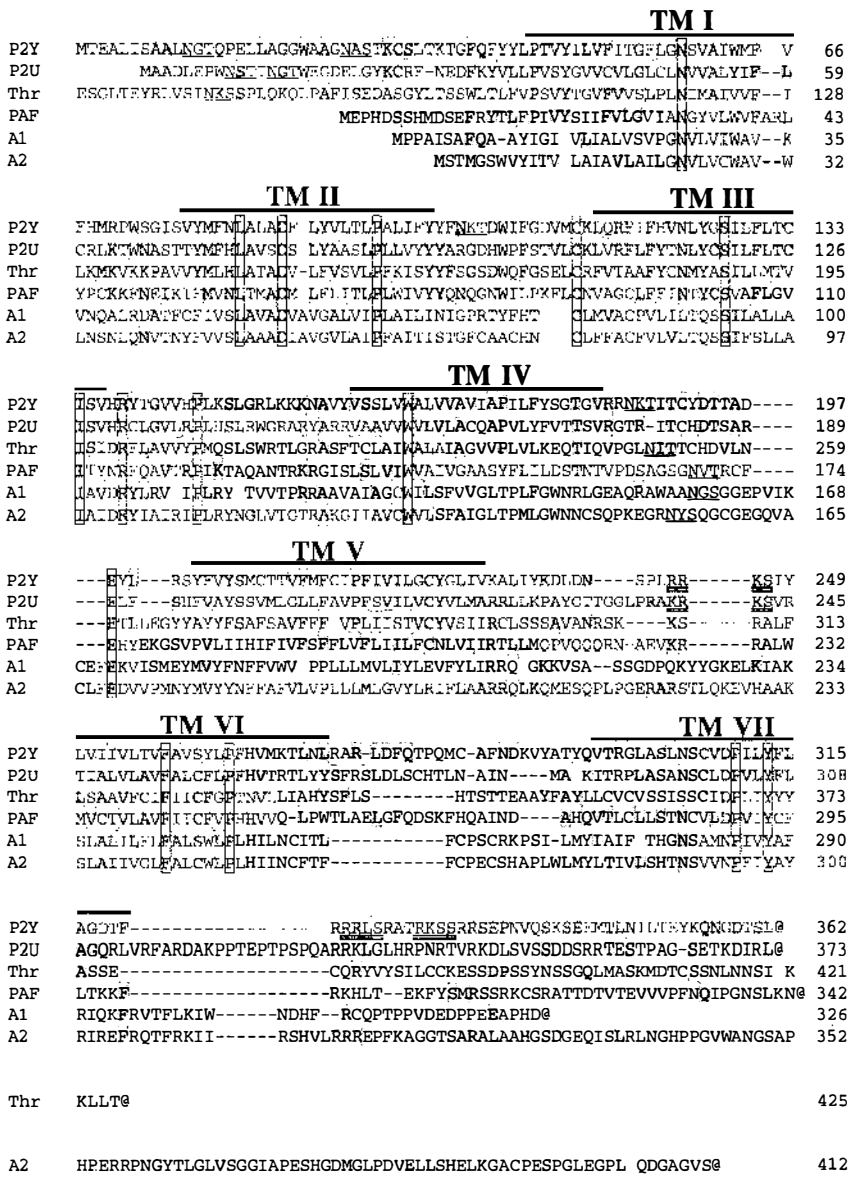



*Figure 4* Alignment of $P_{2Y}$- and $P_{2U}$-purinergic receptors with other related G protein--coupled receptors. The amino acid sequences of the turkey $P_{2Y}$-purinergic receptor (P2Y), the mouse $P_{2U}$-purinergic receptor (P2U), the human thrombin receptor (Thr), the human platelet-activating factor receptor (PAF), and the canine adenosine A1 (A1) and A2 (A2) receptors have been aligned using the pileup command in the GCG sequence analysis program. The shaded amino acids have identity with either the $P_{2Y}$- or $P_{2U}$-purinergic receptor. Dashes represent gaps introduced to increase homology. Amino acids conserved in all six receptors are boxed. Potential glycosylation sites are underlined, and putative protein kinase A sites are double underlined.

putative $P_2$-purinergic receptor, only the $P_{2Y}$- and $P_{2U}$-purinergic receptors are discussed here.

As has been observed for other G protein–coupled receptors (160), most of the homology between $P_{2U}$- and $P_{2Y}$-purinergic receptors occurs in the putative transmembrane-spanning regions (Figure 3), particularly within the putative third and seventh transmembrane domains. Most notably, there is a region of homology in the third transmembrane segment that spans 12 consecutive amino acids. Because this same region was reported to be conserved in the putative ADP receptor mentioned above (109), it may prove to be a signature of $P_2$-purinergic receptors. At least some of the conserved amino acids within the putative transmembrane regions of $P_2$-purinergic receptors are probably involved in the binding of nucleotide ligands and in the subsequent transmission of ligand binding to the activation of the associated G protein. In preliminary experiments, Garrad and coworkers have obtained evidence implicating several positively charged amino acids in the sixth and seventh transmembrane regions in establishing agonist potency and specificity (161).

$P_{2U}$- and $P_{2Y}$-purinergic receptors surprisingly are most similar to G protein–coupled receptors for several peptide ligands. Thus, $P_{2U}$-purinergic receptors have the highest homology to $P_{2Y}$-purinergic receptors, followed in descending order by receptors for somatostatin, thrombin, platelet-activating factor, mu-opiates, and neuropeptide Y. $P_{2Y}$-purinergic receptors have the highest homology to $P_{2U}$-purinergic receptors, followed in descending order by receptors for interleukin-8, thrombin, vasoactive intestinal peptide, platelet-activating factor, angiotensin II, and neuropeptide Y. Figure 4 shows the alignment of the $P_{2Y}$- and $P_{2U}$-purinergic receptors with the human thrombin receptor (162), the human platelet-activating factor receptor (163), and the canine A1- and A2-adenosine receptors (164). Although the adenosine receptors recognize at least part of the same ligand as the $P_2$-purinergic receptors, they have considerably less homology to $P_2$-purinergic receptors than do the peptide receptors. Only a small number of amino acids are conserved in all six receptors, some of which have been found to be important in the function of more well-studied G protein–coupled receptors. These include an asparagine residue in the first transmembrane segment (TM I), an aspartic acid residue in TM II, an arginine residue in the cytoplasmic region of TM III, a tryptophan residue in TM IV, a phenylalanine residue in TM VI, and two proline residues in TM VI and VII (160, 165). The $P_2$-purinergic receptors are the only known G protein–coupled receptors that have a histidine residue at the cytoplasmic region of the third transmembrane segment instead of the usual aspartic acid. This residue may be involved in transmission of ligand binding to the activation of the G protein in other G protein–coupled receptors (166).

There are two consensus sequences in the amino terminus for N-linked

glycosylation in both $P_{2U}$- and $P_{2Y}$-purinergic receptors, and $P_{2Y}$-purinergic receptors have two additional glycosylation consensus sequences, one in the first extracellular loop and another in the second extracellular loop (Figures 3 and 4). It is unclear which of these possible glycosylation signals are used in vivo. There are cysteine residues in the first and second extracellular loops that likely form a disulfide bond (Figure 3). This putative disulfide linkage has been shown to be critical for proper function of several other G protein–coupled receptors (167, 168). $P_{2Y}$-purinergic receptors also contain a possible protein kinase A phosphorylation site within the third intracellular region and the two additional sites in the carboxy terminus (Figure 4). A single protein kinase A site in $P_{2U}$-purinergic receptors is located in the third intracellular region. Several serine and threonine residues that are potential phosphorylation sites by other receptor kinases are present in the carboxy termini of these receptors.

Abbracchio & Burnstock (67) have proposed that the metabotropic G protein–linked $P_2$-purinergic receptors be classified in a single series from $P_{2Y1}$ to $P_{2Y7}$ and so on. The $P_{2U}$-purinergic receptor would become the $P_{2Y2}$-purinergic receptor in this scheme because it was the second G protein–coupled $P_2$-purinergic receptor whose sequence was reported. Although this idea has merit, the low amino acid homology between the cloned $P_2$-purinergic receptors suggests that it may be too early to classify these receptors in a single family. The cloning of additional $P_2$-purinergic receptors will eventually lead to a unifying classification scheme for these signaling proteins that can be based on sequence homology and pharmacological selectivity. The existence of a uridine nucleotide–specific receptor that could have high homology to $P_{2U}$-purinergic receptors (85) adds a potential problem with such a classification scheme. Furthermore, receptors exhibiting a $P_{2Y}$-like pharmacological specificity have been shown to regulate ion channels (60–63), which may or may not involve a G protein, and at least two papers have been reported demonstrating a $P_{2X}$-like pharmacological specificity for a receptor that supposedly regulates adenylyl cyclase (25, 52). In light of these concerns we believe that the receptor classification scheme that has been proposed by the IUPHAR subcommittee on the classification of purinoceptors (17) should be adhered to until much more information is available on receptor structure in the $P_2$-purinergic receptor class of signaling proteins.

## SUMMARY

In the last decade, general acceptance of an important physiological role for extracellular adenine nucleotides has led to remarkable progress in delineation of physiological responses regulated by these molecules, of the subclassification of the cell surface receptors that recognize these extracellular stimuli, and

of the complex signaling mechanisms that underlie their biological activities. This is a rapidly moving field whose knowledge base remains considerably behind that of other receptor-mediated pathways (e.g. those for acetylcholine, catecholamines, serotonin, etc) that are of longer-standing and broader interest. Progress in the $P_2$-purinergic receptor field continues to be retarded by lack of high-affinity, highly specific receptor antagonists, and the availability of receptor-selective agonists remains limited. Truly reliable radioligand-binding assays for these receptors are not available. The impact of molecular biology on the field is at its nascent stage; only two of the G protein–linked $P_2$-purinergic receptor subtypes have been cloned, and very little progress has been made in the application of this new molecular knowledge to delineation of further subtypes, of gene structure and regulation, and of receptor distribution and regulation. This is a field of research that is also plagued by the enormous capacity of tissues to metabolize extracellular adenine nucleotides, and there are hundreds of proteins in addition to $P_2$-purinergic receptors that bind ATP and some of its analogues with high affinity.

The next five years should see an expansion of the members of the $P_2$-purinergic receptor classes, which should allow some resolution of these into a reliable system for receptor subclassification. This will be a complex task. Although $P_{2Y}$- and $P_{2U}$-purinergic receptors principally produce their physiological effects through G protein–coupled activation of second-messenger producing enzymes, there may be exceptions to this idea, as well as to the idea that receptors exhibiting a $P_{2X}$-like specificity are single ion channel proteins. Obviously, the knowledge base is too small to answer these questions, and only by molecular cloning of cDNA and expression and characterization of proteins of defined function are such conundrums likely to be rectified in a unifying classification scheme for the $P_2$-purinergic receptors. There are many unknowns, and the $P_2$-purinergic receptor field represents a fecund area for research, ranging from synthesis of new receptor ligands to definition of the molecular structure and function of new signaling proteins.



## ACKNOWLEDGMENTS

The authors are indebted to John W O'Tuel III and Brenda K Lindsey for their help in preparing the manuscript. The work from the authors' laboratory on structure-activity relationships for $P_2$-purinergic receptor agonists was carried out in collaboration with Drs. Ken Jacobson and Bilha Fischer of the National Institutes of Health and Dr. Geoffrey Burnstock of University College London. The authors are indebted to Drs. G Weisman, B King, G Burnstock, and PPA Humphrey for providing preprints of their work. Work cited from the authors' laboratories was supported by USPHS grants GM38213, GM29536, and HL34322.

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