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PERSPECTIVE

Identification of the $P2Y_{12}$ Receptor: A Novel Member of the P2Y Family of Receptors Activated by Extracellular Nucleotides

ROBERT A. NICHOLAS

Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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The action of extracellular nucleotides, specifically the ability of adenine nucleotides to induce coronary vasodilation and bradycardia, was first described more than 70 years ago (Drury and Szent-Györgyi, 1929). Nearly 30 years later, ADP was shown to promote platelet aggregation (Gaarder et al., 1961; Born, 1962). However, the general perception that nucleotides were extracellular signaling molecules was slow to take root, and it was not until the mid-1970s and early 1980s that the possibility became more generally accepted. Today, we know that extracellular nucleotides are released from cells either from vesicles or in response to stress or hypoxia and evoke a myriad of responses in virtually all cells and tissues. These responses include platelet aggregation, increases in Cl^- secretion and ciliary beat frequency in airway cells, relaxation and contraction of vascular smooth muscle, stimulation of hepatic glycogenolysis, rapid depolarization of central nervous system and sensory neurons, and nociception (Harden et al., 1995; Ralevic and Burnstock, 1998).

Extracellular nucleotides exert their physiological effects through fast, ionotropic P2X receptors, which are intrinsic Ca^{2+}-permeable cation channels with homology to epithelial sodium channels, and slower, metabotropic P2Y receptors, which are G protein-coupled receptors (GPCRs). Currently, seven P2X receptors, designated $P2X_{1–7}$, have been cloned and characterized. With the cloning of the $P2Y_{12}$ receptor (also designated HORK3, SP1999, $P2T_{AC}$, or $P2_T$) reported in this issue (Takasaki et al., 2001) and in two other independent reports (Hollopeter et al., 2001; Zhang et al., 2001), there are currently six functional mammalian P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$ (Table 1). The large gaps in numbering have arisen from the unfortunate inclusion of nonmammalian P2Y receptors (chick p2y3 and *Xenopus laevis* p2y8 receptors) (Webb et al., 1996b; Bogdanov et al., 1997) and receptors (p2y5, p2y7, p2y9, and p2y10) with low homology to P2Y receptors and with no supporting functional data (Herold et al., 1997; Janssens et al., 1997; Li et al., 1997). $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors all couple to the activation of phospholipase C, mobilization of intracellular Ca^{2+}, and activation of protein kinase C, whereas the newly cloned $P2Y_{12}$ receptor couples solely to the inhibition of adenylyl cyclase in a pertussis toxin-sensitive manner (Table 1). The $P2Y_{11}$ receptor is dually coupled to activation of phospholipase C and adenylyl cyclase (Communi et al., 1997). Surprisingly, P2Y receptors are more closely related to GPCRs for peptides, such as somatostatin, angiotensin, and platelet activating factor, than they are to receptors for adenosine or other small molecules (Lustig et al., 1993).

The capacity of ADP to promote platelet aggregation has been known for some time (Gaarder et al., 1961). Early studies demonstrated that ADP inhibited adenylyl cyclase activity in platelet membranes in a GTP-dependent manner, suggesting the existence of an ADP-activated G protein-coupled receptor (Cooper and Rodbell, 1979). Later studies documented that ADP also promoted a rise in intracellular $[Ca^{2+}]$ in intact platelets, but this effect initially was thought to be caused by the same GPCR that inhibited adenylyl cyclase activity, designated the P2T receptor. Subsequently, the $P2Y_1$ receptor was shown to be expressed in platelets (Leon et al., 1997), which was hypothesized to account for the rise in ADP-promoted intracellular $[Ca^{2+}]$. Finally, using $P2Y_1$ and $P2T_{AC}$ receptor-selective antagonists (Table 1) (Humphries et al., 1995; Boyer et al., 1996; Boyer et al., 1998; Camaioni et al., 1998; Ingall et al., 1999), several groups simultaneously demonstrated that the P2T receptor responsible for platelet aggregation was actually two receptors: the $P2Y_1$ receptor, which is coupled solely to mobilization of intracellular Ca^{2+} and mediates platelet shape change, and the $P2T_{AC}$ ($P2Y_{12}$) receptor, which is coupled solely to inhibition of adenylyl cyclase (Daniel et al., 1998; Hechler et al., 1998a; Savi et al., 1998). The sustained aggregation of platelets upon addition of ADP requires the concomitant activa-

ABBREVIATIONS: GPCR, G protein-coupled receptor; 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; 2MeSAMP, 2-methylthio-AMP; HPLC, high-performance liquid chromatography.

tion of both the $P2Y_1$ and $P2Y_{12}$ receptors, which initiates signaling pathways that ultimately trigger the activation of glycoprotein IIb/IIIa by an unknown mechanism (Fig. 1). The activation of glycoprotein IIb/IIIa promotes high-affinity binding to fibrinogen and platelet aggregation. In addition to these two P2Y receptors, a potential role of $P2X_1$ receptors in platelet aggregation also has been suggested (Daniel et al., 1998), although definitive evidence for its participation is lacking.

Because of the therapeutic potential of drugs targeted to the platelet $P2Y_{12}$ receptor for the treatment of thromboembolisms and other clotting disorders, the cloning of this receptor has been an intensive area of research for many years (Boeynaems et al., 2000). These efforts proved futile until the last 6 months, when three laboratories independently identified the elusive platelet ADP receptor coupled to the inhibition of adenylyl cyclase, which was named the $P2Y_{12}$ receptor by Hollopeter et al. (2001). The reports describing the cloning of this receptor used either ligand screening methods with cells expressing an orphan GPCR (Takasaki et al., 2001; Zhang et al., 2001) or an expression cloning technique (Hollopeter et al., 2001). The cloned receptor is 1) activated with a nucleotide selectivity consistent with the $P2T_{AC}$ (2 MeSADP>ADP), 2) inhibited by antagonists selective for the $P2T_{AC}$ receptor, 3) coupled solely to inhibition of adenylyl cyclase, and 4) most highly expressed in platelets. Taken together, these three studies demonstrate conclusively that the cloned receptor is identical to the platelet $P2T_{AC}$ receptor. The conclusion that the cloned receptor is identical to the $P2T_{AC}$ receptor is further supported by studies with patients who have a mild bleeding disorder caused by the inability of their platelets to aggregate in response to ADP. Coincidentally, ADP is unable to promote the inhibition of adenylyl cyclase in these platelets, suggesting that they lack $P2T_{AC}$ receptors (Nurden et al., 1995). This idea was confirmed by Hollopeter et al. (2001), who demonstrated that one of these patients contains a 2-base-pair deletion in one of the $P2Y_{12}$ alleles that results in the loss of platelet P2Y12 receptor mRNA transcripts. Finally, Foster et al. (2001) report direct evidence that SP1999 (Zhang et al., 2001) is the P2Y12 receptor. Platelets from a SP1999 (P2Y12)-knockout mouse show impaired aggregation in response to ADP, a loss of ADP-promoted inhibition of adenylyl cyclase, and a lack of response to the thienopyridine antithrombotic drug clopidogrel.

The article by Takasaki et al. (2001) describes a radioligand binding assay with [^{3}H]2MeSADP. The ^{33}P-labeled congener of this radionucleotide was originally described as a radioligand for the platelet P2T receptor (the idea that ADP acted through two receptors was not known at the time) by Macfarlane et al. (1983) and has been utilized with increasing frequently to quantify levels of $P2Y_1$ and $P2Y_{12}$ receptors in platelets (Gachet et al., 1995; Cattaneo et al., 1997). However, the study by Takasaki et al. (2001) is one of the first studies to demonstrate the appearance of new binding sites in cells upon transfection of each individual receptor into a null cell line [see also Savi et al. (2001)]. The utility of radioligand binding assays to quantify P2Y receptors is controversial. A spurious radioligand binding assay with 3′-deoxyadenosine 5′-[α-^{35}S]thiotriphosphate was used to erro-

TABLE 1
Pharmacological properties and signaling mechanisms of P2Y receptors
The nonselective P2 antagonists suramin and reactive blue 2 inhibit most of the PLC-coupled P2Y receptors. Nonmammalian P2Y receptors are shown in lower case (e.g., p2y3).

P2Y Receptors	Nucleotide Selectivity	Partial Agonists	Antagonists	G Protein Coupling	Effector
Human					
$P2Y_1$	2MeSADP>ADP>2MeSATP	ATP	A3P5P A2P5P A3P5PS MRS2179 PPADS	Gq	↑ PLC
$P2Y_2$	ATP≈UTP ADP, UDP Inactive			Gq, Gi[a]	↑ PLC
$P2Y_4$	UTP≫GTP≈ITP[b] UDP, ADP inactive		ATP	Gq	↑ PLC
$P2Y_6$	UDP≫UTP≫ADP ATP inactive			Gq, Gi[a]	↑ PLC
$P2Y_{11}$	2MeSATP≈ATP[c]	ADP		Gq, Gs	↑ PLC, ↑ AC
$P2Y_{12}$	2MeSADP>ADP	ATP?	ARLC66096 AR-C69931MX Clopidogrel[d] Ticlopidine[d] 2MeSAMP	Gi	↓ AC
Nonmammalian		Mammalian Homologue?			
p2y3 Chick	UDP≫ADP≈UTP≫ATP	$P2Y_6$		Gq	↑ PLC
p2y8 Frog	UTP≈ATP≈ITP≈GTP≈CTP	$P2Y_4$?		Gq?	↑ PLC
tp2y Turkey	UTP≈ATP≈ITP≈GTP≈CTP	$P2Y_4$?		Gq, Gi	↑ PLC, ↓ AC

PLC, phospholipase C; A3P5P, adenosine 3′,5′-diphosphate; A2P5P, adenosine 2′,3′-diphosphate; A3P5PS, adenosine 3′-phosphate 5′-phosphosulfate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
[a] Coupling to Gi is inferred from the pertussis-toxin sensitivity of the PLC response observed in some cells.
[b] The rat $P2Y_4$ receptor homologue is activated by all nucleoside triphosphates, including ATP (Kennedy et al., 2000).
[c] The canine homologue of the $P2Y_{11}$ receptor is activated most potently by nucleoside diphosphates (Qi et al., 2001).
[d] These compounds are inactive in vitro, but irreversibly inhibit the $P2Y_{12}$ receptor after activation by hepatic metabolism (Savi et al., 2000).

neously identify p2y5 and p2y7 receptors as P2Y receptors (Akbar et al., 1996; Webb et al., 1996c) and sparked considerable debate in the literature (Motte et al., 1996; Schachter and Harden, 1997). However, Takasaki et al. (2001) provide compelling data that [^{3}H]2 MeSADP is a useful radioligand for binding to $P2Y_1$ and $P2Y_{12}$ receptors expressed in a cultured cell line. They demonstrate the lack of specific binding of [^{3}H]2MeSADP to membranes from nontransfected cells, that specific binding is a high percentage of the total binding, and that the specific binding is inhibited by nucleotides consistent with the known selectivities of the two receptors. Recently, ^{33}P-labeled MRS2179, a $P2Y_1$-selective antagonist, was also shown to label $P2Y_1$ receptors in platelets and in 1321N1 human astrocytoma cells expressing the $P2Y_1$ receptor (Baurand et al., 2001). It should be noted that the [^{3}H]2MeSADP binding assay cannot be extended to other human P2Y receptors, because 2MeSADP is not an agonist (or antagonist) at these receptors. In addition, whether either of these two binding assays can be extended to more complex tissues (e.g., brain) has yet to be proven.

One of the more peculiar properties of platelets is that ATP blocks ADP-promoted aggregation, presumably by antagonizing either the $P2Y_1$ or $P2Y_{12}$ receptor (or both). There has been considerable debate and investigation on the activity of ATP and its analogs at the $P2Y_1$ receptor. The avian $P2Y_1$ receptor was originally described as an ATP receptor based on heterologous expression studies quantifying inositol phosphate accumulation in 1321N1 cells (Filtz et al., 1994) or Ca^{2+}-activated Cl^- currents in *X. laevis* oocytes (Webb et al., 1993), although later studies with the bovine, human, and turkey $P2Y_1$ receptor homologues reported that 2MeSADP and ADP were more potent agonists than ATP (Henderson et al., 1995; Schachter et al., 1996). However, the notion of whether the $P2Y_1$ receptor was activated by ATP and other triphosphate nucleotides at all was challenged by Leon et al. (1997), who demonstrated in a careful study that ATP and 2MeSATP were weak antagonists at the $P2Y_1$ receptor expressed in human Jurkat cells, provided that the nucleotides were purified from contaminating diphosphates by HPLC before addition to cells. A subsequent study by Palmer et al. (1998) extended these findings by showing that HPLC-purified ATP is a partial agonist at the $P2Y_1$ receptor, and that the response to ATP depends on the level of receptor reserve. Thus, at high levels of receptor reserve, such as in human embryonic kidney 293 and 1321N1-$P2Y_1$ cells, HPLC-purified ATP acts as an agonist, whereas in cells with low levels of reserve, such as platelets and Jurkat-$P2Y_1$ cells, ATP antagonizes the response to ADP. A similar scenario for the $P2Y_{12}$ receptor may also emerge, although ATP would need to antagonize only one receptor (i.e., the $P2Y_1$ receptor) to block ADP-promoted platelet aggregation.

The expression of P2Y receptors coupled to the inhibition of adenylyl cyclase activity in cells other than platelets has been known for some time. ADP and 2MeSADP were shown to inhibit adenylyl cyclase activity in megakaryoblast-like cell lines such as MEG-01, HEL, and DAMI cells (Vittet et al., 1992; Shi et al., 1995). A receptor with a similar nucleotide selectivity to the $P2Y_{12}$ receptor was shown to be negatively coupled to adenylyl cyclase in C6 glioma cells (Pianet et al., 1989; Boyer et al., 1993), and studies have shown that aden-

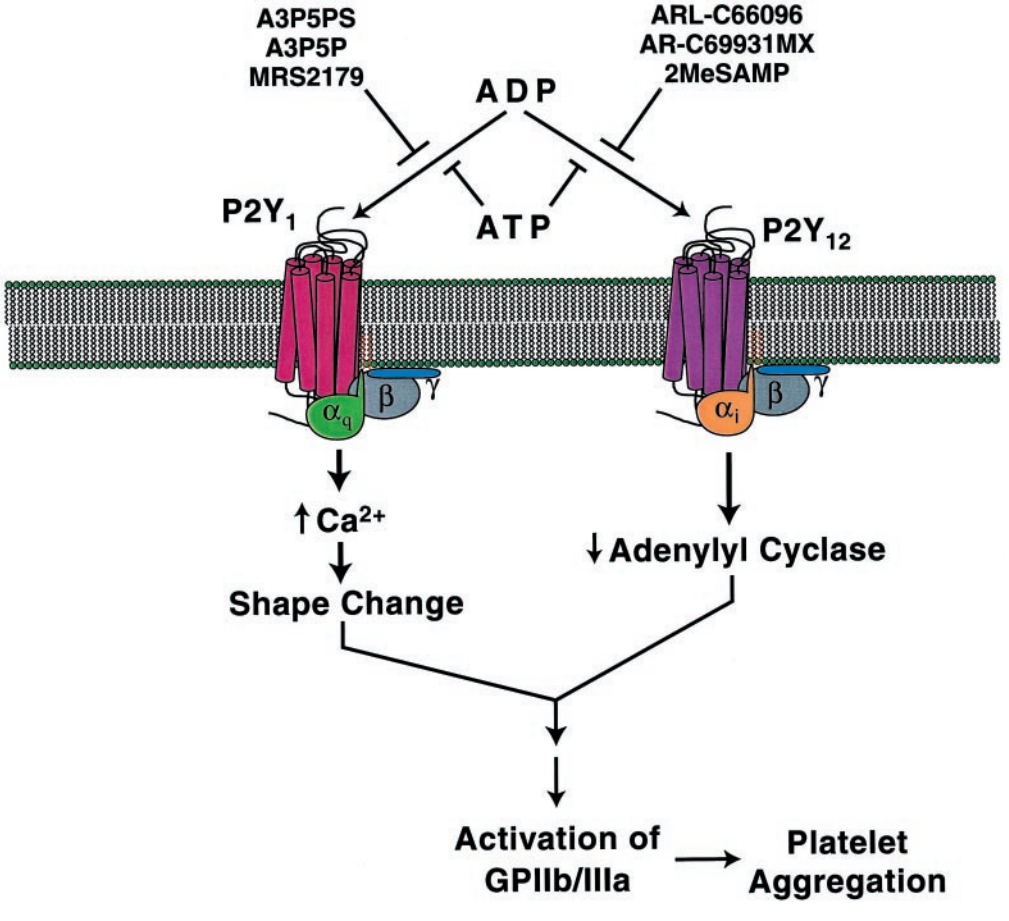

Fig. 1. The role of P2Y receptors in ADP-promoted platelet aggregation. Although originally thought to be caused by the actions of a single receptor, denoted the P2T receptor, it is now known that ADP promotes platelet aggregation through two distinct P2Y receptors: the $P2Y_1$ receptor and the recently identified $P2Y_{12}$ ($P2T_{AC}$) receptor. Activation of the $P2Y_1$ receptor alone causes platelet shape change, but no aggregation occurs unless the $P2Y_{12}$ receptor is activated concomitantly. The signal transduction mechanisms leading to activation of fibrinogen receptors (GP IIb/IIIa) have not been fully delineated.

osine nucleotides cause both inositol phosphate accumulation/Ca^{2+} mobilization and inhibition of adenylyl cyclase activity in bovine aortic endothelial cells (Webb et al., 1996a). A recent study by Jin et al. (2001) showed that the P2Y receptor coupled to inhibition of adenylyl cyclase in C6 glioma cells is identical to the P2Y12 receptor. Whether the P2Y receptor coupled to inhibition of adenylyl cyclase in the other cell types is the P2Y12 receptor awaits similar confirmation.

One of the more intriguing aspects to come out of the cloning of the $P2Y_{12}$ receptor is its extremely low sequence identity to other P2Y receptors. The $P2Y_{12}$ receptor shares only 22% sequence identity with the $P2Y_1$ receptor (its closest P2Y relative), which is one of the lowest sequence identities known for GPCRs that recognize a common agonist. The low sequence identity of the $P2Y_{12}$ receptor with other P2Y receptors suggests why this receptor was not cloned earlier by homology screening and reverse transcription-polymerase chain reaction approaches. The lack of sequence identity is even more unusual given the highly similar nucleotide selectivities of $P2Y_1$ and $P2Y_{12}$ receptors: both receptors are adenine nucleotide-specific, are activated most potently by ADP and other adenosine diphosphate nucleotides (Leon et al., 1997; Hechler et al., 1998b; Palmer et al., 1998), and show a marked preference for 2-thioether substitution of the adenine ring (Boyer et al., 1993, 1995). Their nucleotide selectivities are so similar that in platelets and in cells expressing both the $P2Y_1$ and $P2Y_{12}$ receptors, it was thought at one time that the response to nucleotides was mediated via a single receptor (Hourani and Hall, 1996; Webb et al., 1996a). With the cloning of the $P2Y_{12}$ receptor, there can be no doubt that these earlier results were caused by the action of two distinct receptors. Although $P2Y_1$ and $P2Y_{12}$ receptors have similar agonist selectivities, several antagonists are now available that absolutely distinguish between these receptors (Table 1). Thus, the binding pockets of the two receptors are more readily distinguished by their antagonist selectivities than by their agonist selectivities.

How does the low sequence identity between the $P2Y_1$ and $P2Y_{12}$ receptor impact our understanding of how these proteins recognize nucleotides? Of all the cloned P2Y receptors, the $P2Y_1$ receptor is the best-studied, and this receptor has the largest available repertoire of selective agonists and antagonists. Mutagenesis studies have identified residues in the $P2Y_1$ receptor necessary for agonist binding and/or activation (Jiang et al., 1997; Hoffmann et al., 1999; Moro et al., 1999). Although these studies are complicated by their reliance on "loss of function" in activity assays, amino acids in the $P2Y_1$ receptor from both putative transmembrane domains (R128, K280, Q307, R310, and S314) and the extracellular loops (D204 and S213) seem critical for high potency agonist activation of phospholipase C. In the $P2Y_{12}$ receptor, only S213, R310, and S314 are conserved, whereas K280 is an arginine, a basic amino acid found in this position in other P2Y receptors. The lack of conservation of the other residues suggests that the $P2Y_{12}$ receptor binds nucleotides in a different orientation than that of the $P2Y_1$ receptor. The cloning of the $P2Y_{12}$ receptor puts a molecular stamp on this receptor and paves the way for future studies to identify new antithrombotic compounds capable of inhibiting this important therapeutic target.

What is the function of the $P2Y_{12}$ receptor in cells other than platelets? Quantitative reverse transcription-polymerase chain reaction and Northern blotting indicate that the only other tissue expressing the $P2Y_{12}$ receptor is brain. Hollopeter et al. (2001) provide in situ hybridization data suggesting that $P2Y_{12}$ receptors are primarily expressed in astrocytes and not neurons. Thus, it will be important to delineate the role of the $P2Y_{12}$ receptor in astrocyte function. Along these lines, a recent article has addressed the role of nucleotides in microglial function (Honda et al., 2001). Both ATP and ADP, but not UTP, induced membrane ruffling and enhanced chemotaxis in cultured microglia, which was inhibited by pertussis toxin treatment or addition of the $P2Y_{12}$-selective antagonist AR-C69931 MX. Elimination of extracellular Ca^{2+} or treatment with suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, or adenosine 3′-phosphate 5′-phosphosulfate had no effect. These results suggest that membrane ruffling and chemotaxis in microglial cells are mediated by the $P2Y_{12}$ receptor. Future studies with astrocytes should shed light on the role of the $P2Y_{12}$ receptor in these cells.

Finally, the cloning of the $P2Y_{12}$ receptor invites the question of whether more P2Y receptors remain to be identified. A BLAST search of GenBank identifies the UDP-glucose receptor as its closest relative (~40% identity), but it also identifies several still-orphaned GPCRs that share significant sequence identity with the $P2Y_{12}$ receptor. Certainly in the near future a number of laboratories will express these receptors and test their responses to nucleotides, so the list of current P2Y receptors (Table 1) may quickly expand. The field of P2Y receptors exploded in 1993 after the cloning of the $P2Y_1$ receptor and shows no signs of abating. This is clearly an exciting time to be working with this potentially therapeutically important class of signaling molecules.

After submission of this article, two important studies were published relating to the $P2Y_{12}$ receptor. One report showed that the P2Y receptor coupled to inhibition of adenylyl cyclase in C6 glioma cells is identical to the $P2Y_{12}$ receptor (Jin et al., 2001), and the other report showed that SP1999 ($P2Y_{12}$)-deficient mice displayed highly prolonged bleeding times (Foster et al., 2001). The latter study also demonstrated that ADP neither promoted platelet aggregation nor inhibited adenylyl cyclase in platelets derived from these mice.

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Address correspondence to: Dr. Robert A. Nicholas, Dept. of Pharmacology, University of North Carolina, Chapel Hill, CB #7365, Mary Ellen Jones Building, Chapel Hill, NC, 27599-7365. E-mail: nicholas@med.unc.edu