Deoxyadenosine Bisphosphate Derivatives as Potent Antagonists at $P2Y_1$ Receptors

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Adenosine 3′,5′- and 2′,5′-bisphosphates previously were demonstrated to act as competitive antagonists at the $P2Y_1$ receptor (Boyer et al. *Mol. Pharmacol.* **1996**, *50*, 1323−1329). 2′- and 3′-Deoxyadenosine bisphosphate analogues containing various structural modifications at the 2- and 6-positions of the adenine ring, on the ribose moiety, and on the phosphate groups have been synthesized with the goal of developing more potent and selective $P2Y_1$ antagonists. Single-step phosphorylation reactions of adenosine nucleoside precursors were carried out. The activity of each analogue at $P2Y_1$ receptors was determined by measuring its capacity to stimulate phospholipase C in turkey erythrocyte membranes (agonist effect) and to inhibit phospholipase C stimulation elicited by 10 nM 2-MeSATP (antagonist effect). Both 2′- and 3′-deoxy modifications were well tolerated. The N^6-methyl modification both enhanced antagonistic potency (IC_{50} 330 nM) of 2′-deoxyadenosine 3′,5′-bisphosphate by 17-fold and eliminated residual agonist properties observed with the lead compounds. The N^6-ethyl modification provided intermediate potency as an antagonist, while the N^6-propyl group completely abolished both agonist and antagonist properties. 2-Methylthio and 2-chloro analogues were partial agonists of intermediate potency. A 2′-methoxy group provided intermediate potency as an antagonist while enhancing agonist activity. An N^1-methyl analogue was a weak antagonist with no agonist activity. An 8-bromo substitution and replacement of the N^6-amino group with methylthio, chloro, or hydroxy groups greatly reduced the ability to interact with $P2Y_1$ receptors. Benzoylation or dimethylation of the N^6-amino group also abolished or greatly diminished the antagonist activity. In summary, our results further define the structure−activity of adenosine bisphosphates as $P2Y_1$ receptor antagonists and have led to the identification of the most potent antagonist reported to date for this receptor.

Introduction

The concept of "purinergic neurotransmission" was first proposed 25 years ago by Burnstock.[1] ATP now is widely accepted as an extracellular signaling molecule released from nerves and many other tissues.[2] Adenine nucleotides activate both ligand-gated ion channels of the P2X superfamily and G-protein-coupled receptors of the P2Y superfamily.[3] In addition, uridine nucleotides act as agonists at certain subtypes of P2Y receptors. Seven subtypes of P2X receptors and at least four distinct subtypes of P2Y receptors have been cloned.[4]

$P2Y_1$ receptors activate phospholipase C, which consequently results in the generation of inositol phosphates and diacylglycerol from phosphatidyl inositol 4,5-bisphosphate. We have extensively studied this receptor in turkey erythrocyte membranes, and this system has been applied to identify molecules that have agonistic[5,6] or competitive antagonistic[7,8] properties toward $P2Y_1$ receptors.

Progress in the P2 receptor field has been slowed by the lack of availability of receptor subtype-selective antagonists. Thus, the observation by Boyer et al.[8] that adenosine 3′-phosphate 5′-phosphosulfate (**2**, A3P5PS or PAPS, Figure 1), a cofactor for sulfotransferase enzymes,[9] and adenosine 3′,5′-bisphosphate (**1**, A3P5P or PAP, Figure 1), a metabolite of PAPS, were both partial agonists at the $P2Y_1$ receptor in turkey erythrocyte membranes was of considerable interest. Moreover, A3P5PS and A3P5P were shown to be competitive $P2Y_1$ receptor antagonists at both turkey and human $P2Y_1$ receptors with pK_B values in the micromolar range.[8] The isomeric adenosine 2′,5′-bisphosphate (**3**, A2P5P) had similar antagonistic effects.[8] The effects of these bisphosphate analogues were specific for the phospholipase C-coupled $P2Y_1$ receptor, since neither agonist nor antagonist activities were observed at the yet to be cloned adenylyl cyclase-coupled P2Y receptor of C-6 glioma cells or at recombinant $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.[8]

Since the nucleotides A3P5P and A2P5P displayed competitive antagonistic activities at the $P2Y_1$ receptor, and other similar derivatives, when substituted at the 3′-position with various uncharged groups, resulted in full agonism,[8] the presence of a phosphate at either the 2′- or 3′-position appeared to be crucial. To better understand the structure−activity relationships (SAR) of $P2Y_1$ receptor antagonists and with the goal of identifying molecules that bind with higher affinity, we have synthesized and evaluated pharmacologically at $P2Y_1$ receptors several (deoxy)nucleotides bisphosphory-

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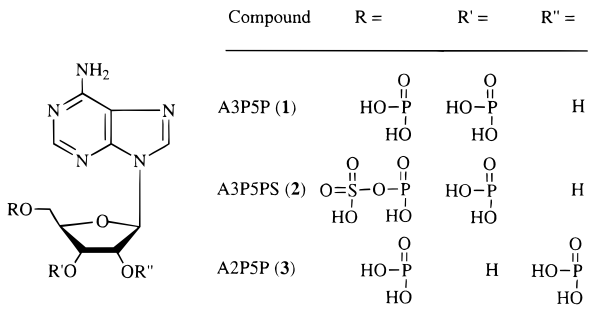
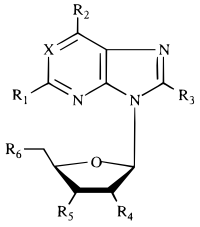


Figure 1. Structures of $P2Y_1$ competitive antagonists (partial agonists) reported by Boyer et al.[8]

Table 1. Chemical Structures of Novel Adenine Nucleotides Synthesized as Potential $P2Y_1$ Receptor Antagonists



no.	X	R_1	R_2	R_3	R_4	R_5	R_6
4	N	H	NH_2	H	H	PO_4H_2	PO_4H_2
5	N^+CH_3	H	NH_2	H	H	PO_4H_2	PO_4H_2
6	N	Cl	NH_2	H	H	PO_4H_2	PO_4H_2
7	N	SCH_3	NH_2	H	H	PO_4H_2	PO_4H_2
8	N	H	NH_2	Br	H	PO_4H_2	PO_4H_2
9	N	H	$NHCH_3$	H	H	PO_4H_2	PO_4H_2
10	N	H	$NHCH_2CH_3$	H	H	PO_4H_2	PO_4H_2
11	N	H	$NH(CH_2)_2CH_3$	H	H	PO_4H_2	PO_4H_2
12	N	H	$NHCOC_6H_6$	H	H	PO_4H_2	PO_4H_2
13	N	H	$N(CH_3)_2$	H	H	PO_4H_2	PO_4H_2
14	N	H	Cl	H	H	PO_4H_2	PO_4H_2
15	N	H	OH	H	H	PO_4H_2	PO_4H_2
16	N	H	SCH_3	H	H	PO_4H_2	PO_4H_2
17	N	H	NH_2	H	PO_4H_2	H	PO_4H_2
18	N	H	$NHCH_3$	H	PO_4H_2	H	PO_4H_2
19	N	H	NH_2	H	PO_4H_2	H	Cl
20	N	H	NH_2	H	OCH_3	PO_4H_2	PO_4H_2
21	N	H	NH_2	H	H	PSO_3H_2	PSO_3H_2

lated at the 5′- and 3′- (or 2′-) positions of the sugar moiety. The removal of an unphosphorylated hydroxyl group from the analogues simplifies the synthesis and interpretation of the SAR. We have found that these hydroxyl groups are not required for recognition of the nucleotide by the $P2Y_1$ receptor.

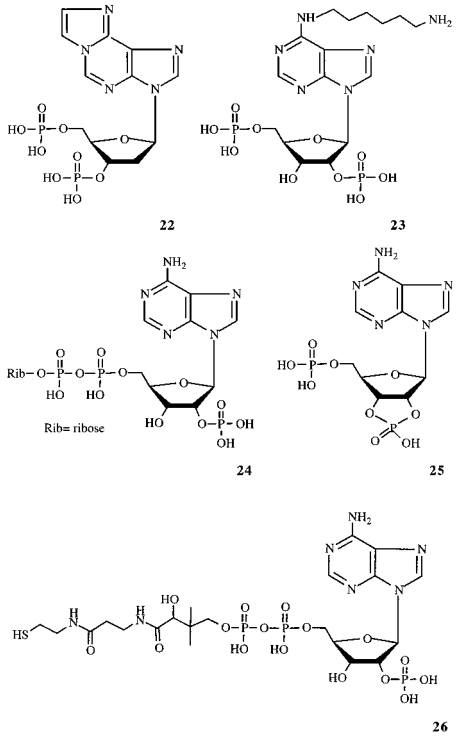
Results and Discussion

Chemical Synthesis. The structures of adenosine and deoxyadenosine nucleotide analogues examined as potential $P2Y_1$ receptor antagonists are shown in Tables 1 and 2. Our synthetic and structural aims were the following: (1) simplify the structure of A3P5P (**1**) to define the minimal requirements for affinity and activity at P2Y receptors; (2) modify in turn different positions of the purine ring system and/or the ribose moiety for enhancement of affinity; (3) replace phosphate groups with thiophosphates to increase the resistance toward metabolic degradation by ectonucleotidases; and (4) screen commercially available bisphosphate analogues (Table 2, compounds **23**−**26**).

For the preparation of novel nucleotide analogues (compounds **4**−**22**), the appropriate nucleosides were either purchased or synthesized and then (thio)phosphorylated. Several such intermediates, **27**−**33**, were prepared in our laboratory by adapting published procedures.[11−15] In particular, 2′-deoxy-2-(methylthio)-adenosine and 2′-deoxy-6-(methylthio)purineriboside, **27** and **28**, were obtained by alkylation of 2-chloro-2′-deoxyadenosine using sodium thiomethoxide.[11] N^6-Ethyl-, N^6-propyl-, and N^6,N^6-dimethyl-2′-deoxyadenosines, **29**−**31**, were synthesized by amination of 6-chloro-2′-deoxypurineriboside with the suitable alkylamine.[11,12] 8-Bromo-2′-deoxyadenosine, **32**, was prepared by direct bromination of 2′-deoxyadenosine using *N*-bromosuccinimide.[13] N^6-Methyl-3′-deoxyadenosine, **33**, was synthesized through a multistep sequence of reactions.[14] Specifically, starting from 3′-deoxyadenosine, the deoxyribose moiety was first protected as the 3′,5′-diacetyl ester, and then the purine exocyclic amine was transformed to iodide[15] by a radical reaction using isopentyl nitrite in diiodomethane. Finally combined deprotection and amination in the presence of aqueous methylamine at room temperature gave the desired compound **33**.

The phosphorylation of 2′- and 3′-deoxyadenosine and their analogues was achieved with a single-step reaction (Scheme 1) using phosphorus oxychloride (or in the case of compound **21** thiophosphoryl chloride), trimethyl phosphate, and Proton Sponge (Aldrich) in an ice bath.

Table 2. Miscellaneous Bisphosphate Analogues Evaluated as Antagonists at $P2Y_1$ Receptors



Scheme 1

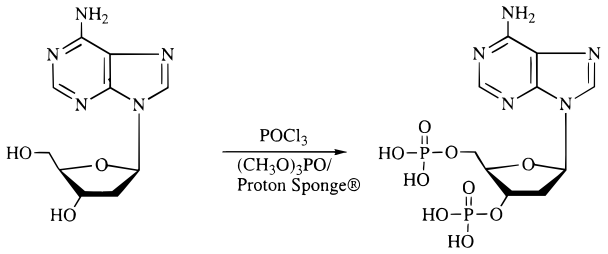


Table 3. Synthetic Data for Nucleotide Derivatives, Including Structural Verification Using High-Resolution Mass Spectroscopy and Purity Verification Using HPLC

		FAB (M − H$^+$)		HPLC (t_R, min)[a]		
no.	formula	calcd	found	system A	system B	yield (%)[b]
4	$C_{10}H_{15}O_9N_5P_2$	410.0267	410.0249	5.98	11.51	44
5	$C_{11}H_{17}O_9N_5P_2$	424.0423	424.0428	3.66	6.42	69
6	$C_{10}H_{14}ClO_9N_5P_2$	443.9877	443.9872	7.20	12.14	58
7	$C_{11}H_{17}O_9N_5P_2S$	456.0144	456.0122	8.14	12.98	48
8	$C_{10}H_{14}BrO_9N_5P_2$	487.9372	487.9355	6.66	12.06	33
9	$C_{11}H_{17}O_9N_5P_2$	424.0423	424.0404	6.93	12.32	41
10	$C_{12}H_{19}O_9N_5P_2$	438.0580	438.0580	7.95	12.83	40
11	$C_{13}H_{21}O_9N_5P_2$	452.0736	452.0734	8.97	13.55	41
12	$C_{17}H_{19}O_{10}N_5P_2$	514.0529	514.0538	9.58	13.67	54
13	$C_{12}H_{19}O_9N_5P_2$	438.0580	438.0582	8.30	12.72	46
14	$C_{10}H_{13}ClO_9N_4P_2$	428.9768	428.9752	6.87	12.54	65
15	$C_{10}H_{14}O_{10}N_5P_2$	411.0107	411.0102	4.58	11.64	22
16	$C_{11}H_{16}O_9N_4P_2S$	441.0035	441.0026	8.48	13.05	39
17	$C_{10}H_{15}O_9N_5P_2$	410.0267	410.0262	5.80	11.51	34
18	$C_{11}H_{17}O_9N_5P_2$	424.0423	424.0437	8.95	12.16	21
19	$C_{10}H_{13}ClO_5N_5P$	348.0265	348.0272	6.89	9.82	22
20	$C_{11}H_{17}O_{10}N_5P_2$	440.0372	440.3660	6.40	12.20	17
21	$C_{10}H_{15}O_7N_5P_2S_2$	441.9810	441.9794	6.60	10.10	18
22	$C_{12}H_{15}O_9N_5P_2$	434.0267	434.0257	7.02	12.33	42

[a] Purity of each derivative was ≥95%, as determined using HPLC with two different mobile phases. System A: gradient of 0.1 M TEAA/CH_3CN from 95/5 to 40/60. System B: gradient of 5 mM TBAP/CH_3CN from 80/20 to 40/60. [b] The percent yield refers to phosphorylation reactions.

The reaction typically was completed after 1 h and quenched by addition of buffer (triethylammoium bicarbonate), and finally the mixture was lyophilized. Purification was performed using a Sephadex ion-exchange column with a linear gradient of water/ammonium bicarbonate (0.01−0.5 M) providing the bisphosphorylated compounds as ammonium salts. The chemical structures of the phosphorylated nucleosides (structures in Tables 1 and 2) were verified using ^{1}H NMR and ^{31}P NMR techniques as well as by high-resolution mass spectroscopy (Table 3). By ^{1}H NMR it was possible to monitor the chemical shift of ribose protons at the 5′- and 2′- (or 3′-) positions and to distinguish them before and after phosphorylation. The presence of bisphosphorylation was indicated by two phosphate signals in the ^{31}P NMR spectra. The assignment of the two peaks (one pseudotriplet and one doublet) was according to the spectra of commercially available A3P5P, 2′-deoxyadenosine 3′-monophosphate (dA3P), and 2′-deoxyadenosine 5′-monophosphate (dA5P) taken as reference compounds. The possibility of N^6-phosphorylation as reported in the literature for NH_2-unprotected nucleosides[10] was also excluded by the ^{31}P NMR spectra. All synthesized compounds gave correct molecular masses (high-resolution FAB, Table 3) and showed more than 95% purity (HPLC). A few of the compounds prepared in this study had been synthesized previously, such as 2′-deoxyadenosine 3′,5′-bisphosphate (**4**)[16] and 3′-deoxyadenosine 2′,5′-bisphosphate (**17**).[17]

Biological Activity

Turkey erythrocytes have been shown previously to express a P2 receptor that markedly stimulates inositol lipid hydrolysis through activation of phospholipase C,[18,19] and this receptor has been positively identified as a P2Y$_1$ receptor.[20] 2-MeSATP has a high potency for stimulation of inositol phosphate accumulation in membranes isolated from [^{3}H]inositol-labeled turkey erythrocytes. The analogues α,β-MeATP and β,γ-MeATP, which are potent as P2X receptor agonists, show little or no effect at the turkey erythrocyte P2Y$_1$ receptor.

The nucleotide analogues prepared in the present study were all tested for agonist and antagonist activity in the PLC assay at the P2Y$_1$ receptor in turkey erythrocyte membranes, and the results are reported in Table 4. Concentration−response curves were obtained for each compound alone and in combination with a given concentration of 2-MeSATP (10 nM). Essentially no basal inositol phosphate activity was observed, and a very small stimulation occurred in the presence of 1 μM GTPγS (not shown). Addition of 10 nM 2-MeSATP resulted in a marked and concentration-dependent activation of the turkey erythrocyte phospholipase C.[18,19] As previously reported,[8] addition of a fixed concentration of either A3P5P or A2P5P shifted the agonist concentration−response curve for 2-MeSATP to the right, as is typically observed with antagonist molecules. The bisphosphates **1** and **3** caused a slight increase in phospholipase C activity in the absence of 2-MeSATP. Thus, as previously reported these two analogues are partial agonists (i.e. having both agonist and antagonist properties) at the turkey P2Y$_1$ receptor.

The removal of the unphosphorylated hydroxyl group from **1** and **3** resulted in compounds **4** and **17**, respectively. These compounds were similar to their parent hydroxylated compounds in biological activity, i.e. they were both partial agonists with slightly less potency as agonists (5−6-fold) at P2Y$_1$ receptors (Figure 2) but were equipotent as antagonists. The 3′,5′-bisphosphate analogue, **4**, was 2-fold more potent as an antagonist compared to the 2′,5′ isomer, **17**. Thus, both 2′- and 3′-deoxy modifications were tolerated in P2Y$_1$ receptor antagonists, and the potencies of each were approximately equal to the corresponding hydroxy analogue.

Modifications of these two lead structures were then studied. Structural modifications were made at the adenine ring at the 2- (**6** and **7**), 8- (**8**), and 6- (**9**−**16** and **18**) positions, on the ribose moiety (**19**, **20**), and on the phosphate groups (**21**). Additional modifications of the purine moiety such as etheno (**22**) and N^1-methyl (**5**) were made, and several commercially available bisphosphates such as the N^6-aminohexyl derivative, **23**, adenosine 2′-monophosphate 5′-diphosphoribose, **24**, adenosine 2′,3′-cyclic monophosphate 5′-monophosphate, **25**, and coenzyme A, **26**, were included in this study.

Substitutions at the 2-position of adenine nucleotides are known to be tolerated and in some cases favored for P2Y receptor agonists.[5] The 2-chloro, **6**, and 2-methylthio, **7**, modifications of compound **4** were nearly 3-fold more potent as P2Y$_1$ antagonists. In both cases the residual agonist stimulation of PLC, as observed for compounds **1**−**4** at the P2Y$_1$ receptor, was still present. The potency of compounds **6** and **7** in activating the P2Y$_1$ receptor was also enhanced by approximately 10-fold vs compound **4**. The 8-bromo-modified analogue, **8**, was 6.4-fold less potent than **4** as an antagonist at the P2Y$_1$ receptor, and agonist activity was abolished. An N^1-methyl analogue, **5**, was a weak antagonist (9.5-fold loss of potency vs **4**) with no agonist activity.

Alkylation of the exocyclic amine had highly consequential effects on biological activity. N^6-Me-2′-deoxy-

Table 4. In Vitro Pharmacological Data for Stimulation of PLC at Turkey Erythrocyte $P2Y_1$ Receptors (Agonist Effect) and the Inhibition of PLC Stimulation Elicited by 10 nM 2-MeSATP (Antagonist Effect) for at Least Three Separate Determinations

compound	agonist effect (% of maximal increase)[a]	EC_{50} (μM)[a]	antagonist effect (% of maximal inhibition)[b]	IC_{50} (μM)[b]
1 (A3P5P)	21 ± 5	1.28 ± 0.28	77 ± 6	4.19 ± 1.22
3 (A2P5P)	25 ± 8	1.37 ± 0.31	75 ± 8	8.46 ± 0.46
4	12 ± 3	6.26 ± 2.52	87 ± 4	5.76 ± 0.68
5	NE		100	54.9 ± 20.0
6	19 ± 3	0.651 ± 0.160	80 ± 3	2.01 ± 0.83
7	22 ± 2	0.550 ± 0.117	77 ± 2	2.11 ± 0.95
8	NE		100	36.7 ± 8.6
9 (MRS 2179)	NE		99 ± 1	0.330 ± 0.059
10	NE		100	1.08 ± 0.38
11	NE		small inhibn at 30−100 μM	
12	NE		NE	
13	NE		70 ± 2	46.7 ± 20.8
14	small or NE		small inhibn or NE	
15	NE		small inhibn at 30−100 μM	
16	NE		78 ± 1	29.1 ± 4.1
17	16 ± 6	8.33 ± 2.22	81 ± 6	11.0 ± 4.8
18	13 ± 2	NC	90 ± 1	0.324 ± 0.054
19	NE		NE	
20	35 ± 7	12.9 ± 3.4	65 ± 7	12.4 ± 2.9
21	9 ± 3	NC	91 ± 3	88.0 ± 30.3
22	NE		NE	
23	NE		NE	
24	~50	≥100	~50	≥100
25	43	40.9	73 ± 5	12.7 ± 3.2
26	43 ± 3	≥100	72 ± 6	73.0 ± 15.0

[a] Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect was achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of 2-MeSATP in the same experiment. [b] Antagonist IC_{50} values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 10 nM 2-MeSATP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration. NE, no effect. NC, not calculated.

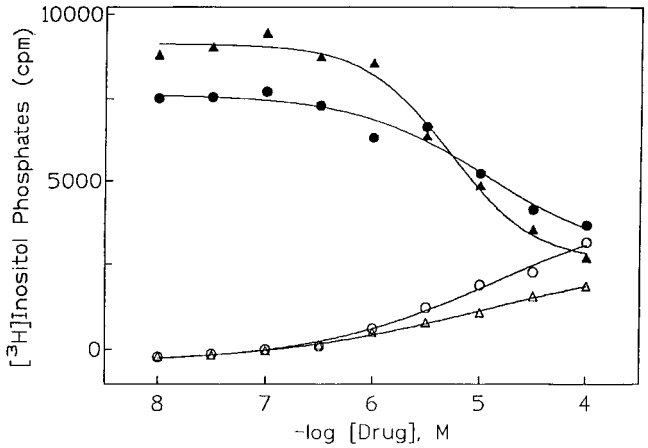


Figure 2. Effects of deoxyadenosine bisphosphate derivatives on phospholipase C in turkey erythrocyte membranes. Both concentration-dependent stimulation of inositol phosphate formation and its inhibition by compounds **4** (triangles) and **17** (circles) were observed. Membranes from [^{3}H]inositol-labeled erythrocytes were incubated for 5 min at 30 °C in the presence of the indicated concentrations of **4** and **17**, either alone (open symbols) or in combination (solid symbols) with 10 nM 2-MeSATP. The data shown are typical curves for at least three experiments carried out in duplicate using different membrane preparations.

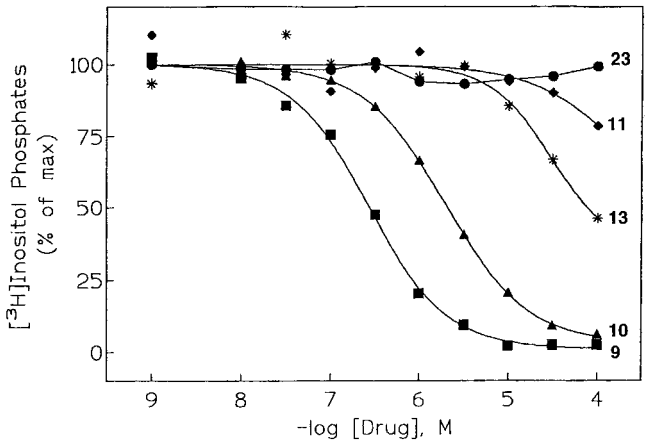


Figure 3. Effects of N^6-alkyl analogues of adenosine and 2′-deoxyadenosine bisphosphate derivatives on agonist-stimulated phospholipase C in turkey erythrocyte membranes. Membranes from [^{3}H]inositol-labeled erythrocytes were incubated for 5 min at 30 °C in the presence of 10 nM 2-MeSATP and the indicated concentrations of compounds **9**−**11**, **13**, and **23**. The data shown are typical curves for at least three experiments carried out in duplicate using different membrane preparations. Key: N^6-methyl analogue, **9** (squares); N^6-ethyl analogue, **10** (triangles); N^6-propyl analogue, **11** (diamonds); N^6-dimethyl analogue, **13** (asterisks); N^6-aminohexyl analogue, **23** (circles).

adenosine 3′,5′-bisphosphate (MRS 2179), **9**, was considerably more potent (IC_{50} 330 nM) as an antagonist than either dA3P5P (17-fold), **4**, or A3P5P (13-fold), **1** (Figure 3A). This analogue displayed only antagonistic properties, since in the absence of 2-MeSATP it did not activate PLC at a concentration as high as 100 μM. A dramatic dependence of the antagonist potency on the size of the N^6-substituent was observed (Figure 3). The methyl analogue, **9**, was 3-fold more potent as an antagonist than the ethyl analogue, **10**. In compound **11** the N^6-propyl group completely abolished both agonist and antagonist properties. Similarly, the longer chain terminating in an amino group in compound **23** (containing also 3′-hydroxyl and thus an analogue of compound **3**) completely abolished interaction with the

$P2Y_1$ receptor. Compound **18**, the 3′-deoxy isomer of **9**, also acted as an antagonist at the turkey $P2Y_1$ receptor and was approximately equipotent to **9**. Compound **18** showed a small activation of the receptor at high concentration. The N^6-benzoyl modification, **12**, completely abolished the ability of the compound to interact with $P2Y_1$ receptors as either an agonist or an antagonist.

Double alkylation of the N^6-amino group in compound **13** resulted in a ≥300-fold loss of potency as an antagonist vs **9**, and no agonist activity was observed. 6-Chloro and 6-hydroxy analogues, **14** and **15**, respectively, were essentially inactive at $P2Y_1$ receptors. The 6-methylthio analogue, **16**, was only a very weak antagonist at $P2Y_1$ receptors.

Replacement of the 5′-phosphate ester with chloro, as in compound **19**, abolished antagonist and agonist activity at $P2Y_1$ receptors. The presence of a 2′-methoxy group on the ribose moiety, **20**, decreased potency by 2.2-fold vs that of **4**, although a restoration of the agonist effect to a very substantial fraction (33% of maximal) was achieved.

Replacement of both phosphate groups with thiophosphate, **21**, diminished potency as a $P2Y_1$ receptor antagonist by 15-fold. The etheno derivative **22** was inactive at the $P2Y_1$ receptor. Cyclization of **1** and **3** to a 2′,3′-cyclic phosphate, in compound **25**, decreased the potency of interaction with the $P2Y_1$ receptor as either agonist (∼30-fold) or antagonist (2−3-fold). Compound **24** and coenzyme A, **26**, both acted as a mixed agonist/antagonist at the $P2Y_1$ receptor, but were very weak in both activities.

Since it is known that 5′-AMP binds weakly to adenosine receptors,[26] selected bisphosphates were examined in binding to the A_1 receptor in rat brain membranes. The lead compound **1** displayed a K_i value of 25.5 ± 5.4 μM vs [^{3}H]-N^6-phenylisopropyladenosine. The 2′-deoxy modification of the N^6-Me analogue, **9**, reduced the affinity at the A_1 receptor, with a K_i value of 94.2 ± 19.8 μM. At rat A_{2A} receptors, compound **9** did not significantly displace the radioligand. Thus, **9** is highly selective in binding to the $P2Y_1$ receptor vs the A_1 adenosine receptor. Compounds **6** and **7** displayed K_i values of 7.9 ± 1.4 and 14.1 ± 1.8 μM, respectively, at rat A_1 receptors.

Conclusions

These results describe competitive antagonism of the $P2Y_1$ receptor by a family of adenine nucleotide derivatives. The most important findings are (1) 2′- and 3′-deoxy modifications are well tolerated and (2) the N^6-methyl modification leads to both enhanced potency and loss of the residual agonist stimulation of the $P2Y_1$ receptor observed for compounds **1**−**4**. Large alkyl modifications at this position or dialkyl substitution[22] are not tolerated for either agonists or antagonists. The steric requirements at this position are extremely restrictive, since *N*-ethyl but not *N*-propyl groups are tolerated. Thus, there appears to be a small and highly sterically restricted hydrophobic pocket at the N^6 binding region of the $P2Y_1$ receptor. It is likely that the exocyclic NH of the antagonists also serves as a hydrogen bond donor to the receptor, as has been proposed for agonists binding to both $P2Y_1$[22] and adenosine receptors.

In general, the structure−activity relationships for adenosine bisphosphates at $P2Y_1$ receptors parallel but are not identical with those for adenine nucleotides as agonists at the same receptor subtype. The N^6-methyl group is well tolerated for agonists[5] at this subtype and in fact has led to $P2Y_1$ receptor selectivity over the P2X receptor.[5] As for agonists,[5] substitution at the C-8 position[21] decreases potency of antagonists, and modifications at the 2-position of the adenine moiety are allowed for antagonists. Specifically, 2-(methylthio)-2′-deoxy and 2-chloro-2′-deoxy analogues were partial agonists of intermediate potency. However, the 2-methylthio group only slightly enhanced antagonist potency, while for agonists (i.e., ATP derivatives[5]) 2-alkyl thioethers enhance potency by approximately 3 orders of magnitude.

On the ribose moiety, the free hydroxyl groups of **1** and **3** are not required for recognition by the $P2Y_1$ receptor, while the 5′-phosphate group is critical. 2′- and 3′-deoxy modifications appear to be completely tolerated in the case of antagonists, while agonists 2′- and 3′-deoxyATP were less potent (6.9- and 27-fold, respectively) than ATP at turkey erythrocyte $P2Y_1$ receptors.[21] Equivalent modifications of A3P5P and A2P5P are very similar in potency, for example, compounds **9** and **18**. The 2′- and 3′-regions on the bisphosphates are particularly associated with promotion of receptor activation. For example, the 2′-methoxy, **20**, and 2′,3′-cyclic phosphate, **25**, derivatives produced a considerably larger percent activation of the receptor than **1**.

The affinity of the most potent antagonist having no agonist properties in the present study, the N^6-methyl analogue **9**, compared favorably with other known P2Y receptor antagonists.[23] At the turkey erythrocyte $P2Y_1$ receptor, pyridoxal-5′-phosphate−6-azophenyl-2,4-disulfonate (PPADS)[24] was a competitive antagonist with a pK_B value of 5.9. Suramin and reactive blue 2 were found to inhibit the PLC effect mediated by the turkey erythrocyte $P2Y_1$ receptor in the high micromolar range. However, the antagonism was not clearly competitive. The likely selectivity of compound **9** for $P2Y_1$ receptors, as predicted from the selectivity of the lead compound A3P5P[8] and not present for other classes of P2 antagonists,[23] suggest that this compound and its congeners are potentially useful pharmacological probes for the study of the $P2Y_1$ receptor. Compound **9** is not of sufficiently high affinity for use as a radioligand in a receptor-binding assay, although it is approaching the required range. Further modifications will be carried out in an effort to achieve this goal, as well as to ensure selectivity for P2 receptors and to enhance stability and bioavailablilty for pharmacological studies.

Experimental Section

Chemical Synthesis. Nucleosides and reagents used for the synthesis were purchased from Sigma (St. Louis, MO) and Aldrich (St. Louis, MO). Other nucleosides, **27**−**33**, were synthesized in our laboratory as mentioned above. Several nucleotides, **23**−**26**, (bisphosphate analogues) were purchased from Sigma (St. Louis, MO).

^{1}H NMR spectra were obtained with a Varian Gemini-300 spectrometer using D_2O as a solvent. ^{31}P NMR spectra were recorded at room temperature by use of Varian XL-300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard.

Purity of compounds was checked by a Hewlett-Packard 1090 HPLC apparatus using SMT OD-5-60 RP-C18 (250 × 4.6 mm; Separation Methods Technologies, Inc., Newark, DE) as

an analytical column in two-solvent systems. System A: linear gradient solvent system: 0.1 M TEAA/CH_3CN from 95/5 to 40/60 in 20 min, and the flow rate was 1 mL/min. System B: linear gradient solvent system: 5 mM TBAP/CH_3CN from 80:20 to 40/60 in 20 min, and the flow rate was 1 mL/min. Peaks were detected by UV absorption using a diode array detector. All derivatives showed more than 95% purity in the HPLC system.

High-resolution FAB (fast atom bombardment) mass spectrometry was performed with a JEOL SX102 spectrometer using 6-kV Xe atoms following desorbtion from a glycerol matrix.

Purification of nucleotides was carried out on DEAE-A25 Sephadex columns as described above.

General Procedure of Phosphorylation. Nucleoside (0.1 mmol) and Proton Sponge (107 mg, 0.5 mmol) were dried for several hours in high vacuum at room temperature and then suspended in 2 mL of trimethyl phosphate. Phosphorus oxychloride (37 μL, 0.4 mmol) was added, and the mixture was stirred for 1 h at 0 °C. The reaction was monitored by analytical HPLC (eluting with gradient from buffer:CH_3CN = 95:5 to 40:60; buffer, 0.1 M triethylammonium acetate (TEAA); elution time, 20 min; flow rate, 1 mL/min; column, SMT OD-5-60 RP-C18; detector, UV, $E_{max} \approx 260-300$ nm). The reaction was quenched by adding 2 mL of triethylammonium bicarbonate buffer and 3 mL of water. The mixture was subsequently frozen and lyophilized. Purification was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, linear gradient (0.01−0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution. All nucleotides (**4**−**23**) were collected, frozen, and lyophilized as the ammonium salts.

2′-Deoxyadenosine 3′,5′-Bis(diammonium phosphate) (4). Starting from 25 mg (0.099 mmol) of 2′-deoxyadenosine and following the general procedure, we obtained 21 mg (0.044 mmol, 44% yield) of the desired compound **4**: ^{1}H NMR (D_2O) ∂ 2.79 (2H, m, CH_2-2′), 4.09 (2H, m, CH_2-5′), 4.47 (1H, bs, H4′), 5.02 (1H, m, H3′), 6.54 (1H, t, J = 6.9 Hz, H1′), 8.25 (1H, s, H2), 8.48 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.13 (d, J = 18.3 Hz, P-3′), 0.63 (pt, P-5′).

2′-Deoxy-N^1-methyladenosine 3′,5′-Bis(ammonium phosphate) (5). Starting from 10 mg (0.025 mmol) of 2′-deoxy-N^1-methyladenosine and following the general procedure we obtained 8.2 mg (0.017 mmol, 69% yield) of the desired compound **5**: ^{1}H NMR (D_2O) ∂ 2.78 and 2.94 (2H, 2m, CH_2-2′), 3.92 (3H, s, N^1-CH_3), 4.08 (2H, bs, CH_2-5′), 4.46 (1H, bs, H4′), 5.01 (1H, m, H3′), 6.59 (1H, t, J = 6.8 Hz, H1′), 8.52 (1H, s, H2), 8.64 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 0.69 (bs, P-3′), 1.18 (bs, P-5′).

2-Chloro-2′-deoxyadenosine 3′,5′-Bis(diammonium phosphate) (6). Starting from 25 mg (0.088 mmol) of 2-chloro-2′-deoxyadenosine and following the general procedure, we obtained 26 mg (0.051 mmol, 58% yield) of the desired compound **6**: ^{1}H NMR (D_2O) ∂ 2.77 (2H, m, CH_2-2′), 4.04 (2H, m, CH_2-5′), 4.42 (1H, bs, H4′), 4.94 (1H, m, H3′), 6.41 (1H, t, J = 6.9 Hz, H1′), 8.45 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 1.44 (d, J = 18.5 Hz, P-3′), 1.82 (pt, P-5′).

2′-Deoxy-2-(methylthio)adenosine 3′,5′-Bis(diammonium phosphate) (7). Starting from 20 mg (0.067 mmol) of 2′-deoxy-2-(methylthio)adenosine, **27**, and following the general procedure, we obtained 17 mg (0.032 mmol, 48% yield) of the desired compound **7**: ^{1}H NMR (D_2O) ∂ 2.51 (3H, s, SCH_3), 2.75 and 2.90 (2H, 2m, CH_2-2′), 4.07 (2H, m, CH_2-5′), 4.42 (1H, bs, H4′), 5.00 (1H, m, H3′), 6.44 (1H, t, J = 6.8 Hz, H1′), 8.27 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.21 (d, J = 17.3 Hz, P-3′), 0.55 (pt, P-5′).

8-Bromo-2′-deoxyadenosine 3′,5′-Bis(diammonium phosphate) (8). Starting from 25 mg (0.076 mmol) of 8-bromo-2′-deoxyadenosine, **32**, and following the general procedure, we obtained 14 mg (0.025 mmol, 33% yield) of the desired compound **8**: ^{1}H NMR (D_2O) ∂ 2.65 and 3.44 (2H, 2m, CH_2-2′), 4.15 (2H, m, CH_2-5′), 4.34 (1H, m, H4′), 5.11 (1H, m, H3′), 6.55 (1H, t, J = 6.8 Hz, H1′), 8.20 (1H, s, H2); ^{31}P NMR (D_2O) ∂ −0.11 (d, J = 18.1 Hz, P-3′), 0.65 (pt, P-5′).

2′-Deoxy-N^6-methyladenosine 3′,5′-Bis(diammonium phosphate) (9). Starting from 25 mg (0.094 mmol) of 2′-deoxy-N^6-methyladenosine and following the general procedure, we obtained 19 mg (0.039 mmol, 41% yield) of the desired compound **9**: ^{1}H NMR (D_2O) ∂ 2.77 (2H, m, CH_2-2′), 2.99 (3H, s, NH-CH_3), 4.07 (2H, bs, CH_2-5′), 4.44 (1H, bs, H4′), 4.97 (1H, m, H3′), 6.40 (1H, t, J = 6.9 Hz, H1′), 8.07 (1H, s, H2), 8.32 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.13 (d, J = 17.0 Hz, P-3′), 0.62 (pst, P-5′).

2′-Deoxy-N^6-ethyladenosine 3′,5′-Bis(diammonium phosphate) (10). Starting from 12 mg (0.043 mmol) of 2′-deoxy-N^6-ethyladenosine, **29**, and following the general procedure, we obtained 8.8 mg (0.017 mmol, 40% yield) of the desired compound **10**: ^{1}H NMR (D_2O) ∂ 1.27 (3H, m, CH_2CH_3), 2.77 (2H, m, CH_2-2′), 3.57 (2H, m, $NHCH_2$), 4.00 (2H, bs, CH_2-5′), 4.40 (1H, bs, H4′), 4.89 (1H, m, H3′), 6.49 (1H, pt, H1′), 8.23 (1H, s, H2), 8.50 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.13 (d J = 16.6 Hz, P-3′), 0.63 (pt, P-5′).

2′-Deoxy-N^6-propyladenosine 3′,5′-Bis(diammonium phosphate) (11). Starting from 13 mg (0.044 mmol) of 2′-deoxy-N^6-propyladenosine, **30**, and following the general procedure, we obtained 9.4 mg (0.018 mmol, 41% yield) of the desired compound **11**: ^{1}H NMR (D_2O) ∂ 0.95 (3H, t, J = 6.8 Hz, CH_2CH_3), 1.65 (2H, q, J = 6.8, 6.8 Hz, CH_2CH_3), 2.76 (2H, m, CH_2-2′), 3.45 (2H, m, $NHCH_2$), 4.06 (2H, bs, CH_2-5′), 4.45 (1H, bs, H4′), 4.98 (1H, m, H3′), 6.48 (1H, t, J = 6.8 Hz, H1′), 8.17 (1H, s, H2), 8.39 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.03 (d, J = 18.1 Hz, P-3′), 0.67 (pt, P-5′).

***N*^{6}-Benzoyl-2′-deoxyadenosine 3′,5′-Bis(diammonium phosphate) (12).** Starting from 25 mg (0.070 mmol) of N^6-benzoyl-2′-deoxyadenosine and following the general procedure, we obtained 22 mg (0.038 mmol, 54% yield) of the desired compound **12**: ^{1}H NMR (D_2O) ∂ 2.85 (2H, m, CH_2-2′), 3.96 (2H, bs, CH_2-5′), 4.43 (1H, bs, H4′), 4.84 (1H, m, H3′), 6.62 (1H, pt, H1′), 7.46 (2H, d, J = 6.8 Hz, Ph), 7.57 (1H, d, J = 6.8 Hz, Ph), 7.88 (2H, d, J = 6.8 Hz, Ph), 8.66 (1H, s, H2), 8.81 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 3.29 (d, J = 16.3 Hz, P-3′), 3.77 (pt, P-5′).

2′-Deoxy-N^6,N^6-dimethyladenosine 3′,5′-Bis(diammonium phosphate) (13). Starting from 18 mg (0.064 mmol) of 2′-deoxy-N^6,N^6-dimethyladenosine, **31**, and following the general procedure, we obtained 15 mg (0.029 mmol, 46% yield) of the desired compound **13**: ^{1}H NMR (D_2O) ∂ 2.75 (2H, m, CH_2-2′), 3.23 (6H, s, $N(CH_3)_2$), 4.04 (2H, m, CH_2-5′), 4.42 (1H, bs, H4′), 4.95 (1H, m, H3′), 6.38 (1H, t, J = 6.8 Hz, H1′), 7.96 (1H, s, H2), 8.28 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 0.40 (bs, P-3′), 1.02 (bs, P-5′).

6-Chloro-2′-deoxypurineriboside 3′,5′-Bis(diammonium phosphate) (14). Starting from 10 mg (0.037 mmol) of 6-chloro-2′-deoxypurineriboside and following the general procedure, we obtained 12 mg (0.024 mmol, 65% yield) of the desired compound **14**: ^{1}H NMR (D_2O) ∂ 2.85 (2H, m, CH_2-2′), 4.00 (2H, bs, CH_2-5′), 4.43 (1H, bs, H4′), 4.91 (1H, m, H3′), 6.65, (1H, t, J = 6.8 Hz, H1′), 8.72 (1H, s, H2), 8.93 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 1.95 (d, J = 18.1 Hz, P-3′), 2.25 (pt, P-5′).

2′-Deoxyinosine 3′,5′-Bis(diammonium phosphate) (15). Starting from 25 mg (0.079 mmol) of 2′-deoxyinosine and following the general procedure, we obtained 8.4 mg (0.017 mmol, 22% yield) of the desired compound **15**: ^{1}H NMR (D_2O) ∂ 2.85 (2H, m, CH_2-2′), 4.09 (2H, bs, CH_2-5′), 4.47 (1H, bs, H4′), 5.01 (1H, bs, H3′), 6.55 (1H, t, J = 6.8 Hz, H1′), 8.22 (1H, s, H2), 8.51 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.23 (d, J = 17.3 Hz, P-3′), 0.54 (pt, P-5′).

2′-Deoxy-6-(methylthio)purineriboside 3′,5′-Bis(diammonium phosphate) (16). Starting from 18 mg (0.064 mmol) of 2′-deoxy-6-(methylthio)purineriboside, **28**, and following the general procedure, we obtained 13 mg (0.025 mmol, 39% yield) of the desired compound **16**: ^{1}H NMR (D_2O) ∂ 2.66 (3H, s, SCH_3), 2.85 (2H, m, CH_2-2′), 4.03 (2H, d, J = 3.9 Hz, CH_2-5′), 4.44 (1H, s, H4′), 4.94 (1H, m, H3′), 6.58 (1H, t, J = 6.8 Hz,

H1′), 8.60 (1H, s, H2), 8.69 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 1.59 (d, J = 17.8 Hz, P-3′), 1.94 (pt, P-5′).

3′-Deoxyadenosine 2′,5′-Bis(diammonium phosphate) (17) and 5′-Chloro-3′-deoxyadenosine 2′-(Diammonium phosphate) (19). Starting from 25 mg (0.099 mmol) of 3′-deoxyadenosine and following the general procedure, we obtained 16 mg (0.033 mmol, 34% yield) of desired compound **17** and 7.2 mg (0.021 mmol, 21% yield) of 5′-chloro derivative **19** as a byproduct. **17**: ^{1}H NMR (D_2O) ∂ 2.42 (2H, m, CH_2-3′), 3.98 (1H, m, CH_2-5′), 4.18 (1H, m, CH_2-5′), 4.75 (1H, bs, H4′), 5.08 (1H, m, H2′), 6.22 (1H, s, H1′), 8.14 (1H, s, H2), 8.41 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 0.34 (d, J = 18.1 Hz, P-3′), 1.22 (t, J = 11.89 Hz, P-5′).

19: ^{1}H NMR (D_2O) ∂ 2.51 (2H, m, CH_2-3′), 3.88 (2H, d AB q, J = 22.5, 12.9, 3.9 Hz, CH_2-5′), 4.84 (1H, bs, H4′), 5.23 (1H, m, H2′), 6.25 (1H, s, H1′), 8.20 (1H, s, H2), 8.33 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 0.31 (d, J = 18.3 Hz, P-3′).

3′-Deoxy-N^6-methyladenosine 2′,5′-Bis(diammonium phosphate) (18). Starting from 25 mg (0.094 mmol) of 3′-deoxy-N^6-methyladenosine, **33**, and following the general procedure we obtained 10.4 mg (0.021 mmol, 22% yield) of the desired compound **18**: ^{1}H NMR (D_2O) ∂ 2.41 (2H, m, CH_2-3′), 3.05 (3H, s, NH-CH_3), 4.01 (1H, m, CH_2-5′), 4.20 (1H, m, CH_2-5′), 4.72 (1H, bs, H4′), 5.07 (1H, m, H2′), 6.22 (1H, s, H1′), 8.20 (1H, s, H2), 8.36 (1H, s, H8); ^{31}P NMR (D_2O) ∂ −0.10 (d, J = 19.6 Hz, P-3′), 0.90 (pt, P-5′).

2′-Deoxy-2′-*O*-methyladenosine 3′,5′-Bis(diammonium phosphate) (20). Starting from 10 mg (0.036 mmol) of 2′-deoxy-2′-*O*-methyladenosine and following the general procedure, we obtained 3 mg (0.006 mmol, 17% yield) of the desired compound **20**: ^{1}H NMR (D_2O) ∂ 3.49 (3H, s, OCH_3), 4.08 (2H, m, CH_2-5′), 4.57 (2H, bs, H2′ + H4′), 4.92 (1H, m, H3′), 6.22 (1H, d, J = 5.9 Hz, H1′), 8.25 (1H, s, H2), 8.59 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 1.47 (d, J = 19.4 Hz, P-3′), 2.02 (pst, P-5′).

2′-Deoxyadenosine 3′,5′-Bis(diammonium thiophosphate) (21). Starting from 50 mg (0.2 mmol) of 2′-deoxyadenosine and following the general procedure, but using thiophosphoryl chloride (81 μL, 0.8 mmol), we obtained 18 mg (0.036 mmol, 18% yield) of the desired compound **21**: ^{1}H NMR (D_2O) ∂ 2.42 (1H, m, CH_2-2′), 2.61 (1H, m, CH_2-2′), 3.83 (2H, pt, CH_2-5′), 4.11 (1H, m, H4′), 4.55 (1H, m, H3′), 6.21 (1H, t, J = 6.8 Hz, H1′), 7.89 (1H, s, H2), 8.32 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 37.72 (pd, P-3′), 43.64 (pt, P-5′).

2′-Deoxy-N^1,N^6-ethenoadenosine 3′,5′-Bis(diammonium phosphate) (22). Starting from 25 mg (0.091 mmol) of 2′-deoxy-N^1,N^6-ethenoadenosine and following the general procedure, we obtained 19.3 mg (0.038 mmol, 42% yield) of the desired compound **22**: ^{1}H NMR (D_2O) ∂ 2.88 (2H, m, CH_2-2′), 4.10 (2H, m, CH_2-5′), 4.46 (1H, s, H4′), 5.02 (1H, m, H3′), 6.51 (1H, t, J = 6.8 Hz, H1′), 7.46 (1H, bs, etheno), 7.84 (1H, bs, etheno), 8.43 (1H, s, H2), 8.93 (1H, s, H8); ^{31}P NMR (D_2O) ∂ 0.11 (d, J = 16.59 Hz, P-3′), 0.78 (pt, P-5′).

Pharmacological Analyses. $P2Y_1$ receptor-promoted stimulation of inositol phosphate formation by adenine nucleotide analogues was measured in turkey erythrocyte membranes as previously described.[18,19] The $K_{0.5}$ values were averaged from three to eight independently determined concentration−effect curves for each compound. Briefly, 1 mL of washed turkey erythrocytes was incubated in inositol-free medium (DMEM; Gibco) with 0.5 mCi of 2-[^{3}H]-*myo*-inositol (20 Ci/mmol; American Radiolabelled Chemicals Inc.) for 18−24 h in a humidified atmosphere of 95% air 5% CO_2 at 37 °C. Erythrocyte ghosts were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA) as described.[19] Phospholipase C activity was measured in 25 μL of [^{3}H]inositol-labeled ghosts (∼175 μg of protein, 200−500000 cpm/assay) in a medium containing 424 μM $CaCl_2$, 0.91 mM $MgSO_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH_2PO_4, and 10 mM Hepes, pH 7.0. Assays (200 μL final volume) contained 1 μM GTPγS and the indicated concentrations of nucleotide analogues. Ghosts were incubated at 30 °C for 5 min, and total [^{3}H]inositol phosphates were quantitated by anion-exchange chromatography as previously described.[18,19]

Radioligand binding at adenosine receptors was carried out as described.[25]

Data Analysis. Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of 2-MeSATP in the same experiment.

Antagonist IC_{50} values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 10 nM 2-MeSATP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration.

All concentration−effect curves were repeated in at least three separate experiments carried out with different membrane preparations using duplicate or triplicate assays.

Abbreviations: ATP, adenosine 5′-triphosphate; A3P5P, adenosine 3′,5′-bisphosphate; DEAE, (diethylamino)ethyl; DMSO, dimethyl sulfoxide; FAB, fast atom bombardment (mass spectroscopy); HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectroscopy; MeATP, adenosine 5′-methylenetriphosphate, (α,β) or (β,γ) isomers; 2-MeSATP, 2-(methylthio)adenosine-5′-triphosphate; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; TBAP, tetrabutylammonium phosphate; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography.

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