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Identification of Competitive Antagonists of the $P2Y_1$ Receptor

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SUMMARY

Although P2 receptors mediate a myriad of physiological effects of extracellular adenine nucleotides, study of this broad class of receptors has been compromised by a lack of P2 receptor-selective antagonist molecules. The adenine nucleotide-promoted inositol lipid hydrolysis response of turkey erythrocyte membranes, which has been used extensively as a model for P2Y receptors, has been applied to identify molecules that competitively block these receptors. Adenosine-3′-phosphate-5′-phosphosulfate (A3P5PS) promoted activation of phospholipase C that was only 10–25% of that observed with the full P2Y receptor agonists ATP, ADP, and 2-methylthio-ATP (2MeSATP). The small stimulatory effects of A3P5PS were saturable. Moreover, these effects were entirely the result of interaction with the P2Y receptor, because A3P5PS had no effect on activation of phospholipase C through the β-adrenergic receptor and produced a concentration-dependent inhibition of 2MeSATP-promoted activity over the same range of A3P5PS concentrations that alone caused a small activation of phospholipase C. Increasing concentrations of A3P5PS produced a rightward shift of the concentration-effect curve for 2MeSATP, and Schild transformation of these data revealed that A3P5PS is a competitive P2Y receptor antagonist with a pK_B of 6.46 ± 0.17. The presence of a phosphate in the 2′- or 3′-position appears to be crucial for antagonist activity, because adenosine-3′-phosphate-5′-phosphate (A3P5P) and adenosine-2′-phosphate-5′-phosphate also exhibited competitive antagonist/partial agonist activities. Other 3′-substituted analogues, such as 3′-amino-ATP and 3′-benzoylbenzoyl-ATP, were full agonists with no antagonist activity. A3P5PS, A3P5P, and adenosine-2′,5′-diphosphate also were competitive antagonists in studies with the cloned human $P2Y_1$ receptor stably expressed in 1321N1 human astrocytoma cells. Moreover, both A3P5PS and A3P5P were devoid of agonist activity at the human $P2Y_1$ receptor. The effects of these 2′- and 3′-phosphate analogues were specific for the phospholipase C-coupled $P2Y_1$ receptor, because no agonistic or antagonistic effects on the adenylyl cyclase-coupled P2Y receptor of C_6 glioma cells or on $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors stably expressed in 1321N1 human astrocytoma cells were observed. These results describe specific competitive antagonism of the $P2Y_1$ receptor by an adenine nucleotide derivative and provide a potential new avenue for P2 receptor drug development.

Extracellular adenine and uridine nucleotides regulate a broad range of physiological responses through an increasingly diverse set of G protein-coupled and ligand-gated ion channel receptors (1–3). Delineation of multiple receptor subtypes initially evolved from tissue- and agonist-specific physiological responses to nucleotide analogues. However, the complexity of the molecular species that respond to adenine and uridine nucleotides has been emphasized recently with reports of the cloning of approximately one dozen different P2 receptor genes (3, 4).

Although progress has been made in identifying adenine nucleotide analogues that exhibit selectivities among P2 receptors, the availability of antagonists for these receptors is limited. Only two compounds, Reactive Blue 2 and suramin, have been available as general P2 receptor antagonists. However, these molecules interact with a broad range of proteins that are unrelated to adenine nucleotide-regulated receptor signaling. Lambrecht *et al.* (5) recently introduced a pyridoxal phosphate analogue, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid, that has been reported to exhibit selectivity as a competitive antagonist of certain P2 receptors (6, 7). The observation that ATP itself is a competitive antagonist at the ADP-activated P2 receptor of platelets suggests the possibility that adenine nucleotide substitutions that lead to selective high affinity antagonists of these receptors might be identified (8). For example, Humphries *et al.* (9, 10) have developed 2-propylthio-β,γ-difluoromethylene-D-ATP as a relatively high affinity antagonist of the ADP receptor on platelets.

We report here the identification of ATP analogues that are partial agonists and consequently are competitive antagonists at the P2Y receptor of turkey erythrocyte membranes. Moreover, two of these molecules are relatively potent competitive antagonists, without any partial agonist activity, at

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ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; A3P5PS, adenosine-3′-phosphate-5′-phosphosulfate; A3P5P, adenosine-3′-phosphate-5′-phosphate; A2P5P, adenosine-2′-phosphate-5′-phosphate; 2MeSATP, 2-methylthio-ATP; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the human $P2Y_1$ receptor stably expressed in 1321N1 human astrocytoma cells. These molecules exhibit higher apparent affinities of interaction with this P2Y receptor than does ATP, and they exhibit absolute selectivity for binding to the $P2Y_1$ receptor over four other G protein-linked P2 receptor subtypes.

Materials and Methods

Cell culture. C_6 rat glioma cells were grown in DMEM supplemented with 5% fetal calf serum, in a humidified atmosphere of 95% air/5% CO_2. Cells were passaged by trypsinization. Experiments were carried out with confluent cell cultures 2–4 days after plating in 12-well clusters, as described previously (11). 1321N1 human astrocytoma cells stably expressing the human (12) or turkey (13) $P2Y_1$ receptor,[1] the human $P2Y_2$ receptor (P_{2U} purinergic receptor) (14), the human $P2Y_4$ receptor (15), or the rat $P2Y_6$ receptor (15) were grown in DMEM supplemented with 5% fetal calf serum and 600 μg/ml G-418.

Turkey erythrocyte labeling. Fresh blood was obtained from female turkeys by venous puncture and collected into a heparinized syringe. Erythrocytes were washed twice by centrifugation and resuspension in sterile DMEM, followed by a final wash with inositol-free DMEM. One milliliter of washed packed erythrocytes was resuspended in a final volume of 4.2 ml of inositol-free DMEM, in the presence of 0.5 mCi of [^{3}H]inositol. Cells were incubated in a stirred glass vial for 16–20 hr at 37°, in a humidified atmosphere of 95% air/5% CO_2, as described previously (16).

Phospholipase C assay. Erythrocyte ghost membranes were prepared from [^{3}H]inositol-labeled cells by hypotonic lysis in 15 volumes of a buffer containing 5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA (lysis buffer). The membranes were washed three times by centrifugation and resuspension with lysis buffer. The final resuspension was in 20 mM HEPES, pH 7.0, to a concentration of 6 mg of protein/ml. This preparation was used immediately for assay of phospholipase C. Twenty-five microliters of labeled membranes (~150 μg of protein, 200,000 cpm) were combined in a final volume of 200 μl of medium containing 0.91 mM $MgSO_4$, 115 mM KCl, 5 mM potassium phosphate, 0.424 mM $CaCl_2$, 2 mM EGTA, and 10 mM HEPES, pH 7.0 (free Ca^{2+} concentration was ~1 μM). Because receptor- and G protein-promoted activation of phospholipase C in turkey erythrocyte membranes is strictly dependent on the presence of guanine nucleotides (16), the nonhydrolyzable GTP analogue guanosine-5′-*O*-(3-thio)triphosphate (1 μM) was included in the assay. Membranes were incubated for 5 min at 30° with the indicated concentrations of adenine nucleotide analogues. Incubations were terminated by addition of 1 ml of ice-cold chloroform/methanol (1:2, by volume), followed by 350 μl of chloroform and 350 μl of water. Samples were mixed and centrifuged, and 1 ml of the aqueous upper phase was diluted with 8 ml of water and transferred onto Dowex AG-1X8 columns (formate form). Columns were washed with 8 ml of 200 mM ammonium formate, 100 mM formic acid, and the eluant was discarded. Total inositol phosphates (inositol bisphosphate, inositol trisphosphate, and inositol tetrakisphosphate) were eluted with 5 ml of 1.2 M ammonium formate, 100 mM formic acid, and collected in scintillation vials (16). [^{3}H]Inositol phosphates were quantitated by scintillation counting. Inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing cloned human $P2Y_1$, $P2Y_2$, or $P2Y_4$ or rat $P2Y_6$ receptors was measured as described previously (17).

Data analysis. Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). All concentration-effect curves were repeated in at least three separate experiments using duplicate or triplicate assays.

Materials. A3P5PS, A3P5P, A2P5P, and (−)-isoproterenol (+)-bitartrate were obtained from Sigma Chemical Co. (St. Louis, MO). 2MeSATP was obtained from Research Biochemicals Inc. (Natick, MA); *myo*-[2-^{3}H]inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Inositol-free DMEM was from Gibco BRL (Grand Island, NY). The sources of other materials have been reported previously (11, 16, 17).

Results

As we have previously reported in detail (16, 18), 2MeSATP (Fig. 1) and other chain-extended 2-thioether derivatives of ATP and ADP are potent and efficacious activators of the P2Y receptor of turkey erythrocytes. Although ATP and ADP are equally efficacious, compared with 2MeSATP, they are much less potent (16) (Fig. 1). Our interest in determining whether sulfate-containing analogues of adenine nucleotides might be potent P2Y receptor agonists that are more resistant to hydrolysis by ectonucleotidases led us to determine the activity of A3P5PS at the turkey erythrocyte P2Y receptor. Relatively small effects of A3P5PS on inositol phosphate formation occurred (Fig. 1), although detailed analyses illustrated that this stimulation was both concentration-dependent and apparently saturable, with an EC_{50} of 0.83 ± 0.08 μM (Fig. 1; Table 1). The maximal effect of A3P5PS, relative to those of 2MeSATP, ATP, and ADP, was somewhat variable but typically ranged from 10 to 25% of the maximal full agonist effect (Table 1). Because, to our knowledge, these results could represent the first observation of partial agonist

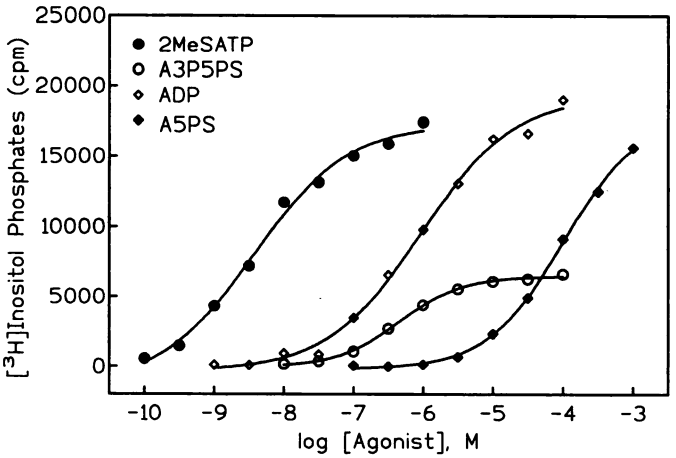

Fig. 1. Effect of sulfate-substituted adenine nucleotide analogues on inositol lipid hydrolysis in turkey erythrocyte membranes. The capacity of the indicated concentrations of 2MeSATP (●), A3P5PS (○), ADP (◇), and adenosine-5′-phosphosulfate (♦) to stimulate the hydrolysis of inositol lipids by turkey erythrocyte membranes was determined as described in Materials and Methods. The data are the mean of duplicate assays from a representative experiment, which was repeated at least three times for each agonist.

[1] The nomenclature of purinergic/pyrimidinergic receptors is provisional and likely will be revised; thus, the terms used here are provisional. The IUPHAR Nomenclature Committee has provided general recommendations that G protein-coupled nucleotide receptors be designated as P2Y receptors (4) and that receptors in each subfamily be denoted by numbers that reflect the chronological order in which the sequences of functional receptors have become available in the public domain. However, the receptor cloned by Chang *et al.* (24) has been referred to as the $P2Y_6$ receptor (see Website http://mgddk1.niddk.nih.gov:800/nomenclature.html), even though it was the third functional P2Y receptor for which a sequence was published. To avoid confusion in the current study, we refer to this receptor as the $P2Y_6$ receptor. The $P2Y_2$ receptor has been previously referred to in the literature as the P_{2U} purinergic receptor.

TABLE 1

Potency and efficacy of adenine nucleotide analogues at the P2Y receptor of turkey erythrocytes

EC_{50} values were obtained as indicated in Materials and Methods. Each value represents the mean ± standard error of at least four experiments carried out with different membrane preparations.

Nucleotide	EC_{50}	Relative efficacy[a]
	μM	
2MeSATP	0.013 ± 0.003	
ADP	6.86 ± 1.51	1
A5PS[b]	221 ± 61	1
A3P5PS	0.83 ± 0.08	0.17 ± 0.03
A3P5P	2.23 ± 0.84	0.17 ± 0.05
A2P5P	1.65 ± 0.35	0.21 ± 0.06

[a] Relative efficacies of adenine nucleotides were determined by comparison with the effect produced by a maximal effective concentration of 2MeSATP in the same experiment.
[b] A5PS, adenosine-5′-phosphosulfate.

activity of a molecule at a P2Y receptor, the properties of A3P5PS were investigated further.

The observed small effects of A3P5PS on inositol phosphate production possibly could involve an effect unrelated to occupation of the P2Y receptor of turkey erythrocyte membranes. Therefore, the capacity of A3P5PS to augment or inhibit the effects of a submaximal concentration of 2MeSATP was determined. A3P5PS antagonized the stimulatory effect of 10 nM 2MeSATP (Fig. 2 and data not shown) over the same concentration range necessary to observe the stimulatory effects of A3P5PS alone. This result suggested that the effect of A3P5PS occurred as a consequence of binding to the P2Y receptor, rather than as a result of interaction with another of the components that comprise the receptor-regulated phospholipase C response. To further assess the potential interaction of A3P5PS with the P2Y receptor, the concentration dependence of the effects of A3P5PS was examined over a broad range of concentrations (1–1000 nM) of 2MeSATP (Fig. 2). A3P5PS produced a concentration-dependent antagonism of the effects of 2MeSATP at all concentrations of 2MeSATP above 3 nM (Fig. 2). Moreover, A3P5PS caused a concentration-dependent parallel rightward shift of the concentration-effect curve for 2MeSATP (Fig. 3A). Schild analysis (Fig. 3B) confirmed that the antagonism was apparently competitive, and the pK_B of A3P5PS was 6.46 ± 0.17. This apparent potency of A3P5PS was 10-fold greater than that of ATP (4.23 ± 1.52 μM) for stimulation of inositol lipid hydrolysis in the same membranes.

Activities of compounds with structures related to A3P5PS were examined to establish whether sulfate substitution at the 5′-position of ATP or substitution at the 3′-position is important for conferring antagonist activity. Antagonism appears to be unrelated to 5′-sulfate substitution, because A3P5P also was a partial agonist, with an EC_{50} for activation of 2.23 ± 0.84 μM and a maximal effect that ranged from 4 to 35% of the maximal effect observed with 2MeSATP (Table 1). A3P5P also caused a parallel rightward shift of the 2MeSATP concentration-effect curve (Fig. 3C), and a calculated pK_B of 5.66 ± 0.21 was determined from Schild regressions with slopes not significantly different from unity (Fig. 3D). Thus, A3P5P also interacts with the P2Y receptor with a potency that is greater than or equal to that of the parent ATP molecule. Effects identical to those of A3P5P were observed with the isomer A2P5P (Table 1 and data not shown).

Partial agonist activity also was observed with other adenosine-3′- and 2′-phosphate analogues, such as adenosine-2′-phosphate-5′-phosphosulfate and adenosine-2′-phosphate-5′-phosphoribose (data not shown). Adenosine-5′-phosphosulfate (Fig. 1) and adenosine-5′-diphosphoribose were full agonists (data not shown), indicating that substitution with phosphate in the 2′- or 3′-positions of adenine nucleotides is required for antagonistic activity. Other 2′ or 3′ substitutions did not confer antagonistic properties to adenine nucleotides. For example, 3′-amino-ATP and 3′-benzoyl-benzoyl-ATP were full agonists, and 3′-*N*-benzylamino-ATP was a very weak agonist devoid of any antagonist activity (data not shown) (19).

The selectivity of the effects of A3P5PS and A3P5P was confirmed by examining their effects at the phospholipase C-coupled β-adrenergic receptor natively expressed in turkey erythrocytes (20, 21). In contrast to the effects of A3P5PS and A3P5P on 2MeSATP-stimulated inositol lipid hydrolysis, the agonist effects of these 3′-phosphate-substituted analogues were additive with those of a maximally effective concentration of the β-adrenergic agonist isoproterenol (Fig. 4). These results indicate that 3′-phosphate adenine nucleotide analogues interact specifically with the P2Y receptor of turkey

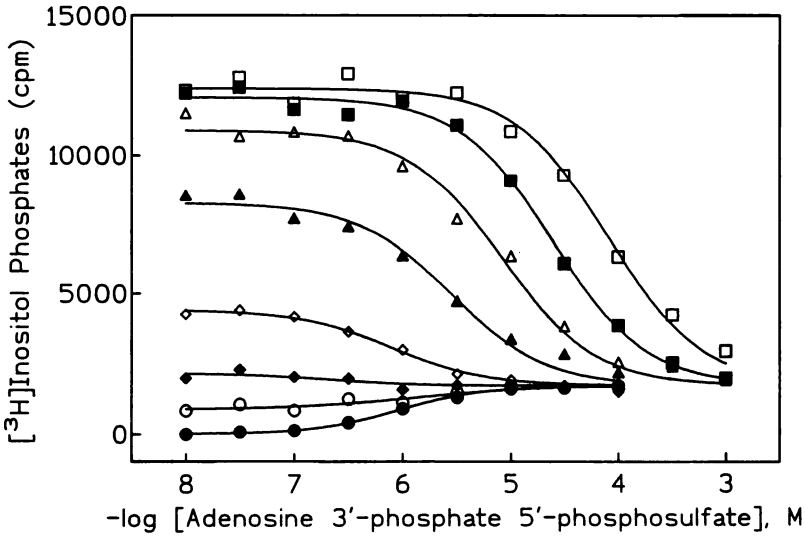

Fig. 2. Effect of A3P5PS on P2Y receptor-mediated activation of phospholipase C in turkey erythrocytes. [^{3}H]-Inositol-labeled turkey erythrocyte membranes were incubated with the indicated concentrations of A3P5PS in the absence (●) or presence of 1 (○), 3 (♦), 10 (◇), 30 (▲), 100 (△), 300 (■), or 1000 (□) nM 2MeSATP. Inositol phosphate formation was determined as indicated in Materials and Methods. The data are the mean of duplicate assays from a representative experiment, which was repeated three times.

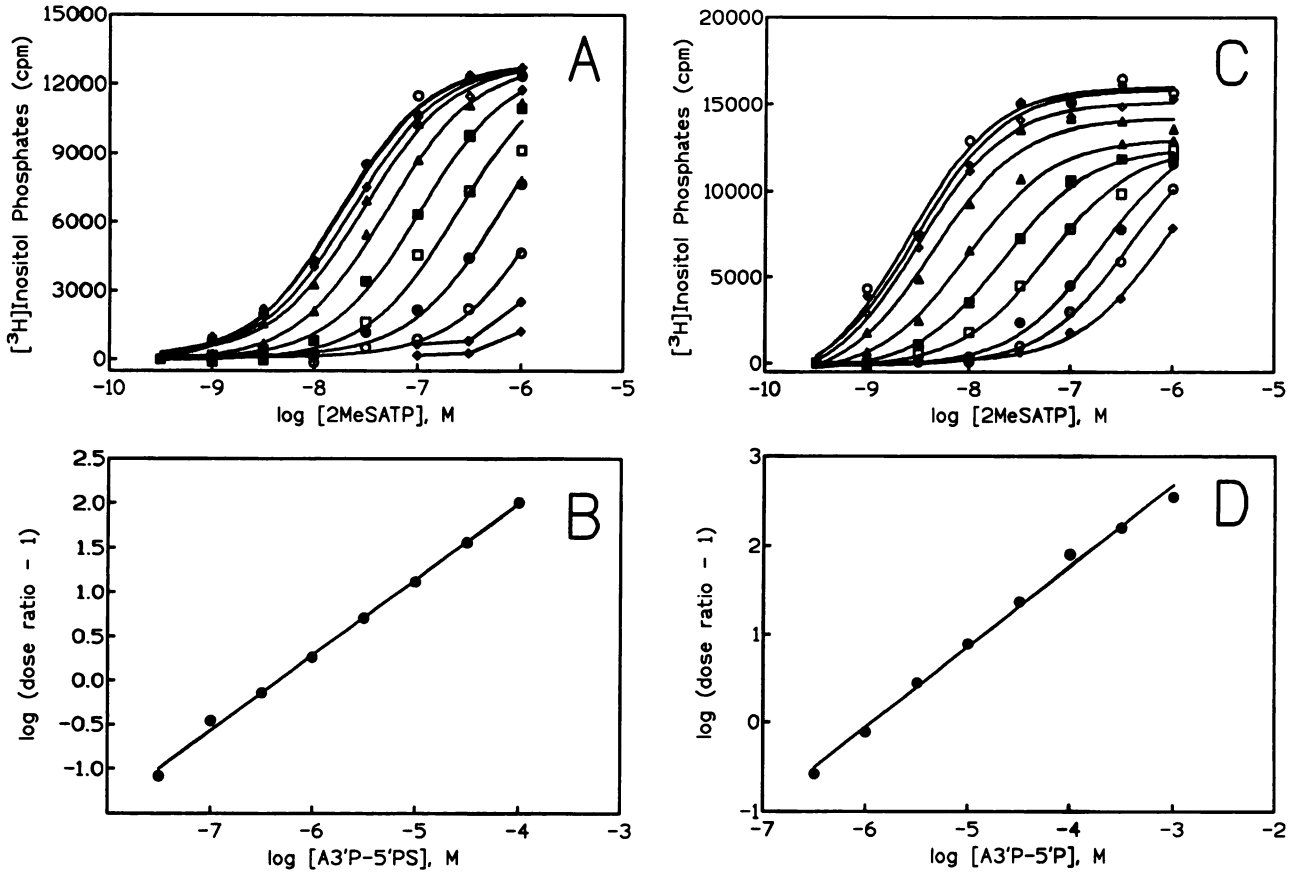

Fig. 3. Competitive inhibition of 2MeSATP-mediated activation of P2Y receptors by A3P5PS and A3P5P. A and C, [^{3}H]Inositol-labeled turkey erythrocyte membranes incubated with the indicated concentrations of 2MeSATP in the absence (●) or presence of 0.1 (○), 0.3 (▲), 1 (△), 3 (■), 10 (□), 30 (●), 100 (○), 300 (♦), or 1000 (◇) μM A3P5PS (A) or A3P5P (C). Inositol phosphate formation was determined as indicated in Materials and Methods. Inositol phosphate accumulation induced by A3P5PS or A3P5P alone was subtracted from the accumulation in the presence of the indicated concentrations of 2MeSATP. B and D, Schild regression analyses of data shown in A and C, respectively. The data are the mean of duplicate assays from a representative experiment, which was repeated at least three times.

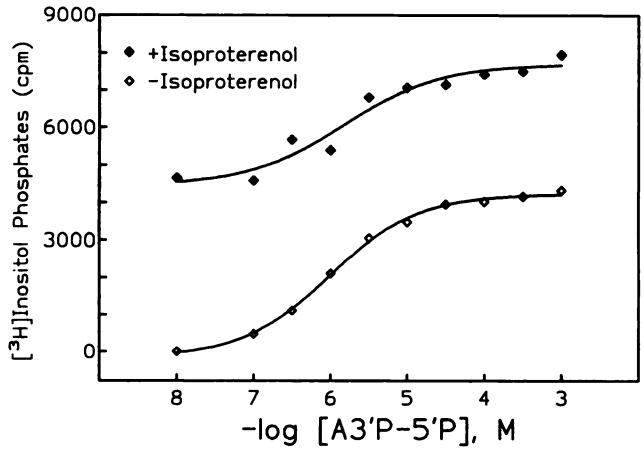

Fig. 4. Effect of A3P5P on the activation of phospholipase C by β-adrenergic receptors in turkey erythrocyte membranes. The capacity of the indicated concentrations of A3P5P to stimulate the accumulation of inositol phosphates in the absence (◇) or presence (♦) of 10 μM isoproterenol was determined as described in Materials and Methods. The maximal response induced by 2MeSATP in the same experiment was 11,980 ± 290 cpm. Data shown are from a representative experiment, which was repeated three times with similar results.

erythrocyte membranes and not with other components of the receptor-phospholipase C cascade.

We have cloned the DNA encoding the turkey erythrocyte P2Y receptor ($P2Y_1$ receptor) (13) and also have cloned its human homologue (12). Therefore, the effects of A3P5PS and A3P5P were determined in 1321N1 human astrocytoma cells stably expressing the cloned receptors. Antagonist activities very similar to those observed with turkey erythrocyte membranes were observed with the expressed turkey $P2Y_1$ receptor (data not shown). In contrast to the results with the turkey erythrocyte P2Y receptor, A3P5PS (data not shown) and A3P5P (Fig. 5) exhibited no partial agonist activity and were simple competitive antagonists at the human $P2Y_1$ receptor. The calculated pK_B (6.05 ± 0.01) for A3P5P was essentially the same as that observed in the turkey erythrocyte membrane preparation (Fig. 5).

The selectivity of A3P5PS and A3P5P for the $P2Y_1$ receptor was tested by examining their effects at other P2 receptors. No effect of either compound as agonist or antagonist was observed at the P2Y receptor of C_6 rat glioma cells (Fig. 6). We previously showed that this adenylyl cyclase-linked P2 receptor differs from the phospholipase C-coupled $P2Y_1$ receptor in the specificity of second messenger signaling and pharmacological selectivity (11, 12). A3P5PS and A3P5P also were neither antagonists nor agonists at the human $P2Y_2$ (Table 2), human $P2Y_4$ (data not shown), or rat $P2Y_6$ (data not shown) receptors stably expressed in 1321N1 cells.

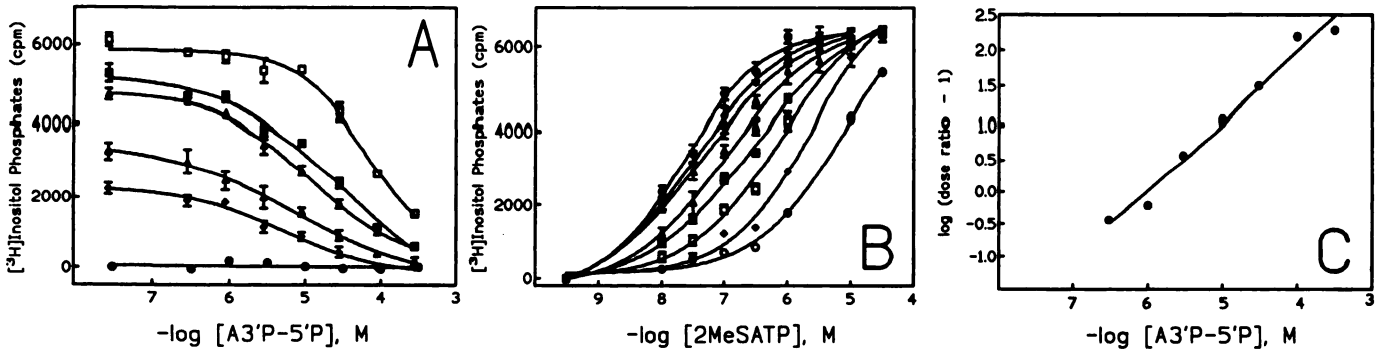

Fig. 5. A3P5P is a competitive antagonist without agonistic activity at the human P2Y$_1$ receptor stably expressed in 1321N1 human astrocytoma cells. A, Cells incubated with the indicated concentrations of A3P5P in the absence (●) or presence of 10 (◇), 30 (▲), 100 (△), 300 (■), or 1000 (□) nM 2MeSATP. B, Concentration dependence of 2MeSATP for the activation of phospholipase C in 1321N1 cells, determined in the absence (●) or presence of 0.3 (◇), 1 (▲), 3 (△), 10 (■), 30 (□), 100 (◆), or 300 (○) μM A3P5P. C, Schild regression analysis of data shown in B. The slope value was not significantly different from unity. Data shown are the mean ± standard error of triplicate determinations from a representative experiment, which was repeated three times with similar results.

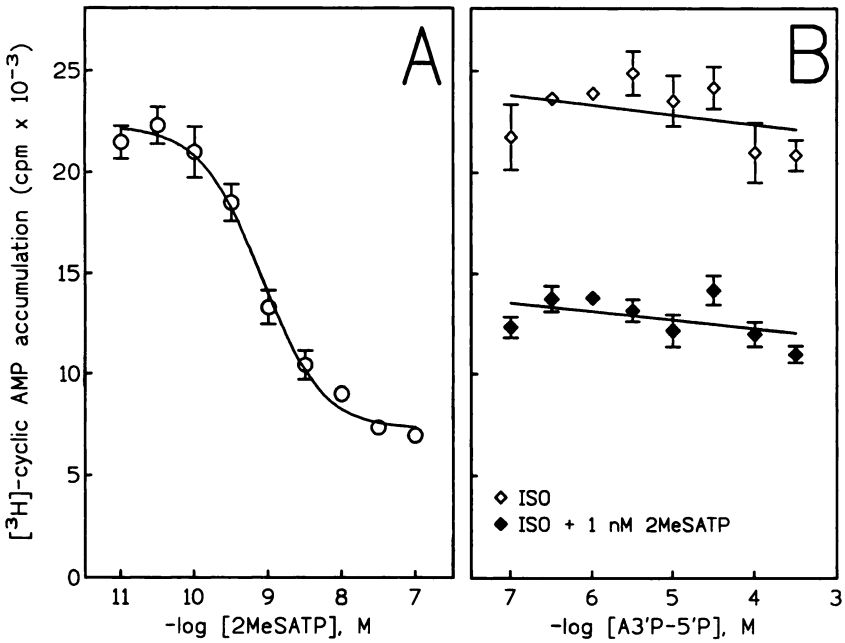

Fig. 6. Lack of agonist or antagonist effects of A3P5P on the adenylyl cyclase-coupled P2Y receptor of C$_6$ cells. The capacity of the indicated concentrations of 2MeSATP to inhibit isoproterenol-stimulated cAMP accumulation (A) or the capacity of A3P5P to affect the response of 10 μM isoproterenol-induced (◇) or 10 μM isoproterenol/1 nM 2MeSATP-induced (◆) accumulation of cAMP (B) was studied as described in Materials and Methods. Data shown are the mean of triplicate assays, and the results are representative of those obtained in three separate experiments.

TABLE 2

Effect of A3P5PS and A3P5P on the human P2Y$_2$ receptor stably expressed in 1321N1 cells

Accumulation of inositol phosphates in response to UTP (1 μM), A3P5PS (100 μM), or A3P5P (100 μM), in the absence or presence of UTP (1 μM), was determined as described in Materials and Methods. Data shown are the mean ± standard error of values from at least three different experiments.

Nucleotide	Inositol phosphates
	cpm
Basal	5,534 ± 679
UTP	25,730 ± 1,445
A3P5PS	5,129 ± 727
A3P5PS + UTP	34,875 ± 7,176
A3P5P	4,553 ± 458
A3P5P + UTP	27,952 ± 675

Discussion

The results of this study illustrate that A3P5PS and A3P5P are competitive antagonists at the P2Y$_1$ receptor. This observation is significant for several reasons. The increasingly broad class of P2 receptors has been difficult to study, in part because of the lack of receptor-specific antagonists. Although certain molecules have been useful as P2 receptor antagonists, these compounds have little if any selectivity for interaction with P2 receptors over a host of other proteins with which they interact, and these nonselective drugs in general do not distinguish among various P2 receptor subtypes. The receptor for ADP on platelets has provided a notable exception, because ATP is a competitive antagonist rather than an agonist at this receptor (8, 22). An analogue of ATP was recently developed as a competitive antagonist of the platelet ADP receptor (9, 10). The three molecules that were previously applied to studies of P2Y receptors are not direct structural analogues of ATP. Thus, the results obtained with A3P5PS and A3P5P (and A2P5P) provide the first observation of competitive antagonism of a P2Y receptor with molecules derived from the parent ATP molecule. Moreover, the antagonist activity of these molecules provides a structural basis on which potentially more potent P2Y receptor antagonists can be developed.

Antagonism of the effects of 2MeSATP occurred at surprisingly low concentrations of A3P5PS and A3P5P, and both compounds exhibited potencies that were greater than that of the natural receptor agonist ATP and that were similar to the

potency of ADP. The antagonism observed with both 3′-phosphate analogues at the P2Y receptor was apparently competitive, because partial agonist activity and inhibition of the stimulatory effects of 2MeSATP were observed over the same range of concentrations (Fig. 2). The EC_{50} for the partial agonist effect of A3P5P was 2.23 ± 0.84 μM, and the pK_B for antagonism of the 2MeSATP effect was 5.66 ± 0.21. Additionally, no effect of these molecules on β-adrenergic receptor-stimulated phospholipase C activity was observed in the same membranes. Schild slopes near unity support the idea that the blockade of the P2Y receptor of turkey erythrocyte membranes is competitive in nature. The calculated pK_B values for both A3P5PS (pK_B = 6.46 ± 0.17) and A3P5P (pK_B = 5.66 ± 21) likely represent accurate values, because of the low intrinsic efficacy of these molecules as agonists at the avian receptor (Table 1). Essentially identical pK_B values were obtained in multiple experiments in which the observed maximal effect of the compounds ranged from 10 to 25% of that observed with the full agonist 2MeSATP. Moreover, the results with the turkey erythrocyte P2Y receptor led to studies of the human homologue of this $P2Y_1$ receptor stably expressed in 1321N1 cells. The two 3′-phosphate adenine nucleotide analogues exhibited no agonist activity at the human $P2Y_1$ receptor, and the calculated pK_B values were essentially the same as those calculated for the receptor on avian erythrocytes.

The results obtained with A3P5P, A2P5P, and other ribose-substituted adenine nucleotide analogues indicate that it is the phosphate substitution of the ribose that confers partial agonist/competitive antagonist activity to these molecules. The observation that adenosine-5′-phosphosulfate was a full agonist (Fig. 1) that exhibited a potency 30-fold lower than that of ADP also has significant implications. First, replacement of the β-phosphate of ADP with a sulfate group results in loss of affinity, relative to ADP, but conserves the full agonist properties of ADP for the $P2Y_1$ receptor. Second, the 3′-phosphate substitution in adenosine-5′-phosphosulfate results in a 200-fold increase in agonist potency (0.83 ± 0.08 μM versus 221 ± 61 μM for A3P5PS and adenosine-5′-phosphosulfate, respectively) and, more importantly, 3′-phosphate substitution confers antagonistic properties to the molecule.

The turkey erythrocyte P2Y receptor model has been instrumental in elucidation of both the pharmacological selectivity of P2 receptor agonists and the second messenger signaling properties of an adenine nucleotide-activated receptor. This receptor has been cloned (13) and is the turkey homologue of the $P2Y_1$ receptor, which has been cloned also from chick (23) and several mammalian species including humans (12). The pharmacological selectivity and second messenger signaling specificity of the human receptor are essentially indistinguishable from those observed with the P2Y receptor of turkey erythrocytes (12). However, in contrast to partial agonist/competitive antagonist activities at the avian receptor, 2′- and 3′-phosphate analogues were competitive antagonists, with no demonstrable partial agonist activity, at the human $P2Y_1$ receptor. These results potentially could be explained by a larger receptor reserve in the turkey erythrocyte membrane preparation than in 1321N1 cells stably expressing the cloned human $P2Y_1$ receptor. However, the receptor reserve, if any, is apparently similar for both human and turkey $P2Y_1$ receptors stably expressed in 1321N1 cells. That is, both the order of potency and the actual potencies of 20 different agonists are essentially identical for the human and turkey $P2Y_1$ receptors stably expressed in 1321N1 cells (12). Because the amino acid sequence of the human $P2Y_1$ receptor is 84% identical to that of the turkey receptor, differences in activity of antagonist molecules between the two species homologues may reside in the differences in amino acid sequence between the two receptors.

The activity of the 3′-phosphate analogues has not been exhaustively examined with all P2 receptors. However, these compounds are notably inactive at a G_i/adenylyl cyclase-linked P2Y receptor that is natively expressed in C_6 rat glioma cells (Fig. 6) and at the human $P2Y_2$ receptor stably expressed in 1321N1 cells (Table 2). As expected, the 2′- and 3′-phosphate-substituted adenine nucleotide analogues also exhibited no antagonistic effects at the pyrimidine nucleotide-preferring $P2Y_4$ and $P2Y_6$ receptors, which are recently cloned (24–26) members of the G protein-coupled P2Y receptor subfamily.[1] The activities of these antagonist molecules at the ligand-gated ion channel class of P2X receptors will need to be investigated.

In collaboration with Jacobson and colleagues, we have identified substitutions on adenine nucleotides that increase agonist potencies of these compounds by up to 300,000-fold, compared with the parent adenine nucleotide molecule. For example, chain-extended 2-thioether substitution results in remarkable increases in agonist potency of adenine di- and triphosphates (11, 18). Moreover, such substitutions result in potent (in some cases in the nanomolar range of concentrations) AMP analogues; this activity sharply contrasts with the complete lack of P2Y receptor activity of AMP itself (27). Similar substitutions in A3P5PS or A3P5P potentially could lead to remarkably potent competitive antagonists for P2Y receptors.

In summary, 2′- and 3′-phosphate derivatives of ATP are adenine nucleotide-based competitive antagonists of P2Y receptors. These molecules should prove very useful for further delineating the physiological roles of extracellular adenine nucleotides and P2 receptors in target tissues and may be heuristic in the development of therapeutic agents that interfere with the signaling proteins used by extracellular adenine nucleotides.

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