British Journal of Pharmacology (1996) 118, 1959–1964

Identification of potent P_{2Y}-purinoceptor agonists that are derivatives of adenosine 5′-monophosphate

*José L. Boyer, †Suhaib Siddiqi, †[2]Bilha Fischer, *Teresa Romero-Avila, †Kenneth A. Jacobson & *[1]T. Kendall Harden

*Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, and †Molecular Recognition Section, NIDDK, NIH, Bethesda, MD 20892-1008, U.S.A.

1 A series of chain-extended 2-thioether derivatives of adenosine monophosphate were synthesized and tested as agonists for activation of the phospholipase C-linked P_{2Y}-purinoceptor of turkey erythrocyte membranes, the adenylyl cyclase-linked P_{2Y}-purinoceptor of C6 rat glioma cells, and the cloned human P_{2U}-receptor stably expressed in 1321N1 human astrocytoma cells.

2 Although adenosine monophosphate itself was not an agonist in the two P_{2Y}-purinoceptor test systems, eleven different 2-thioether-substituted adenosine monophosphate analogues were full agonists. The most potent of these agonists, 2-hexylthio AMP, exhibited an EC_{50} value of 0.2 nM for activation of the C6 cell receptor. This potency was 16,000 fold greater than that of ATP and was only 10 fold less than the potency of 2-hexylthio ATP in the same system. 2-hexylthio adenosine was inactive.

3 Monophosphate analogues that were the most potent activators of the C6 cell P_{2Y}-purinoceptor were also the most potent activators of the turkey erythrocyte P_{2Y}-purinoceptor. However, agonists were in general more potent at the C6 cell receptor, and potency differences varied between 10 fold and 300 fold between the two receptors.

4 Although 2-thioether derivatives of adenosine monophosphate were potent P_{2Y}-purinoceptor agonists no effect of these analogues on the human P_{2U}-purinoceptor were observed.

5 These results support the view that a single monophosphate is sufficient and necessary for full agonist activity at P_{2Y}-purinoceptors, and provide insight for strategies for development of novel P_{2Y}-purinoceptor agonists of high potency and selectivity.

Keywords: P_{2Y}-purinoceptors; cyclic AMP accumulation; inositol phosphate formation; adenylyl cyclase inhibition; activation of phospholipase C; C6 rat glioma cells; turkey erythrocytes; 2-thioether derivatives of adenosine monophosphate

Introduction

Extracellular purine and pyrimidine nucleotides interact with members of the P_{2X}- and P_{2Y}-purinoceptor families to elicit a broad range of physiological responses (Burnstock & Kennedy, 1985; Dubyak & El-Moatassim, 1993; Harden *et al.*, 1995). Although multiple receptors exist within the two purinoceptor classes, pharmacological resolution of these receptor subtypes has been difficult due to lack of availability of purinoceptor antagonists and subtype-selective agonists. Molecular cloning of DNA sequences encoding G protein-coupled P_2-purinoceptors is beginning to shed light on the molecular complexity of the receptor proteins that comprise this important class of signaling proteins (Lustig *et al.*, 1993; Webb *et al.*, 1993; Parr *et al.*, 1994; Filtz *et al.*, 1994; Lazarowski *et al.*, 1995).

We have recently developed two model systems for biochemical and pharmacological analyses of P_{2Y}-purinoceptors. The turkey erythrocyte expresses a P_{2Y}-purinoceptor that markedly activates a phospholipase C-β isoenzyme through the G-protein, G_{11} (Boyer *et al.*, 1989; Waldo *et al.*, 1991; Maurice *et al.*, 1993). This purinoceptor has been cloned (Filtz *et al.*, 1994) and shown to be the turkey homologue of the P_{2Y1}-purinoceptor that also has been cloned from chick (Webb *et al.*, 1993) and from several mammalian species, including man (Schachter *et al.*, 1996). C6 rat glioma cells express a P_{2Y}-purinoceptor that inhibits adenylyl cyclase but does not activate phospholipase C (Boyer *et al.*, 1993; 1994; 1995). Differences in specificity of activation of second messenger signaling cascades between the P_{2Y}-purinoceptors of C6 and turkey erythrocytes are not explained by differences in the cell type in which these receptors are expressed. For example, expression of the human or turkey P_{2Y1}-purinoceptor in C6 cells confers a purinoceptor-stimulated inositol lipid response with no evidence of a change in cyclic AMP response of these cells (Schachter *et al.*, 1996). Marked differences in pharmacological selectivities also have been observed between the P_{2Y}-purinoceptors on turkey erythrocytes and C6 cells (Boyer *et al.*, 1994; 1995).

Using these two model systems for quantification of activities of drugs at two different P_{2Y}-purinoceptors, our laboratories have initiated a systematic synthesis and analysis of adenine nucleotide analogues that are potent and receptor-selective agonists for P_{2Y}-purinoceptors. We have observed previously that whereas adenosine monophosphate (AMP) is not a P_{2Y}-purinoceptor agonist, a base-substituted adenosine monophosphate analogue exhibited full agonist activity at the turkey erythrocyte P_{2Y}-purinoceptor (Fischer *et al.*, 1993). Here we expand these observations to a broad range of analogues of adenosine monophosphate, and examine the receptor subtype selectivity of these molecules. Monophosphate analogues were identified that are 10,000 fold more potent than adenosine 5′-triphosphate (ATP) at the P_{2Y}-purinoceptor of C6 glioma cells.

Methods

Cell culture

C6 rat glioma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum in

[1] Author for correspondence.
[2] Present address: Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel.

a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged by trypsinization. Experiments were carried out in confluent cultures 2–4 days after plating in 12-well clusters as previously described (Boyer *et al.*, 1995).

Cyclic AMP accumulation

Cells were labelled for 2 h with 1 μCi of [^{3}H]-adenine ml^{-1} (Meeker & Harden, 1983). Cells were washed twice with HEPES (20 mM, pH 7.5)-buffered Eagle's medium and then preincubated for 10 min at 37°C with HEPES-Eagle's medium containing 200 μM 3-isobutyl-1-methylxanthine. Agonist incubations were initiated by the simultaneous addition of 10 μM isoprenaline and various concentrations of P_{2Y}-purinoceptor agonists. The reactions were stopped after 10 min by aspiration of the drug-containing medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. [^{3}H]-adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation was determined by Dowex and alumina chromatography as previously described (Harden *et al.*, 1982).

Turkey erythrocyte labelling

Fresh blood was obtained from female turkeys by venous puncture and collected into a heparinized syringe. Erythrocytes were washed twice by centrifugation and resuspension with sterile DMEM, followed by a final wash in inositol-free DMEM. One ml 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 [^{3}H]-inositol. Cells were incubated in a stirred glass vial for 16–20 h at 37°C in a humidified atmosphere of 95% air, 5% CO_2 as previously described in detail (Harden *et al.*, 1988; Boyer *et al.*, 1989).

Phospholipase C assay

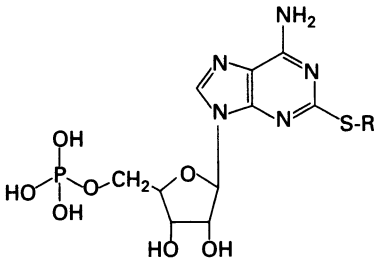
Erythrocyte ghost membranes were prepared from [^{3}H]-inositol-labelled cells by hypotonic lysis in 15 volumes of a buffer containing 5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA (lysis buffer). The membranes were washed three times by centrifugation and resuspension with lysis buffer. The final resuspension was in 20 mM HEPES, pH 7.0, to a concentration of 6 mg protein ml^{-1}. This preparation was used immediately for assay of phospholipase C. Twenty five μl of [^{3}H]-inositol-labelled membrane preparation ($\approx$150 μg protein; 200,000 c.p.m.) was combined in a final volume of 200 μl of a medium containing 0.91 mM $MgSO_4$, 115 mM KCl, 5 mM potassium phosphate, 0.424 mM $CaCl_2$ 2 mM EGTA, 10 mM HEPES, pH 7.0 (free Ca^{2+} concentration was $\approx$1 μM). Since receptor-and G-protein activation of phospholipase C in turkey erythrocyte membranes is strictly dependent on the presence of guanine nucleotides (Boyer *et al.*, 1989), the non-hydrolyzable analogue of guanosine 5′-triphosphate (GTP), GTPγS (1 μM) was included in the assay. Membranes were incubated for 5 min at 30°C with the indicated concentrations of P_{2Y}-purinoceptor agonists. Incubations were terminated by the addition of 1 ml of ice cold 5% trichloroacetic acid. [^{3}H]-inositol phosphates were isolated as previously described (Harden *et al.*, 1988; Boyer *et al.*, 1989).

Assay of activity of agonists at the human P_{2U}-purinoceptor

The activity of agonists was also tested in 1321N1 human astrocytoma cells stably expressing the cloned human P_{2U}-purinoceptor (Parr *et al.*, 1994; Lazarowski *et al.*, 1995). Inositol phosphate accumulation was measured as described previously (Lazarowski *et al.*, 1995).

Synthesis of 2-thioether analogues of AMP

A series of chain extended 2-thioether substituted analogues of AMP were synthesized, purified, and chemically characterized by the general methods we previously described (Fischer *et al.*, 1993; van Rhee *et al.*, 1995).



Compound	R
2-Pentylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-
2-Hexylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-
2-Heptylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-
2-Octylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-
2-Decylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-
2-Undecylthio AMP	CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-
2-(2-Ethylthio-ethylthio) AMP	CH_3-CH_2-S-CH_2-CH_2-
2-Cyclopentylthio AMP	
2-(5-Hexenylthio) AMP	CH_2=CH-CH_2-CH_2-CH_2-CH_2-
2-(6-Cyanohexylthio) AMP	NC-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-
2-(4-methyl-4-pentenyl)thio AMP	CH_2=C(CH_3)-CH_2-CH_2-CH_2-
2-(*p*-nitrophenylethyl)thio AMP	O_2N-C$_6$H$_4$-CH_2-CH_2-

Figure 1 Structure of 2-thioether adenosine monophosphates.

Data analysis

Agonist potencies were calculated by 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 in duplicate or triplicate assays.

Materials

2-Methylthio adenosine triphosphate (2-MeSATP) was obtained from Research Biochemicals Inc. (Natick, MA); (−)-isoprenaline (+)-bitartrate was from Sigma Chemical Co. (St. Louis, MO); 2-[^{3}H]myo-inositol (20 Ci mmol^{-1}) and [8-^{3}H]-adenine (27 Ci mmol^{-1}) were obtained from American Radiolabelled Chemicals, Inc. (St. Louis, MO); inositol-free DMEM was from Gibco BRL (Grand Island, NY). The sources of other materials have been stated previously (Boyer *et al.*, 1989; 1994; 1995).

Results

A series of derivatives of adenosine 5'-monophosphate were synthesized as chain-extended thioether-substituted molecules at the 2-position of the adenine base (Figure 1). These 2-thioether derivatives of AMP included molecules of alkyl chain length ranging up to eleven carbons and analogues with alkenyl-, thioether-, cyano-, isopropyl-, or p-nitrophenylethyl-thioether substitutions. Analogues of AMP and ATP, with substitutions in the N^6-position of the adenine base, were synthesized (van Rhee *et al.*, 1995) or obtained from commercial sources. The activities of these analogues were quantified in two different tests of P_{2Y}-purinoceptor activity. The capacity of agonists to activate phospholipase C was determined in membranes prepared from turkey erythrocytes, and the capacity of the analogues to inhibit isoprenaline-stimulated cyclic AMP accumulation was determined in C6 glioma cells.

Although AMP itself was not an agonist in either of the P_{2Y}-purinoceptor test systems, 2-thioether-substituted analogues of AMP were remarkably potent full agonists i.e. produced the same maximal response as 2MeSATP or ATP (Figure 2). The most potent agonists were 2-hexylthio AMP, 2-(5-hexenylthio) AMP, and 2-pentylthio AMP, which activated the C6 cell purinoceptor with EC_{50} values of 0.2 nM, 2 nM and 4 nM, respectively (Figure 2, Table 1). Not only were these analogues full agonists at a purinoceptor in which statistically significant activation of the receptor was not observed with the parent molecule, AMP, but they also were 16,000 fold, 1600 fold, and 800 fold more potent, respectively, than the natural receptor agonist, ATP (Figures 2 and 3). These derivatives of AMP also were remarkably potent compared to the very potent corresponding structural analogues of ATP. For example, the potencies of 2-hexylthio ATP and 2-(5-hexenylthio) ATP (EC_{50} values = 28 and 117 pM, respectively), which we have previously obtained for the C6 cell P_{2Y}-purinoceptor (Boyer *et al.*, 1995), were only approximately 10 fold greater than the potencies of the corresponding AMP derivatives (Table 1).

The monophosphate analogues that were the most potent agonists at the C6 cell P_{2Y}-purinoceptor also were the most potent agonists for activation of the turkey erythrocyte P_{2Y}-purinoceptor (Table 1). Although the order of potency of eight full agonist monophosphate analogues was essentially the same for the C6 cell and turkey erythrocyte receptors, the absolute potencies were consistently higher for the C6 cell receptor than for the receptor on turkey erythrocytes (Table 1). The largest difference in agonist potency between the two test systems was for 2-hexylthio AMP, which was approximately 300 fold more potent for the C6 cell P_{2Y}-purinoceptor than for the turkey erythrocyte receptor. In contrast, the potency of 2-cyanohexylthio AMP differed by only 10 fold between the two receptors.

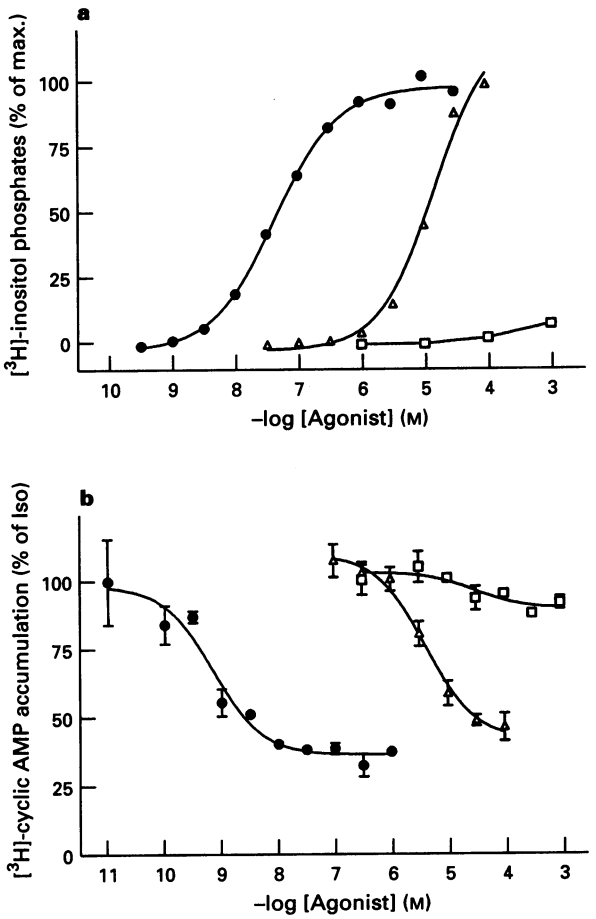


Figure 2 Effects of 2-hexylthio AMP, ATP and AMP on phospholipase C- and adenylyl cyclase-coupled P_{2Y}-purinoceptors. The capacity of the indicated concentrations of 2-hexylthio AMP (●), ATP (Δ), or AMP (□) to stimulate the accumulation of inositol phosphates in turkey erythrocyte membranes (a), or to inhibit isoprenaline-stimulated cyclic AMP accumulation in C6 rat glioma cells (b) was determined as described in Methods. pEC_{50} for ATP was 5.36±0.17 in turkey erythrocyte membranes and 5.51±0.07 in C6 rat glioma cells (from Boyer *et al.*, 1995). Data shown are the mean of triplicate (cyclic AMP) or duplicate (inositol phosphate) assays and the results are representative of those obtained in three separate experiments. The maximal response (100%) of inositol phosphates in turkey erythrocytes was obtained with saturating concentrations of 2MeSATP (21263±958 c.p.m.). For C6 cells, 100% value corresponds to the cyclic AMP accumulation obtained in response to 10 μM isoprenaline in the absence of adenine nucleotide analogue (35705±2563 c.p.m.).

Based on the high potency of 2-hexylthio AMP, a series of analogues were prepared with alkyl chains ranging in length up to eleven carbons. Although all of these analogues were full agonists with potencies greater than that of ATP in the two test systems, none of these agonists were more potent than 2-hexylthio AMP (Table 1). Introduction of a double bond in the alkyl side chain of 2-hexylthio AMP, i.e. 2-(5-hexenylthio) AMP, decreased potency by approximately 10 fold compared to the high potency of the corresponding analogue with a saturated side chain (Figures 2 and 3).

Introduction of other functional groups into the 2-thioether-substituted position of AMP produced variable changes in potency relative to 2-thioether derivatives bearing an alkyl side chain. For example, the potencies of the ethylthio-ethylthio- derivative were similar to those observed with the standard alkyl chain-extended analogues such as 2-pentyl-, 2-hexyl, 2-octylthioAMP (Table 1). In contrast, cyanohexyl-substitution resulted in an AMP analogue with potencies 500 and 25,000 lower than 2-hexylthioAMP in turkey erythrocytes and C6 cells, respectively (Table 1).

Table 1 2-Thioether adenine nucleotide monophosphates are potent and selective agonists of the phospholipase C- and adenylyl cyclase-coupled P_{2Y}-purinoceptors

Nucleotide	*Turkey erythrocytes* pEC_{50}	*C6 rat glioma cells* pEC_{50}	*hP_{2U}-1321N1 cells* pEC_{50}
5'-AMP	No effect	No effect	No effect
2-Pentylthio AMP	6.04±0.06	8.30±0.21	Small or no effect
2-Hexylthio AMP	7.23±0.07	9.88±0.35	10% at 100 μM
2-Heptylthio AMP	6.19±0.05	N/A	No effect
2-Octylthio AMP	6.53±0.14	N/A	No effect
2-Decylthio AMP	6.52±0.14	7.57±0.32	No effect
2-Undecylthio AMP	5.60±0.16	N/A	No effect
2-(2-Ethylthio-ethylthio) AMP	6.20±0.29	7.47±0.08	No effect
2-Cyclopentylthio AMP	5.12±0.13	8.41 (n=1)	No effect
2-(5-Hexenylthio) AMP	6.47±0.05	8.56±0.17	No effect
2-(6-Cyanohexylthio) AMP	4.52±0.22	5.48±0.24	N/A
2-(4-Methyl-4-pentenyl)thio AMP	15–30% at 1 mM	N/A	No effect
2-(*p*-Nitrophenylethyl)thio AMP	5.60±0.21	7.03±0.26	N/A
N^6 Me-2-hexenylthio AMP	<4	5.50±0.30	N/A

pEC_{50} values were determined as indicated in Methods, and all values are the mean±s.e.mean of values from at least three different determinations. Whenever the potency of the drug allowed, the maximal response induced by the AMP analogue was identical to that of a maximally effective concentration of 2MeSATP. Where a partial effect is indicated, it was observed only with the highest concentration of drug tested (no partial agonism was observed). N/A: not assayed; hP_{2U}-1321N1 cells: 1321N1 cells stably expressing the human P_{2Y}-purinoceptor.

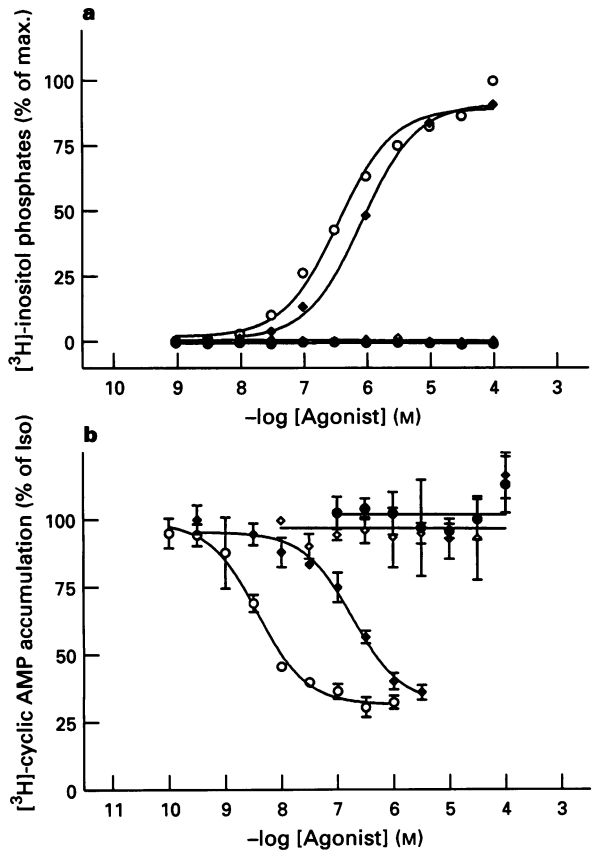


Figure 3 Effects of 2-thioether adenosine and 2-thioether AMP analogues on phospholipase C- and adenylyl cyclase-coupled P_{2Y}-purinoceptors. The capacity of the indicated concentrations of 2-(*p*-nitrophenylethyl)thio AMP (◆), 2-(*p*-nitrophenylethyl)thio adenosine (◇), 2-(5-hexenylthio) AMP (○), and 2-(5-hexenylthio) adenosine (●) to stimulate the accumulation of inositol phosphates in turkey erythrocyte membranes (a), or to inhibit isoprenaline-stimulated cyclic AMP accumulation in C6 rat glioma cells (b) was determined as described in Methods. Data shown are the mean of triplicate (cyclic AMP) or duplicate (inositol phosphate) assays and the results are representative of those obtained in three separate experiments. Values for the maximal response (100%) of inositol phosphates and cyclic AMP accumulation were 12964±492 and 35482±655 c.p.m. respectively. See legend of Figure 2 for details.

Neither 2-(p-nitrophenylethyl)thio adenosine nor 2-(5-hexenylthio) adenosine were agonists at the two P_{2Y}-purinoceptors (Figure 3). Thus, optimization of structure sufficient to produce potent full agonist molecules that possess a single phosphate was not sufficient for production of a non-nucleotide agonist.

We have previously shown that whereas N^6-substituted ATP analogues retain their potency relative to ATP at P_{2Y}-purinoceptors, these compounds are not agonists at P_{2X}-purinoceptors present on various smooth muscle preparations (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). Substitution in the N^6-position alone, e.g. N^6-dimethyl AMP and N^6-phenylethyl AMP, was insufficient to produce AMP derivatives that exhibited P_{2Y}-purinoceptor activity (Table 2). However, the doubly substituted analogue, N^6-methyl-2-(5-hexenylthio) AMP was a full agonist at the C6 cell P_{2Y}-purinoceptor, although its potency (EC_{50}=3 μM) was approximately 1000 fold less than the potency of 2-hexenylthio AMP at this receptor. N^6-methyl-2-(5-hexenlylthio) AMP was also an agonist at the turkey erythrocyte P_{2Y}-purinoceptor, although its relatively low potency precluded full concentration-effect analysis.

Although the chain-extended analogues of adenosine monophosphate were potent full agonists at P_{2Y}-purinoceptors, none of the molecules activated the human P_{2U}-purinoceptor (P_{2Y2}-purinoceptor) stably expressed in 1321N1 human astrocytoma cells (Table 1; Figure 4). Under these same conditions ATP and uridine 5'-triphosphate (UTP) produced marked activation of the P_{2U}-purinoceptor.

Discussion

P_{2X}- and P_{2Y}-purinoceptors initially were delineated by analogues of ATP (Burnstock & Kennedy, 1985). However, the existence of a receptor on platelets that is activated by adenosine 5'-diphosphate (ADP), but antagonized by ATP, provided support for the idea that purinoceptors need not be selective for ATP and triphosphate analogues (Hourani & Cusack, 1991). This idea has been expanded by subsequent work that has illustrated that ADP is a potent P_{2Y}-purinoceptor agonist, and with at least one cloned P_{2Y}-purinoceptor subtype, the P_{2Y1}-purinoceptor, ADP is one order of magnitude more potent that ATP (Filtz *et al.*, 1994; Schachter *et al.*, 1996). The work described here confirms the idea that neither a tri- nor diphosphate is necessary for full P_{2Y}-purinoceptor agonist activity, and that derivatives of adenosine

Table 2 N^6-derivatives of adenine nucleotide monophosphates as agonists of the phospholipase C- and adenylyl cyclase-coupled P_{2Y}-purinoceptors

Nucleotide	*Turkey erythrocytes* pEC_{50}	*C6 rat glioma cells* pEC_{50}
N^6-phenylethyl ATP	5.15±0.21	N/A
N^6-phenylethyl AMP	No effect	No effect $n=2$
N^6-dimethyl AMP	$<<4$	No effect*
N^6 Me-2-hexenylthio AMP	<4	5.50±0.30

pEC_{50} values were obtained as indicated in Methods. Except when indicated, all values are the mean±s.e.mean from at least 3 separate determinations. N/A: not assayed. *$n=1$.

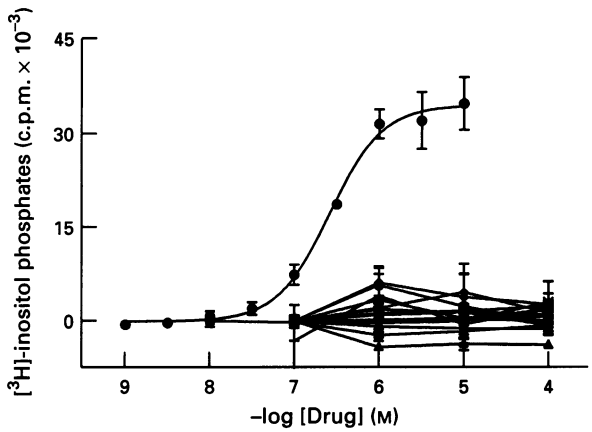


Figure 4 Lack of effect of 2-thioether adenosine monophosphates on the activation of phospholipase C in 1321N1 cells expressing the human P_{2U}-purinoceptor. Inositol phosphate responses to UTP (●) or the 2-thioether derivatives of AMP shown in Table 1 (all other curves) were studied in 1321N1 cells stably expressing the human P_{2U}-purinoceptor. pEC_{50} value for UTP is 6.46±0.10. Data shown are the mean of triplicate assays, and the results are representative of those obtained in three separate experiments.

monophosphate can be synthesized that exhibit potencies up to 3000 fold greater than that of ATP at the same receptor.

The observed lack of agonist activity of AMP at P_{2Y}-purinoceptors in various tissue preparations, in the model P_{2Y}-purinoceptor systems that we have used in this study, and with cloned and expressed P_{2Y}-purinoceptors suggests that AMP itself is not an endogenous agonist for P_{2Y}-purinoceptors. However, the identification by Cusack and colleagues (see Cusack *et al.*, 1988) over a decade ago of 2MeSATP as a potent P_{2Y}-purinoceptor agonist formed the initial basis on which full agonists could be generated with molecules containing only a single phosphate group. The strategy we have applied is to optimize structure in a series of chain-extended 2-thioether analogues of ATP, and then to apply this knowledge of optimized substitution to adenine-containing analogues bearing less than three phosphates. Optimization was initially accomplished with analogues of ADP, which to no surprise based on the relative activities of ADP, ATP, ADPβS, and ATPγS at P_{2Y}-purinoceptors, resulted in diphosphate analogues that were at least as potent as the corresponding triphosphate analogues (Fischer *et al.*, 1993). However, extension of these syntheses to derivatives of adenosine monophosphate produced surprising results. Analogues that are full agonists exhibiting remarkable potencies were derived from the parent AMP molecule, which is inactive at P_{2Y}-purinoceptors. Thus, substitutions, e.g. hexylthio-, that resulted in up to 100,000 fold increases in potency of triphosphate analogues compared to the parent ATP molecule, resulted in generation of adenosine monophosphate analogues that are P_{2Y}-purinoceptor agonists. Not only are these monophosphate molecules full agonists, but their potencies are in most cases in the nM range of EC_{50} values and these molecules are only 10–30 fold less potent than the corresponding ATP analogues. Substitutions that resulted in the greatest increase in potency of ATP analogues in general resulted in the most potent monophosphate analogues. For example, we have previously demonstrated the remarkable high potency ($EC_{50}=30$ pM) of 2-hexylthio ATP (Boyer *et al.*, 1995), and 2-(5-hexenylthio) AMP exhibits an equally remarkable high potency ($EC_{50}=200$ pM).

The possibility that 2-thioether derivatives of AMP used in this study are converted into their di- or triphosphate forms under our assay conditions seems unlikely, especially in the turkey erythrocyte membrane preparation where there is no source of ATP for phosphorylation of the monophosphate analogue. Interconversion is more likely to occur in incubations with intact cells since ATP can be released in significant amounts. However, h.p.l.c. analyses indicated that 2-cyclopentylthio AMP was not converted to a di- or triphosphate derivative during incubation with turkey erythrocyte membranes or C6 glioma cells. Therefore, the pharmacological effects of these 2-thioether AMP analogues are not a consequence of conversion of the compounds to more active di- or triphosphate derivatives.

The turkey erythrocyte and C6 glioma cell model systems provide two measures of P_{2Y}-purinoceptor activity that we have previously shown to involve two different P_{2Y}-purinoceptors that exhibit different specificities of coupling to second messenger signaling cascades and different pharmacological selectivities (Boyer *et al.*, 1993; 1994; 1995). Similar results were obtained with monophosphate analogues at each of these two P_{2Y}-purinoceptors. However, as has been the case in previous studies, the observed absolute potencies differed between the two receptors. Monophosphate agonists were in general more potent at the C6 cell P_{2Y}-purinoceptor, but the difference in potencies between the two receptors ranged from 10 fold to 300 fold, which provides additional pharmacological evidence of differences in these two signaling proteins.

Although there is no unambiguous evidence to suggest that any molecule other than ATP or ADP exists as a physiologically important agonist for P_{2Y}-purinoceptors, the results indicate that a single phosphate group is sufficient for P_{2Y}-purinoceptor activity if the adenine nucleotide molecule is appropriately modified. The simplest interpretation of these observations is that 2-thioether-substitution of the adenine base stabilizes the configuration of the resulting analogue in a conformation favourable for interaction of the single phosphate within the receptor binding pocket. This stabilization was sufficient for full agonist activity with all of the 2-thioether-substituted analogues, and was most favourable with certain substitutions, e.g. 2-hexylthio, that also produced the most favourable increase in potency for the corresponding ATP analogue. The presence of 5′-phosphate is essential for effective binding to the receptor, since the adenosine analogues of two potent thioether monophosphates were devoid of agonistic or antagonistic activity at these receptors (Figure 3 and data not shown).

ATP and ATPγS are potent full agonists (Parr *et al.*, 1994; Lazarowski *et al.*, 1995) at the human P_{2U}-purinoceptor (P_{2Y2}-receptor). Optimization of substitutions of ATP that generate potent P_{2Y}-purinoceptor agonist and extension of this knowledge to produce potent monophosphate agonists resulted in molecules that, nevertheless, have no activity at the human P_{2U}-purinoceptor. Thus, the nucleotide binding site for the ATP-preferring P_{2Y}-purinoceptors can be readily targeted by the chain-extended 2-thioether substitutions of the monophosphates studied here. Although the interactions of these analogues of AMP with nucleotidases, ATPases, or other ATP binding proteins has not been examined, we speculate that

these molecules will exhibit high selectivity for the ATP-preferring P_{2Y}-purinoceptors over other nucleotide receptors and other triphosphate binding proteins.

In summary, the results presented here confirm that a single phosphate group is sufficient and necessary for full activity of two different P_{2Y}-purinoceptor subtypes. Although the receptor selectivity of these novel P_{2Y}-purinoceptor agonists has not been fully investigated, these compounds are likely to be highly selective for P_{2Y}-purinoceptors and therefore, represent a new structural template on which selective and perhaps more specific P_{2Y}-purinoceptor agonists, and potentially antagonists, can be designed.

The authors are indebted to Dr Eduardo Lazarowski for critical discussions and assistance on h.p.l.c. analysis, and to Brenda Lindsey Asam for typing the manuscript. This work was supported by USPHS grants GM38213 and HL32322.

References

BOYER, J.L., DOWNES, C.P. & HARDEN T.K. (1989). Kinetics of activation of phospholipase C by P_2 purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.* **264,** 884–890.

BOYER, J.L., LAZAROWSKI, E.R., CHEN, X.-H. & HARDEN, T.K. (1993). Identification of a P_{2Y}-purinergic receptor that inhibits adenylyl cyclase but does not activate phospholipase C. *J. Pharmacol. Exp. Ther.*, **267,** 1140–1146.

BOYER, J.L., O'TUEL, J.W., FISCHER, B., JACOBSON, K.A. & HARDEN, T.K. (1995). 2-Thioether derivatives of adenosine nucleotides are exceptionally potent agonists at adenylylcyclase-linked P_{2Y}-purinoceptors. *Br. J. Pharmacol.*, **116,** 2611–2616.

BOYER, J.L., ZOHN, I., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of putative P_2-purinoceptor antagonists on adenylyl cyclase- and phospholipase C-coupled P_{2Y}-purinoceptors. *Br. J. Pharmacol.*, **113,** 614–620.

BURNSTOCK, G., FISCHER, B., HOYLE, C.H.V., MAILLARD, M., ZIGANSHIN, A.U., BRIZZOLARA, A.L., VON ISAKOVICS, A., BOYER, J.L., HARDEN, T.K. & JACOBSON, K.A. (1994). Structure activity relationships for derivatives of adenosine-5'-triphosphate as agonists at P_2-purinoceptors: heterogeneity within P_{2X} and P_{2Y} subtypes. *Drug Dev. Res.*, **31,** 206–219.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptors? *Gen. Pharmacol.*, **16,** 433–440.

CUSACK, N.J., WELFORD, L.A. & HOURANI, S.M.O. (1988). Studies on the P_2-purinoceptor using adenine nucleotide analogues. In *Adenosine and Adenine Nucleotides: Physiology and Pharmacology*. ed D.M. Paton. pp. 73–84. New York: Taylor & Francis.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265,** C577–C606.

FILTZ, T.M., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1994). Expression of a cloned P_{2Y}-purinergic receptor that couples to phospholipase C. *Mol. Pharmacol.*, **46,** 8–14.

FISCHER, B., BOYER, J.L., HOYLE, C.H.V., ZIGANSHIN, A.U., BRIZZOLARA, A.L., KNIGHT, G.E., ZIMMET, J., BURNSTOCK, G., HARDEN, T.K. & JACOBSON, K.A. (1993). Identification of potent, selective P_{2Y}-purinoceptor agonists: structure activity relationships for 2-thioether derivatives of adenosine-5'-triphosphate. *J. Med. Chem.*, **36,** 3937–3946.

HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. P_2-purinergic receptors: subtype-associated signaling responses and structure. *Ann. Rev. Pharmacol. Toxicol.*, **35,** 541–579.

HARDEN, T.K., HAWKINS, P.T., STEPHENS, L., BOYER, J.L. & DOWNES, C.P. (1988). Phosphoinositide hydrolysis by guanosine 5'-0-(3-thiotriphosphate)-activated phospholipase C of turkey erythrocyte membranes. *Biochem. J.*, **252,** 583–593.

HARDEN, T.K., SCHEER, A.G. & SMITH, M.M. (1982). Differential modification of the interaction of cardiac muscarinic cholinergic and β-adrenergic receptors with a guanine nucleotide binding site(s). *Mol. Pharmacol.*, **21,** 570–580.

HOURANI, S.M.O. & CUSACK, N.J. (1991). Pharmacological receptors on blood platelets. *Pharmacol. Rev.*, **43,** 243–298.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the cloned human phospholipase C-linked P_{2U}-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.*, **116,** 1619–1627.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 5113–5117.

MAURICE, D.H., WALDO, G.L., MORRIS, A.J., NICHOLAS, R.A. & HARDEN, T.K. (1993). Identification of $G\alpha_{11}$ as the phospholipase C-activating G-protein of turkey erythrocytes. *Biochem. J.*, **290,** 765–770.

MEEKER, R.B. & HARDEN, T.K. (1983). Muscarinic cholinergic receptor-mediated control of cyclic AMP metabolism. *Mol. Pharmacol.*, **23,** 384–392.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacology. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 3275–3279.

SCHACHTER, J., QING, L., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human P_{2Y1}-purinoceptor. *Br. J. Pharmacol.*, **118,** 167–173.

VAN RHEE, A.M., FISCHER, B., VAN GALEN, P.J.M. & JACOBSON, K.A. (1995). Modelling the P_{2Y} purinoceptor using rhodopsin as template. *Drug Design and Discovery*, **13,** 133–154.

WALDO, G.L., BOYER, J.L., MORRIS, A.J. & HARDEN, T.K. (1991). Purification of an AlF_4- and G-protein $\beta\gamma$-subunit-regulated phospholipase C-activating protein. *J. Biol. Chem.*, **261,** 14217–14225.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett.*, **324,** 219–225.

(Received February 8, 1996
Revised April 25, 1996
Accepted April 29, 1996)