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Potent agonist action of 2-thioether derivatives of adenine nucleotides at adenylyl cyclase-linked P_{2Y}-purinoceptors

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1 Analogues of adenine nucleotides inhibited β-adrenoceptor-stimulated cyclic AMP accumulation in C6 rat glioma cells with a pharmacological selectivity consistent with that for involvement of a P_{2Y}-purinoceptor.

2 The inhibitory effect of adenine nucleotides was completely prevented by pretreatment of cells with pertussis toxin.

3 The capacity of a series of recently synthesized 2-thioether analogues of adenine nucleotides to inhibit cyclic AMP accumulation was examined. Several ATP analogues, e.g. 2-cyclohexylthio and 2-hexylthio ATP, inhibited cyclic AMP accumulation with EC_{50} values of approximately 30 pM. These values represent 100,000 fold increases in potency over ATP.

4 Analogues of ADP exhibited the same remarkable increase in potency relative to their natural congener and diphosphates were at least as potent as the corresponding triphosphates at the C6 cell P_{2Y}-purinoceptor.

5 The relative potencies of a broad series of agonists at the C6 cell receptor did not correspond to the relative potencies of the same compounds for activation of P_{2Y}-purinoceptors on turkey erythrocyte membranes. Some agonists, particularly 2-thioether derivatives were more potent for stimulation of the C6 cell receptor, whereas other agonists were more potent in the turkey erythrocyte system.

6 These results add further support to the view that the adenylyl cyclase-linked P_{2Y}-purinoceptor of C6 rat glioma cells is a different subtype from the phospholipase C-linked P_{2Y}-purinoceptor of turkey erythrocyte membranes and several mammalian tissues

Keywords: P_{2Y}-purinoceptors; cyclic AMP accumulation; adenylyl cyclase inhibition; C6 rat glioma cells; 2-thioether derivatives of adenine nucleotides

Introduction

Extracellular adenine nucleotides interact with cell surface P_2-purinoceptors in the central nervous system and peripheral tissues to produce a broad range of physiological responses (Dubyak & El-Moatassim, 1993). Classification of P_2-purinoceptors has proved difficult since no selective P_2-receptor antagonists are available. Nonetheless, observation of differential pharmacological effects of analogues of ATP led Burnstock & Kennedy (1985) to propose that at least two subtypes of P_2-purinoceptors exist. Pharmacological responses mediated through P_{2X}-purinoceptors, e.g. contraction of smooth muscle, occurred with the potency order of α,β-methyleneATP > β,γ-methyleneATP > ADP > 2-methylthioATP. Pharmacological responses mediated through P_{2Y}-purinoceptors, e.g. relaxation of smooth muscle, occurred with the potency order of 2-methylthioATP > ATP > > α,β-methyleneATP = β,γ-methyleneATP. P_2-purinoceptors in addition to the P_{2X}- and P_{2Y}-subtypes exist (Dubyak & El-Moatassim, 1993; Fredholm *et al.*, 1994; Harden *et al.*, 1995). The P_{2T}-purinoceptor is activated by ADP but not ATP and is expressed on platelets (Haslam & Cusack, 1981; Hourani & Cusack, 1991). The P_{2U}-purinoceptor responds to ATP, UTP, and ATPγS, but only weakly or not at all to other analogues of ATP including those that are selective for P_{2X}- and P_{2Y}-purinoceptors (Dubyak *et al.*, 1988; Fine *et al.*, 1989; Brown *et al.*, 1991; Stutchfield & Cockcroft, 1990). High concentrations of ATP permeabilize cells through the so-called P_{2Z}-purinoceptor (Cockcroft & Gomperts, 1980; Dubyak & El-Moatassim, 1993), but the general physiological significance of this entity is not known.

Evidence also exists for the need for subclassification within the major P_2-purinoceptor subtypes. For example, differences in the relative potencies of a series of adenine nucleotide analogues in several preparations led to the suggestion that multiple P_{2X}- and P_{2Y}-purinoceptors occur (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). cDNAs encoding two different P_{2X}-purinoceptors that exhibit different pharmacological selectivities have been cloned recently (Valera *et al.*, 1994; Brake *et al.*, 1994). Studies with two model cell systems also suggest the existence of multiple P_{2Y}-purinoceptor subtypes. The P_{2Y}-purinoceptor on C6-2B rat glioma cells inhibits adenylyl cyclase, but has no effect on phospholipase C activity (Boyer *et al.*, 1993), whereas the P_{2Y}-purinoceptor on turkey erythrocyte membranes activates phospholipase C but does not regulate adenylyl cyclase (Boyer *et al.*, 1989). These results are reminiscent of the G-protein/effector enzyme coupling selectivities in other receptor families. For example, m_1, m_3, and m_5 muscarinic cholinoceptors activate phospholipase C, whereas m_2 and m_4 muscarinic cholinoceptors inhibit adenylyl cyclase (Hulme *et al.*, 1990). The idea that the P_{2Y}-purinoceptor on C6 glioma cells differs from that on turkey erythrocytes also has been supported by the fact that pyridoxal phosphate 6-azophenyl 2',4'-disulphonic acid (PPADS) and reactive blue 2 are markedly different in their effectiveness as antagonists in the two model systems (Boyer *et al.*, 1994).

Fischer *et al.* (1993) recently have reported the synthesis and relative pharmacological activities of a set of analogues of adenine nucleotides substituted on the purine ring as chain-extended 2-thioethers. We have expanded our study of these compounds to C6 rat glioma cells in which several analogues of ATP displayed remarkable potency in the pM range for P_{2Y}-purinoceptor activation. The relative potencies of a broad

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range of agonists at this adenylyl cyclase-coupled P_{2Y}-purinoceptor differed substantially from the relative potencies of these agonists for activation of the phospholipase C-coupled P_{2Y}-purinoceptor of turkey erythrocytes.

Methods

Cell culture

C6 rat glioma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum in 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.*, 1993).

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]-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^{++} 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 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. Samples were centrifuged, the supernatants were transferred to a fresh tube, and the trichloroacetic acid was extracted three times with 3 ml of ethyl ether. Neutralized samples of the aqueous layer were diluted with 8 ml of water and transferred onto Dowex AG-X8 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 (IP_2 through IP_4) were eluted with 5 ml of 1.2 M ammonium formate, 100 mM formic acid, and collected in scintillation vials (Harden *et al.*, 1988; Boyer *et al.*, 1989). [^{3}H]-inositol phosphates were quantitated by scintillation spectroscopy.

Materials

2-Methylthio adenosine triphosphate (2-MeSATP) was obtained from Research Biochemicals International. (Natick, MA, U.S.A.); (−)-isoprenaline (+)-bitartrate and 8-(6-aminohexylamino)ATP were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 2-[^{3}H]-*myo*-inositol (20 Ci mmol^{-1}) and [8-^{3}H]-adenine (27 Ci mmol^{-1}) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.); inositol-free DMEM was from Gibco BRL (Grand Island, NY, U.S.A.). The sources of other materials have been reported previously (Boyer *et al.*, 1989; 1993; 1994).

Synthesis of 2-thioether analogues of ATP and ADP

A series of chain extended 2-thioether substituted analogues of ATP and ADP were synthesized, purified and chemically characterized as described previously (Fischer *et al.*, 1993).

Data analysis

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

Results

We have reported previously that ATP, ADP, and several adenine nucleotide analogues inhibit isoprenaline-stimulated cyclic AMP accumulation in C6-2B and C6 rat glioma cells with a pharmacological specificity consistent with that of a P_{2Y}-purinoceptor (Boyer *et al.*, 1993). The parent C6 glioma cell from which C6-2B cells were subcloned (de Vellis & Brooker, 1973) also expresses P_{2Y}-purinoceptors as evidenced by potent inhibition of cyclic AMP accumulation by the P_{2Y}-purinoceptor agonist, 2-MeSATP (Boyer *et al.*, 1994). The maximal inhibition observed with 2-MeSATP was consistently much greater (60–80% inhibition) than we previously observed with C6-2B cells (Figure 1a and Boyer *et al.*, 1993). Pretreatment of cells with pertussis toxin completely blocked the capacity of 2-MeSATP to inhibit cyclic AMP accumulation (Figure 1a), and a concentration-effect curve revealed that this inhibition occurred at low concentrations of pertussis toxin (Figure 1b). Although 2-MeSATP was a very potent agonist for inhibition of cyclic AMP accumulation, the P_{2X}-purinoceptor agonists, α,β-methyleneATP and β,γ-methyleneATP, had little or no effect (Figure 2). As was the case with C6-2B cells, ADP was approximately 50 fold more potent that ATP for inhibition of cyclic AMP accumulation in C6 glioma cells. The overall comparative potencies of the eight adenine nucleotide analogues were consistent with the involvement of a P_{2Y}-purinoceptor (Figure 2).

We earlier reported the synthesis of a series of 2-thioether derivatives of ATP and ADP (Fischer *et al.*, 1993). The activities of these compounds were tested at the adenylyl cyclase-linked P_{2Y}-purinoceptor on C6 glioma cells. Concentration effect curves for 2-(5-hexenylthio)ATP, 2-(5-hexenylthio)ADP and ATP are compared in Figure 3. The tri- and diphosphate analogues exhibited remarkable potencies with EC_{50} values in the 100 pM range. These analogue potencies represent 10,000 and 30,000 fold increases in potency over the parent ADP and ATP congeners, respectively.

In light of the high potencies of the two hexenylthio analogues, the effects on cyclic AMP accumulation of other 2-

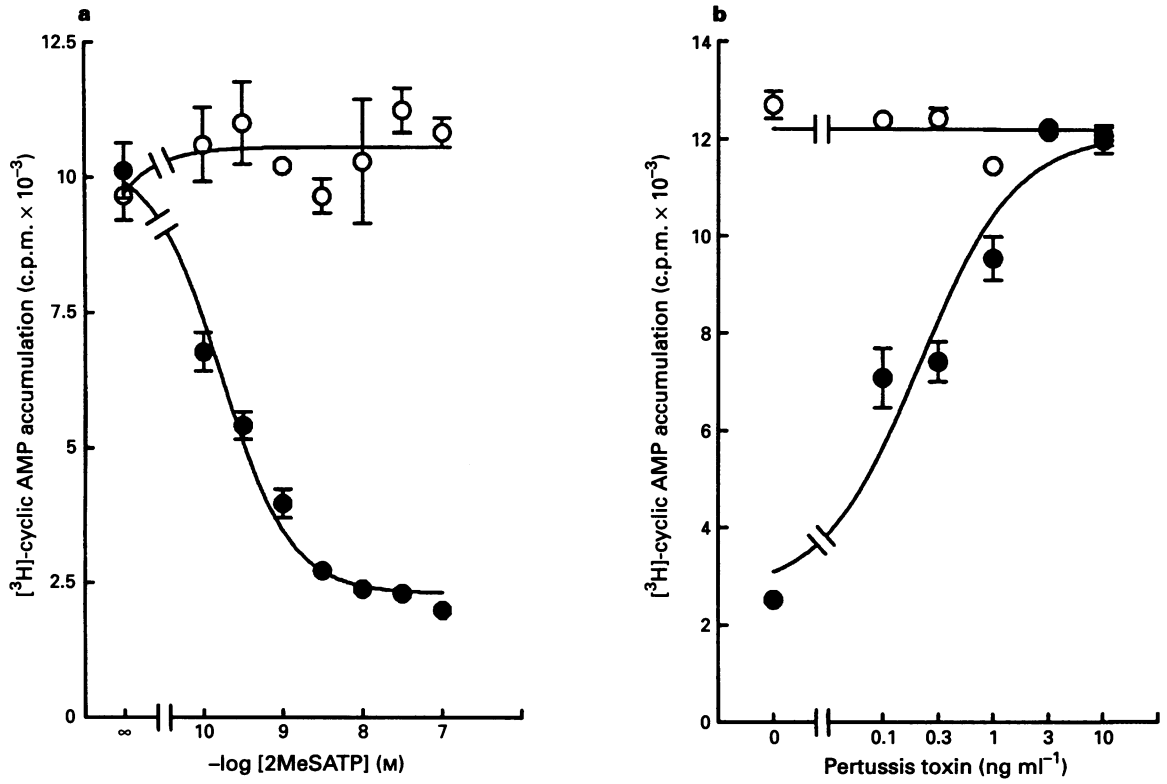

Figure 1 Pertussis toxin-sensitive inhibition of isoprenaline-stimulated cyclic AMP accumulation by 2-MeSATP in C6 rat glioma cells. (a) C6 cells were incubated overnight in the absence (●) or presence (○) of pertussis toxin (10 ng ml^{-1}). The capacity of the indicated concentrations of 2-MeSATP to inhibit isoprenaline-stimulated cyclic AMP accumulation was then determined. The data are the mean ± s.e.mean of three determinations. (b) C6 cells were incubated overnight with the indicated concentrations of pertussis toxin. Isoprenaline (10 μM)-stimulated cyclic AMP accumulation then was quantitated in the absence (○) or presence (●) of 0.1 μM 2-MeSATP as described in Methods. The data are the mean ± s.e.mean of three determinations.

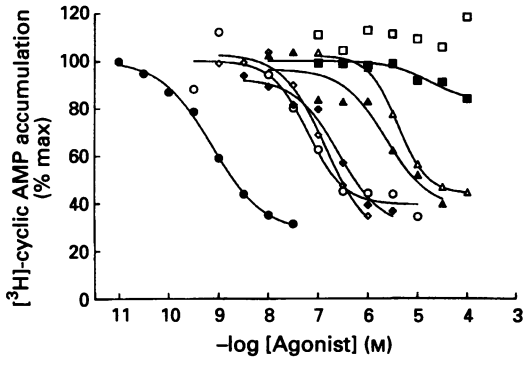

Figure 2 Inhibition of cyclic AMP accumulation by adenine nucleotide analogues. The capacity of the indicated concentrations of 2-MeSATP (●), ADPβS (○), 2-ClATP (◆), ADP (◇), ATPγS (▲), ATP (Δ), β,γMeATP (■), and α,βMeATP (□) to inhibit isoprenaline-stimulated cyclic AMP accumulation was determined as described in Methods. Data shown are the mean of triplicate determinations and the results are representative of those obtained in three separate experiments.

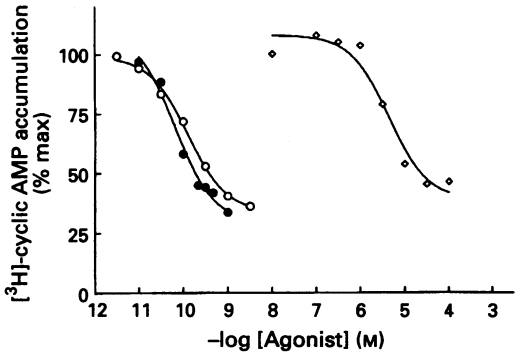

Figure 3 Inhibition of cyclic AMP accumulation by chain-extended 2-thioether derivatives of ATP and ADP. The capacity of the indicated concentrations of 2-(5-hexenylthio)ATP (●), 2-(5-hexenylthio)ADP (○), and ATP (◇) to inhibit isoprenaline-stimulated cyclic AMP accumulation was determined as described in Methods. Data shown are the mean of triplicate determinations and the results are representative of those obtained in three separate experiments.

thioether-substituted adenine nucleotide analogues were determined. The most potent agonists were 2-cyclohexylthio-ATP and 2-hexylthioATP, which both exhibited EC_{50} values of approximately 30 pM. Thus, 2-thioether substitution of a number of analogues resulted in an increase in potency of greater than 5 orders of magnitude relative to that of ATP (Table 1 and Figure 3). However, 2-(7-aminoheptylthio)ATP, which was the least potent of the eight 2-thioether derivatives of ATP that were tested, was approximately as potent as ATP itself. Although only a single diphosphate analogue was tested (2-(5-hexenylthio)ADP), it exhibited at least as great a potency as the corresponding triphosphate. This near equipotency was shown to hold for other di- and triphosphate pairs in other P_{2Y}-purinoceptor test systems (Fischer *et al.*, 1993).

We have reported earlier that N^6-substitution of adenine nucleotides results in a loss of potency of these compounds at P_{2X}-purinoceptors with little change occurring in relative potency for P_{2Y}-purinoceptors (Fischer *et al.*, 1993). One such compound, N^6-methyl-2-(5-hexenylthio)ATP, was tested and shown to retain its high potency at the C6 cell P_{2Y}-purinoceptor, although the corresponding analogue lacking the N^6-methyl group was 3 fold more potent (Table 1). N^6-me-

Table 1 Selectivity of adenine nucleotide analogues for activation of P_{2Y}-purinoceptor-regulated responses in turkey erythrocyte membranes (TE) and C6 glioma cells (C6)

	Nucleotide analogue	*Turkey erythrocyte (nM)*	*C6 glioma cell (nM)*	*Selectivity TE/C6*
1	ATP	4230 ± 1530	3190 ± 220	1.3
2	ADP	8020 ± 2070	69 ± 28	116
3	AMP	No effect	No effect	–
4	α,β-MeATP	> 100,000	No effect	–
5	β,γ-MeATP	> 100,000	*	–
6	2-ClATP	71.8 ± 19***	132 ± 13	0.54
7	2-MeSATP	9.46 ± 1.60	1.10 ± 0.30	8.6
8	ATPγS	1260 ± 560	1300 ± 628	0.97
9	ADPβS	123 ± 35	82.0 ± 39	1.5
10	8-(6-aminohexylamino)ATP	8770 ± 120	41500 ± 18800	0.21
11	3′-NH_2-ATP	164 ± 35	Little or no effect	< < 1
12	N^6-MeATP	19100 ± 6000***	Little or no effect	< 1
13	2-hexylthioATP	4.82 ± 0.91***	0.0282 ± 0.0050	171
14	2-(5-hexenylthio)ATP	8.98 ± 3.34	0.117 ± 0.067	76
15	2-cyclohexylthioATP	24.3 ± 4.4***	0.058 ± 0.036	418
16	N^6-Me-2-(5-hexenylthio)ATP	25.8 ± 7.0***	0.320**	81
17	2-cyanohexylthioATP	10.1 ± 4.7	3.10 ± 1.70	3.2
18	2-(p-nitrophenylethyl)thioATP	9.35 ± 3.54***	1.06 ± 0.47	9
19	2-(5-hexenylthio)ADP	6.70 ± 2.90***	0.0828 ± 0.0248	80
20	2-(7-aminoheptylthio)ATP	69.5 ± 44.4	2400 ± 790	0.03

EC_{50} values were determined as described in Methods. Except where indicated all values are the mean ± s.e.mean of values from at least three different determinations. *Partial inhibition at 100 μM; **$n = 1$; ***taken from Fischer *et al.* (1993).

thylATP had little or no inhibitory effect on cyclic AMP accumulation in C6 cells, which contrasts with the inhibitory effect observed with ATP alone. Since we have shown previously that N^6-substituted analogues of ATP are not active at P_{2X}-purinoceptors (Fischer *et al.*, 1993), N^6-methyl-2-(5-hexenylthio)ATP represents a very high potency P_{2Y}-purinoceptor-selective agonist.

The P_{2Y}-purinoceptor on C6 glioma cells is different from that on turkey erythrocytes, based on both the specificity of second messenger coupling of these receptors to signalling responses (Boyer *et al.*, 1989; 1993) and the relative selectivity of antagonism by two purinoceptor antagonists (Boyer *et al.*, 1994). The data with 2-thioether derivatives of adenine nucleotides also were consistent with the involvement of two different receptors in the two test systems (Table 1 and Figure 4). Thus, some agonists exhibited similar potencies in the two model systems, e.g. 2-ClATP and ADPβS, some agonists were much more potent at the C6 cell receptor than at the turkey erythrocyte receptor, e.g. 2-cyclohexylthioATP and 2-hexylthioATP, and several agonists were less potent in the C6 than in the turkey erythrocyte system, e.g. 2-(7-aminoheptylthio)ATP, 3′NH_2-ATP, N^6-methylATP, and 8-(6-aminohexylamino)ATP.

The presence of a long-chain thioether at the 2-position of adenosine di- and triphosphates increased potency in both functional receptor subtypes. However, this structural feature resulted in increased selectivity of triphosphate analogues for the adenylyl cyclase linked receptor. The degree of selectivity depended very much on the structure of the thioether substitutent. A small alkyl group, e.g. 2-MeSATP, provided only 8.1 fold selectivity. Larger alkyl thioethers, such as hexenyl and hexyl, resulted in selectivity for the C6 cell P_{2Y}-receptor of roughly 100 fold. Curiously, the presence of a cyano functional group at the distal position of hexyl alkyl chain reduced the degree of selectivity from 170 to 3.2 fold, entirely as a result of the change in potency in C6 cells. A nitrophenylethyl thioether also diplayed only moderate selectivity for C6 cell receptors. The enhancement of selectivity for the adenylyl cyclase-linked receptor associated with the presence of a long chain 2-thioether was not observed for the 2-Cl analogue or for modification of ATP at other sites, e.g. N^6, 3′, or on the triphosphate group (Table 1). The N^6-methyl substitution did not preclude the appearance of selectivity for the adenylyl cyclase-linked receptor, since N^6-methyl-2-(5-hexenylthio)ATP was 81 fold selective. ADP was 120 fold selective for the adenylyl cyclase linked receptor, and approximately the same degree of selectivity was observed for a long chain (hexyl) thioether of ADP.

Discussion

Pianet and coworkers (1989) first reported that ADP and ATP inhibited cyclic AMP accumulation in C6 rat glioma cells, which was subsequently confirmed by others (Valeins *et al.*, 1992; Lin & Chuang, 1993; Boyer *et al.*, 1993). Our previous

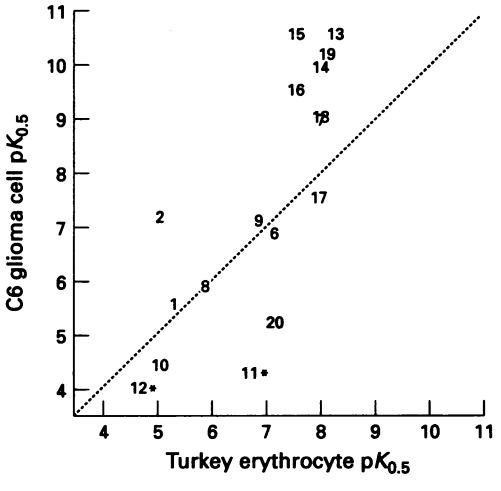

Figure 4 Comparison of the relative potencies of adenine nucleotide analogues for stimulation of two different P_{2Y}-purinoceptor-regulated responses. Concentration-effect curves for 20 compounds were generated for the adenylyl cyclase-linked P_{2Y}-purinoceptor of C6 glioma cells and the phospholipase C-linked P_{2Y}-purinoceptor of turkey erythrocytes. The average $pK_{0.5}$ for at least three different experiments in each case is plotted for the turkey erythrocyte response (abscissa scale) and C6 cell response (ordinate scale). Numbers in the plot correspond to nucleotides as indicated in Table 1. *$pK_{0.5}$ values for 3′-NH_2ATP and N^6-MeATP (nucleotides 11 and 12, respectively) on C6 cells are approximate, since only a partial effect was observed at 100 μM. Compounds 7 and 18 are superimposed in the plot.

data (Boyer *et al.*, 1993) and data presented here illustrate that this inhibitory effect of adenine nucleotides is mediated through a P_{2Y}-purinoceptor. Analogues of adenine nucleotides modified on the purine ring as chain-extended 2-thioethers exhibited remarkable potencies for the adenylyl cyclase-coupled P_{2Y}-purinoceptor of C6 glioma cells. Several of these 2-thioether derivatives exhibited potencies at C6 cell P_{2Y}-purinoceptors that were at least 30 fold greater than that of 2-MeSATP, which was the initially useful compound for pharmacologically delineating P_{2Y}-purinoceptors. Compared to the potency of ATP, some of these agonists represent increases in potencies of at least 5 orders of magnitude. 2(5-Hexenylthio) ADP, the single ADP analogue that was tested, also exhibited a potency that was 3 orders of magnitude more potent than ADP itself. The fact that ADP and its analogues are at least as potent as ATP and the corresponding triphosphate analogues suggests that analogues of ADP might be preferable to those of ATP as P_{2Y}-purinoceptor agonists. That is, ADP analogues are much less likely to interact with P_{2X}- (Fischer *et al.*, 1993; Burnstock *et al.*, 1994; Valera *et al.*, 1994) or P_{2U}-purinoceptors (Dubyak *et al.*, 1988; Fine *et al.*, 1989; Brown *et al.*, 1991; Lustig *et al.*, 1993) or with ATP-binding enzymes.

The cyclic AMP-regulating P_{2Y}-purinoceptor on C6 glioma cells apparently represents a different receptor type from the P_{2Y}-purinoceptor on turkey erythrocytes. No evidence for coupling of the C6 cell receptor to phospholipase C has been observed even though this effector system is present in the cells, and stimulation of the erythrocyte receptor results in activation of phospholipase C with no effects on adenylyl cyclase. These results have been corroborated with data from the cloned turkey P_{2Y}-purinoceptor heterologously expressed in 1321N1 human astrocytoma cells (Filtz *et al.*, 1994) and after expression of the human homologue of this receptor (J.L. Schachter, Q. Li, R.A. Nicholas & T.L. Harden, unpublished observations). The expressed receptor promoted inositol lipid hydrolysis without producing any effects on cyclic AMP levels. The idea that different P_{2Y}-purinoceptors couple to different second messenger pathways was also supported by differential effects of two antagonists of P_2-purinoceptors in the two model test systems (Boyer *et al.*, 1994). Although the delineation of multiple subtypes within a receptor class are often difficult to make by use of receptor agonists alone, the data presented here comparing relative agonist affinities between the two P_{2Y}-purinoceptor-regulated second messenger systems add further support to the notion that these are pharmacologically distinct P_{2Y}-purinoceptor subtypes. For example, a number of the chain-extended 2-thioether derivatives of ATP were much more potent activators of the C6 cell than of the turkey erythrocyte purinoceptor. In contrast N^6-methylATP was essentially inactive at the C6 cell P_{2Y}-purinoceptor, but was equipotent with ATP at the P_{2Y}-purinoceptor of turkey erythrocytes and of several mammalian smooth muscle preparations (Fischer *et al.*, 1993). Although the agonist potencies at the C6 cell P_{2Y}-purinoceptor only have been compared to potencies at turkey erythrocytes these differences also will hold for mammalian P_{2Y}-purinoceptors. That is, we have reported previously that the potencies of agonists at the turkey erythrocyte receptor are closely aligned with those for the same agonists at the guinea-pig taenia coli (Fischer *et al.*, 1993).

Perhaps the most remarkable observation made in this study is that agonists can be synthesized that activate P_{2Y}-purinoceptors in the pM range of concentrations. The potential contribution of receptor reserve to this very high potency of agonists has not been examined. The fact that so many of the chain-extended 2-thioether derivatives were more potent at C6 cells than in erythrocyte membranes is suggestive that considerable receptor reserve may exist for these adenylyl cyclase-regulating receptors. However, the similar potencies between the two systems observed with the classically used P_{2Y}-purinoceptor agonists, 2-ClATP and ADPβS, and of ATP itself, make this argument less tenable. An alternate explanation is that the differences reflect differences in conformation or in crucial amino acids in the receptor binding site responsible for ligand recognition. The potential existence of receptor reserve in the C6 cells also does not fit with the observation that certain agonists, e.g. 2-(7-aminoheptylthio)ATP and 3'-NH_2-ATP, were more potent activators of the turkey erythrocyte than the C6 cell receptor. Means of irreversibly inactivating or down-regulating the C6 cell P_{2Y}-purinoceptor will need to be delineated to address this issue adequately.

In summary, C6 glioma cells provide an excellent model for the study of an adenylyl cyclase-linked P_{2Y}-purinoceptor. Assuming that receptor reserve is not a major contributor, the remarkably high potencies observed with adenine nucleotide analogues at C6 cell P_{2Y}-purinoceptors represent some of the largest substitution-promoted increases in potencies over that of a natural receptor agonist of which we are aware. The possibility of obtaining agonist potencies in the pM range raises a number of possibilities. For example, we now have identified substitutions that may allow us to synthesize P_2-purinoceptor agonists that are not nucleotides.

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