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An examination of deoxyadenosine 5′(α-thio)triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the $P2Y_1$ receptor

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1 The functional activity of deoxyadenosine 5′(α-thio)triphosphate (dATPαS) was assessed at the cloned human $P2Y_1$ receptor stably expressed in 1321N1 human astrocytoma cells and transiently expressed in Cos-7 cells.

2 Cells expressing the receptor responded to adenine nucleotides with an increase in [^{3}H]-inositol phosphate accumulation. Half-maximal responses were obtained at approximately 30 nM for 2-methylthioadenosine-5′-triphosphate (2MeSATP), 300 nM for dATPαS, and 1000 nM for adenosine 5′-triphosphate (ATP). dATPαS produced a maximal response that was only $37 \pm 4\%$ of that produced by ATP or 2MeSATP. dATPαS also competitively antagonized the phospholipase C response to 2MeSATP with a K_B of 644 ± 14 nM. Thus dATPαS acts as a low potency partial agonist at $P2Y_1$ receptors.

3 The selectivity of dATPαS for $P2Y_1$ receptors was determined by examining its capacity to activate $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors also stably expressed in 1321N1 cells. Although dATPαS was a partial agonist at $P2Y_1$ receptors it was a full agonist at $P2Y_2$ receptors, albeit with a potency that was two orders of magnitude lower than at $P2Y_1$ receptors. No agonist or antagonist activity was observed at $P2Y_4$ and $P2Y_6$ receptors.

4 Although [^{35}S]-dATPαS bound to a relatively high density (ca 10 pmol mg^{-1} protein) of binding sites in membranes from 1321N1 or Cos-7 cells expressing the $P2Y_1$ receptor, no difference in the total density of sites was observed between membranes from wild-type, empty vector-transfected, or $P2Y_1$ receptor-expressing cells. Moreover, adenine nucleotide analogues inhibited [^{35}S]-dATPαS binding with an order of potency that differed markedly from that for the accumulation of inositol phosphates in intact transfected $P2Y_1$ receptor-expressing cells. Saturation binding experiments demonstrated multiple affinity states for [^{35}S]-dATPαS binding in wild-type Cos-7 cell membranes. These data from 1321N1 and Cos-7 cells suggest that cellular membranes exhibit a large number of high affinity binding sites for [^{35}S]-dATPαS that are not related to P2Y receptor subtypes.

Keywords: dATPαS; ATP; P_2 receptor; inositol phosphates; radioligand binding; human $P2Y_1$ receptor

Introduction

Extracellular receptors for adenine nucleosides and nucleotides were initially divided into two families based on the relative potencies of adenosine (P_1-receptors) and adenosine 5′-triphosphate (ATP; P_2-receptors) in various tissue responses. Recognition that methylxanthines are potent inhibitors of responses to adenosine (Sattin & Rall, 1970) prompted the development of a series of antagonist molecules that have been used to characterize and radiolabel several subtypes of P_1 receptors.

Subtypes of P_2-receptors (P_{2X} and P_{2Y}) were originally defined pharmacologically based on the relative potencies of 2-methylthio-ATP (2MeSATP), ATP and α,β methylene-ATP in contractile responses of a variety of peripheral tissues (Burnstock & Kennedy, 1985). In contrast to the situation with adenosine receptors, the lack of selective, high affinity ligands for P_2 receptors has prevented unambiguous identification and characterization of P_2 receptor subtypes in intact tissues. The recent molecular cloning of a number of P2X and P2Y receptor subtypes (the subscripted terms P_{2X} and P_{2Y} are used here to designate receptor classes that have been defined pharmacologically by Burnstock and Kennedy (1985) whereas $P2X_n$ and $P2Y_n$ (where n = 1, 2, 3 . . .) refer to molecularly defined P2 receptor subtypes) now provides the opportunity to determine the potencies of various compounds at structurally defined receptors.

With the exception of chain-extended 2-thioether derivatives of ATP and ADP (Fischer *et al*., 1993), no P_{2Y} receptor agonists have been found that exhibit potencies in the range of concentrations, i.e. nanomolar, that conventionally have been necessary for reliable receptor radioligands. Further, molecules known to act as P_{2Y} receptor antagonists have been shown to do so with only micromolar affinity. Thus, it is of interest that [^{35}S]-2′ deoxyadenosine-5′-(α-thio)triphosphate ([^{35}S]-dATPαS) has been utilized recently in attempts to radiolabel selectively and pharmacologically define native P_{2Y} receptors in brain tissue and recombinant P2Y receptors expressed in cultured cells (Simon *et al*., 1995a,b; Webb *et al*., 1996; Akbar *et al*., 1996; Vallejo *et al*., 1996). It has been proposed that [^{35}S]-dATPαS exhibits high affinity, specific binding to three different putative P2-receptor subtypes (Simon *et al*., 1995a; Webb *et al*., 1996; Akbar *et al*., 1996). Since the pharmacological activity of dATPαS has not been documented previously, we have determined and compared both the activity and the ligand binding characteristics of dATPαS at the cloned human $P2Y_1$ receptor. Our data are not consistent with a selective, high affinity interaction of dATPαS with the human $P2Y_1$ receptor and suggest that radioligand binding of the compound is predominantly to endogenous ATP-binding proteins that are distinct from the expressed $P2Y_1$ receptor.

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Methods

Expression of the human P2Y$_1$ receptor

The molecular cloning of the human $P2Y_1$ receptor has been demonstrated previously (Schachter *et al.*, 1996). The receptor was subcloned into the plasmid pcDNA3 with EcoR1 and Xho1 restriction sites and expressed in 1321N1 human astrocytoma cells by the calcium phosphate precipitation transfection method (Chen & Okayama, 1987). These cells were utilized because they do not exhibit functional responses to agonists of any of the known P2Y receptors. All of the experiments described here for the $P2Y_1$ receptor in 1321N1 cells were performed in a stably expressing cell clone (clone 6T) that showed the largest magnitude of functional response to purinoceptor agonists among eleven clones examined.

The $P2Y_1$ receptor also was expressed transiently in Cos-7 cells with Lipofectamine reagent (Gibco BRL). Optimized conditions involved treating cells at approximately 70% confluency in 100 mm plates with 21 μl of Lipofectamine reagent and 210 ng of plasmid DNA in serum-free Dulbecco's modification of Eagle's medium (DMEM) for 12 h. Following readdition of serum for 12 h, the cells were washed and either grown in the 100 mm plates for two days before harvest for membrane binding assays or split into 24 well plates for assay of inositol phosphate accumulation 48 h later.

Quantitation of inositol lipid hydrolysis

The presence and pharmacological selectivity of the $P2Y_1$ receptor was assessed by quantitation of inositol lipid hydrolysis in response to purinoceptor agonists. Cells expressing recombinant receptor were replated at a density of 1×10^5 cells/well in 24 well plates and grown to confluence (two to three days in culture) before the assay. The culture medium was replaced 24 h before the assay with 200 μl of serum-free, inositol-free DMEM supplemented with 0.4 μCi [^{3}H]-*myo*-inositol (20 Ci mmol^{-1}, American Radiolabeled Chemicals). Drugs were added as 5X concentrated stocks in 50 μl of 250 mM HEPES, pH 7.3, containing 50 mM LiCl. Incubations were stopped by rapidly aspirating the medium and the cell lysate was applied directly to Dowex AG1-X8 columns for chromatographic isolation of inositol phosphates (Berridge *et al.*, 1985). Assays were performed at least three times in triplicate.

Membrane binding of [^{35}S]-dATPαS

Membranes were prepared from 100 mm plates of transfected or wild type cells either exactly as described (Simon *et al.*, 1995a; Webb *et al.*, 1996) or with the following minor modifications. The freeze/thaw method for cell lysis used in the previous studies was replaced by hypotonic swelling and homogenization in 5 mM Tris, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, and protease inhibitors (100 μg ml^{-1} bacitracin, 10 μg ml^{-1} soy bean trypsin inhibitor, 10 μg ml^{-1} aprotinin, 1 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride). Following cell disruption with a Brinkman Polytron (setting 5.5 for 10 s on ice) the membranes were centrifuged at 25,000 × *g* and resuspended three times in 10 ml of the above buffer containing 50 mM Tris, pH 7.4 (buffer A). The pellets were gently resuspended each time in a hand-held glass/glass homogenizer, replacing the practice of trituration with a 22 gauge needle. Membranes were rapidly frozen and stored at −80°C. Before assay the thawed membranes were homogenized in a motor-driven glass/teflon homogenizer. All manipulations were performed rapidly with ice-cold solutions and instruments. Direct comparison of the freeze/thaw/trituration method and the alternative method described above gave similar results, but use of the modified method resulted in greater reproducibility.

Binding assays were initiated by addition of 50 μl of membranes (10 μg protein) to 100 μl of buffer A containing the radioligand with or without competing drug, giving a final concentration of 10 nM [^{35}S]-dATPαS (0.15 μCi/assay adjusted to 10 nM with unlabelled dATPαS). Following incubation for 60 min at room temperature with constant shaking, the assay was terminated by rapidly adding 5 ml of ice-cold 20 mM Tris, pH 7.4, and immediately filtering over glass fibre filters (Scheicher & Schuell #30) presoaked with 20 mM pyrophosphate, 20 mM Tris, pH 7.4. Filters were washed twice before being dried and counted.

Materials

[^{35}S]-dATPαS was obtained from Amersham. Unlabelled dATPαS was from New England Nuclear. Lipofectamine reagent and cell culture media were from Gibco. [^{3}H]-myo-inositol was from American Radiolabeled chemicals. Nucleotides and HEPES buffer were purchased from Boehringer Manheim. Other reagents were from Sigma.

Results

Functional activity of dATPαS in 1321N1 cells expressing the human P2Y$_1$ receptor

Although [^{35}S]-dATPαS is purported to radiolabel the $P2Y_1$ receptor and other putative P2Y receptors (Simon *et al.*, 1995a,b; Webb *et al.*, 1996; Akbar *et al.*, 1996; Vallejo *et al.*, 1996), the pharmacological activity of the compound at P2Y receptors has not, to our knowledge, been defined. Therefore, we have examined the activity of dATPαS at the recombinant human $P2Y_1$ receptor. We previously characterized the pharmacological selectivity of the human $P2Y_1$ receptor expressed in 1321N1 cells by examining the phospholipase C response to approximately 20 adenine nucleotides (Schachter *et al.*, 1996). Most of these nucleotides produced the same maximal response, despite considerable differences in EC_{50} values. Here we compared the effect of dATPαS to those of 2MeSATP and ATP, which appeared to act as full agonists in 1321N1-h$P2Y_1$ cells (Schachter *et al.*, 1996). As presented in Figure 1, dATPαS promoted inositol lipid hydrolysis, but the maximal effect observed was only 37.2±4.3% of the activity observed with

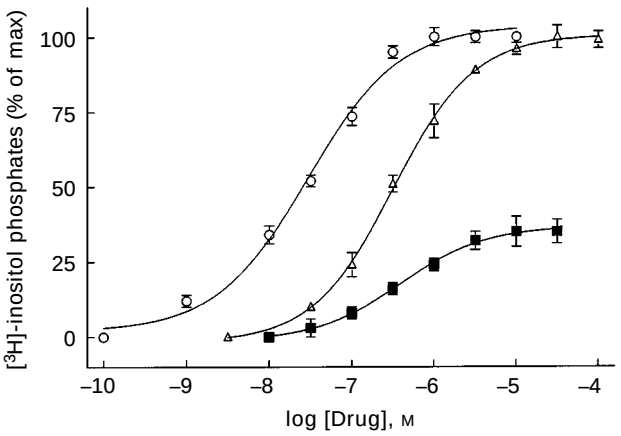

Figure 1 Inositol phosphate accumulation in 1321N1 cells stably transfected with the human $P2Y_1$ receptor. A stable transfectant was clonally selected for purinergic responsiveness and inositol phosphate accumulation was assessed after a 10 min incubation with 2MeSATP (○), ATP (△) or dATPαS (■). Data for each nucleotide concentration (mean with s.e.mean (vertical lines) of three experiments performed in triplicate) are normalized to the inositol phosphate response of 3 μM 2MeSATP (4–6 fold above the blank or basal level) and are shown with the basal value (usually about 2000 c.p.m.) subtracted.

either ATP or 2MeSATP. Several possible explanations were considered for the smaller maximal response of dATPαS. The time course of inositol phosphate accumulation in the presence of a maximally effective concentration of dATPαS was a constant fraction of that of 2MeSATP at all times of incubation examined (Figure 2). Additionally, preincubation of cells with dATPαS for 20 min did not alter the subsequent response to a supramaximal concentration of 2MeSATP. Thus, dATPαS did not appear to desensitize rapidly or nonspecifically inhibit the activity of the $P2Y_1$ receptor. Similar results were obtained with 1321N1 cells expressing the recombinant turkey $P2Y_1$ receptor (data not shown), indicating that the low potency and partial agonist behaviour of dATPαS is not simply a reflection of differences in avian versus mammalian homologues of the $P2Y_1$ receptor.

To determine whether dATPαS exhibited the characteristics of a competitive antagonist of the $P2Y_1$ receptor, we generated concentration-effect curves for dATPαS in the presence of several concentrations of 2MeSATP (Figure 3a). dATPαS produced a concentration-dependent inhibition of 2MeSATP-stimulated inositol phosphate accumulation down to the level induced by the partial agonist alone. Concentration-effect curves also were established for 2MeSATP in the presence of various concentrations of dATPαS (Figure 3b). Increasing concentrations of dATPαS caused a shift to the right of the agonist concentration-response curve for 2MeSATP. Analysis of these data according to the method of Schild (1947) gave a K_B value of 644 ± 14 nM for dATPαS as a $P2Y_1$ receptor antagonist (Figure 3c).

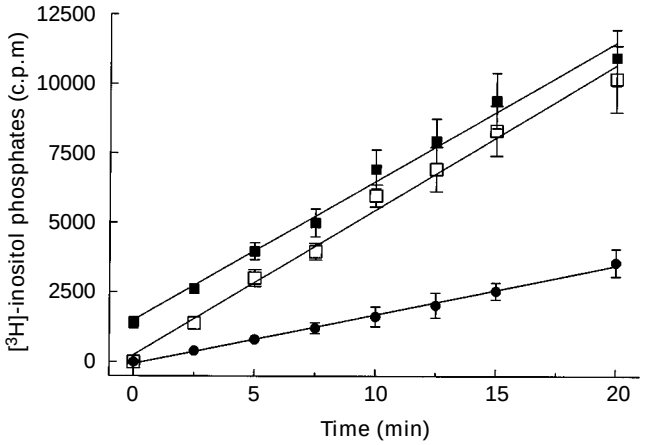

Figure 2 Time course of inositol phosphate accumulation in the presence of dATPαS and 2MeSATP. LiCl (10 mM final concentration) was added simultaneously with 10 μM dATPαS (●) or 30 μM 2MeSATP (□) to 1321N1-hP2Y_1 cells. A third set of cells was preincubated with 10 μM dATPαS for 20 min in the absence of LiCl before the addition of 30 μM 2MeSATP plus LiCl (■). The basal (or blank) value did not change over the time of the assay and was subtracted from each time point.

To establish the selectivity of dATPαS among other G protein-coupled P2Y receptors, we examined its capacity to activate $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, each stably expressed in 1321N1 cells. dATPαS was fully efficacious in activating the human $P2Y_2$ receptor, but did so with low potency ($EC_{50} = 23.1 \pm 3.6$ μM, Figure 4a) compared with the potency of ATP or UTP (230 or 140 nM, (Lazarowski *et al.*, 1995)) in the same $P2Y_2$ receptor-expressing cells. Neither agonist nor antagonist activity was observed with dATPαS at the $P2Y_4$ or $P2Y_6$ receptors (Figure 4b) or at a P2Y receptor on C6 rat glioma cells (Boyer *et al.*, 1993) that couples to the inhibition of adenylyl cyclase (not shown).

Radioligand binding of [^{35}S]-dATPαS to membranes from transfected cells

In light of the application of [^{35}S]-dATPαS in studies designed to radiolabel the $P2Y_1$ receptor (Simon *et al.*, 1995a), and two other putative P2 receptors (Webb *et al.*, 1996; Akbar *et al.*, 1996), we carried out similar binding assays with membranes from 1321N1 cells engineered to express stably the $P2Y_1$ receptor. Although a high level of [^{35}S]-dATPαS binding was observed, similar amounts of total [^{35}S]-dATPαS binding occurred with membranes obtained from both wild type 1321N1 cells and from 1321N1 cells expressing high concentrations of the $P2Y_1$ receptor (based on the marked inositol phosphate response of these cells to 2MeSATP). The binding of [^{35}S]-dATPαS to membranes from 1321N1-hP2Y_1 cells was inhibited by adenine nucleotide analogues, but the order of potency (2′,3′-dideoxy ATP > ATP > 2MeSATP ⩾ ADP > 2MeSADP; Figure 5) was markedly different from the order of potency of these compounds for activation of inositol lipid hydrolysis (2MeSADP > 2MeSATP > ADP > ATP > > 2′,3′-dideoxyATP; see Table 1). Additionally, two compounds that are competitive antagonists of the $P2Y_1$ receptor at low micromolar concentrations, PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) and adenosine-3′,5′-bisphosphate (Boyer *et al.*, 1994; 1996), only inhibited [^{35}S]-dATPαS binding at millimolar concentrations. Thus, no evidence for radiolabelling of $P2Y_1$ receptors was observed with membranes obtained from 1321N1-hP2Y_1 cells.

Several of the previously published studies of [^{35}S]-dATPαS binding were performed in transiently transfected Cos-7 cells (Simon *et al.*, 1995a; Webb *et al.*, 1996; Akbar *et al.*, 1996).

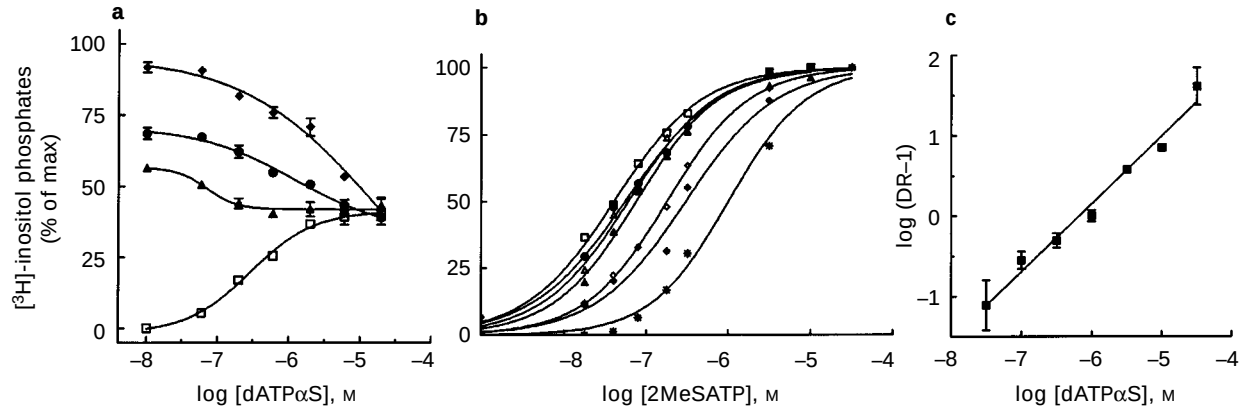

Figure 3 Antagonist activity of dATPαS at the human $P2Y_1$ receptor. Inositol phosphate accumulation in 1321N1-hP2Y_1 cells was determined in the presence of various concentrations of dATPαS and 2MeSATP alone and in combination. (a) Concentration-response profile of dATPS in the absence (□) or presence of 40 nM (▲), 100 nM (●) or 400 nM (◆) 2MeSATP. (b) Concentration-response profile of 2MeSATP in the absence (□), or presence of 0.1 (●), 0.3 (△), 1 (▲), 3 (◇), 10 (◆) or 30 μM dATPαS (*). (c) Schild plot of the data from (b). The average of three such plots gave a K_i value of 644 ± 14 nM and a slope of 1.1 ± 0.15.

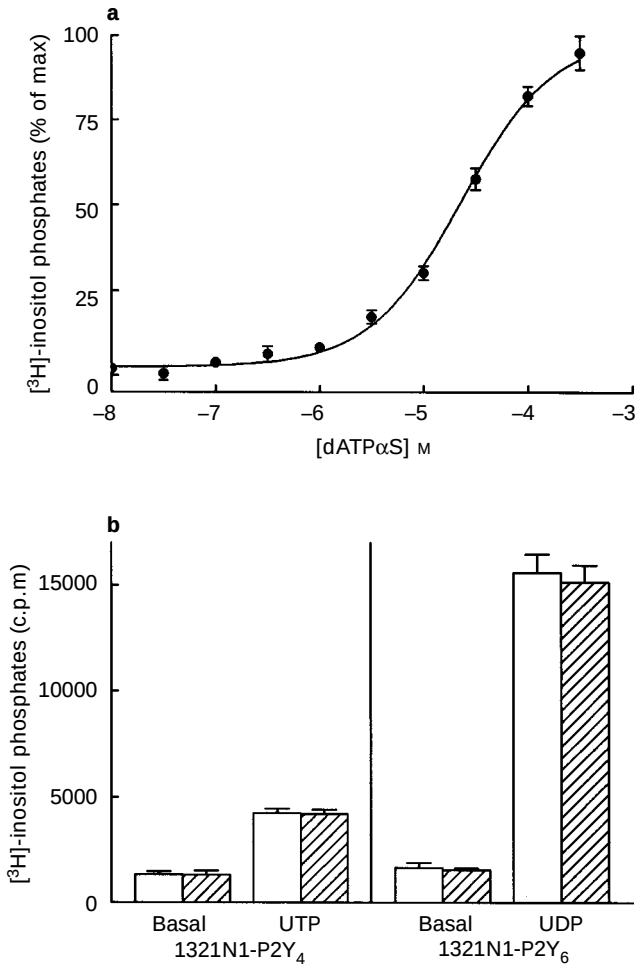

Figure 4 Inositol phosphate accumulation in 1321N1 cells expressing the $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors. (a) The response of 1321N1-h$P2Y_2$ cells to dATPαS is expressed as a percentage of the maximal response to 10 μM UTP. (b) The effects of 30 μM dATPαS alone or in the presence of 2 μM UTP (in $P2Y_4$-expressing cells) or to 0.5 μM UDP (in $P2Y_6$-expressing cells) were quantitated. These uridine nucleotide concentrations elicited 60–65% of the maximal response for $P2Y_4$ or $P2Y_6$ receptor-expressing cells. Values shown are the mean±s.e.mean in the absence (open column) or presence (hatched columns) of 30 μM dATPαS.

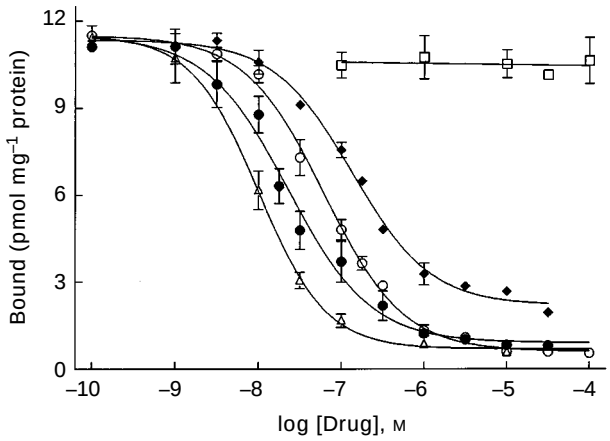

Figure 5 Inhibition of [^{35}S]-dATPαS binding to membranes from 1321N1-h$P2Y_1$ cells. Membranes prepared from 1321N1-h$P2Y_1$ cells were incubated with 10 nM [^{35}S]-dATPαS and the indicated concentrations of competing drug as described in Methods. Data shown are for 2′3′-dideoxy ATP (△), ATP (●), ADP (○), 2MeSADP (◆) and PPADS (□).

Table 1 Comparison of EC_{50} values for inositol lipid hydrolysis with IC_{50} values for the inhibition of [^{35}S]-dATPαS binding in 1321N1-h$P2Y_1$ cells

Drug	*Inositol phosphates* EC_{50} (nM)	*Ligand binding* IC_{50} (nM)
Agonists		
2MeSADP	13.6±2.1	358±112
2MeSATP	51.4±6.0	24±3.2
ADP	257±14	62±26
ATP	1520±210	17±6.1
ATPγS	795±210	21±1.1
2′,3′-ddATP	>100,000	10±0.9
Antagonists		
A-3′, 5′-DP	1000±150	>>100,000
PPADS	4300±900	>100,000

EC_{50} values (in nanomolar) are shown in the middle column for the stimulation of inositol phosphate accumulation by adenine nucleotides in 1321N1 cells stably transfected with the human $P2Y_1$ receptor. IC_{50} values are shown in the righthand column for inhibition of [^{35}S]-dATPαS binding in membranes from these cells. K_i values for two $P2Y_1$ receptor antagonists were also compared with their IC_{50} values for inhibition of binding.

Therefore, we also carried out [^{35}S]-dATPαS binding experiments in Cos-7 cells transfected with the human $P2Y_1$ receptor. Control or vector-transfected cells did not respond to dATPαS, 2MeSADP or 2MeSATP with an increase of inositol phosphate accumulation. However, both ATP and UTP promoted inositol phosphate accumulation in a nonadditive manner (Figure 6a), suggesting the presence in Cos-7 cells of an endogenous nucleotide receptor. The response to UTP (or ATP, data not shown) was not affected by the presence of 30 μM dATPαS (Figure 6b). Transfection of Cos-7 cells with DNA encoding the human $P2Y_1$ receptor conferred a responsiveness to 2MeSADP, 2MeSATP and dATPαS (Figure 7a). As was the case with 1321N1 cells expressing the $P2Y_1$ receptor, dATPαS elicited only a fraction of the response observed for 2MeSADP and 2MeSATP. The response to ATP in $P2Y_1$ receptor-expressing Cos-7 cells was approximately twice as large as the 2MeSATP response, due to the additive effects of ATP at the transfected $P2Y_1$ receptor and the endogenous nucleotide receptor (Figure 7a).

Radioligand binding assays were carried out with [^{35}S]-dATPαS in membranes prepared from $P2Y_1$ receptor-transfected Cos-7 cells. As with membranes from 1321N1 cells, no difference in total [^{35}S]-dATPαS binding was observed between membranes from wild type and $P2Y_1$ receptor-expressing Cos-7 cells. The order of potency for the inhibition of [^{35}S]-dATPαS binding by adenine nucleotides was markedly different from that for the stimulation of inositol phosphate accumulation in intact cells (compare Figure 7a and b), but was not different from the order of potency for the inhibition of binding to membranes from nontransfected Cos-7 cells (Figure 7c). [^{35}S]-dATPαS binding in membranes from non-transfected Cos-7 cells was also inhibited by suramin and reactive blue-2, which have been commonly used to suggest the involvement of purinoceptors (Table 2). Since Cos-7 cells express an endogenous PLC-coupled nucleotide receptor that is sensitive to both uridine 5′-triphosphate (UTP) and ATP (Figure 5a), the possibility that this receptor is responsible for the binding of [^{35}S]-dATPαS was considered. However, the endogenous nucleotide receptor of Cos-7 cells was neither activated nor antagonized by dATPαS (Figure 5), and much higher concentrations of UTP were necessary to inhibit [^{35}S]-dATPαS binding than to stimulate inositol phosphate accumulation (see Table 2).

The previous studies of [^{35}S]-dATPαS binding utilized a narrow range of radioligand concentrations and concluded that binding was to a single population of sites in transfected Cos-7 membranes (Simon *et al.*, 1995a; Webb *et al.*, 1996; Akbar *et al.*, 1996). Results from saturation binding assays

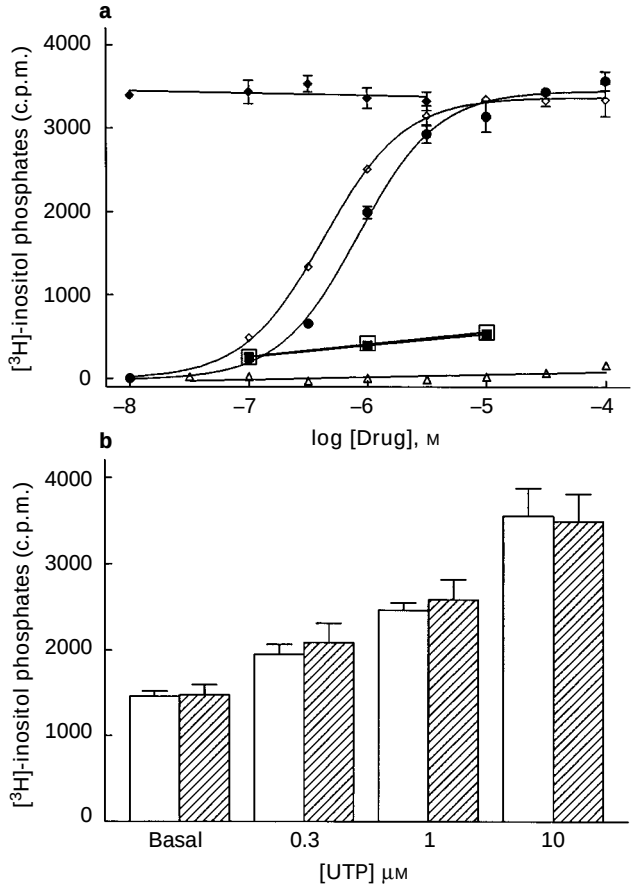

Figure 6 Lack of activity of dATPαS at the endogenous nucleotide receptor in wild type Cos-7 cells. (a) Inositol phosphate accumulation in wild type Cos-7 cells was measured in response to dATPαS (△), 2MeSADP (■), 2MeSATP (□), ATP (●), UTP (◇) and to ATP in the presence of 10 μM UTP (◆). (b) UTP-stimulated inositol phosphate accumulation was assessed in wild-type Cos-7 cells in the absence (open columns) or presence (hatched columns) of 30 μM dATPαS. Data in (b) are shown without subtraction of basal inositol phosphate accumulation. Data are shown as the mean ± s.e.mean of three experiments performed in triplicate.

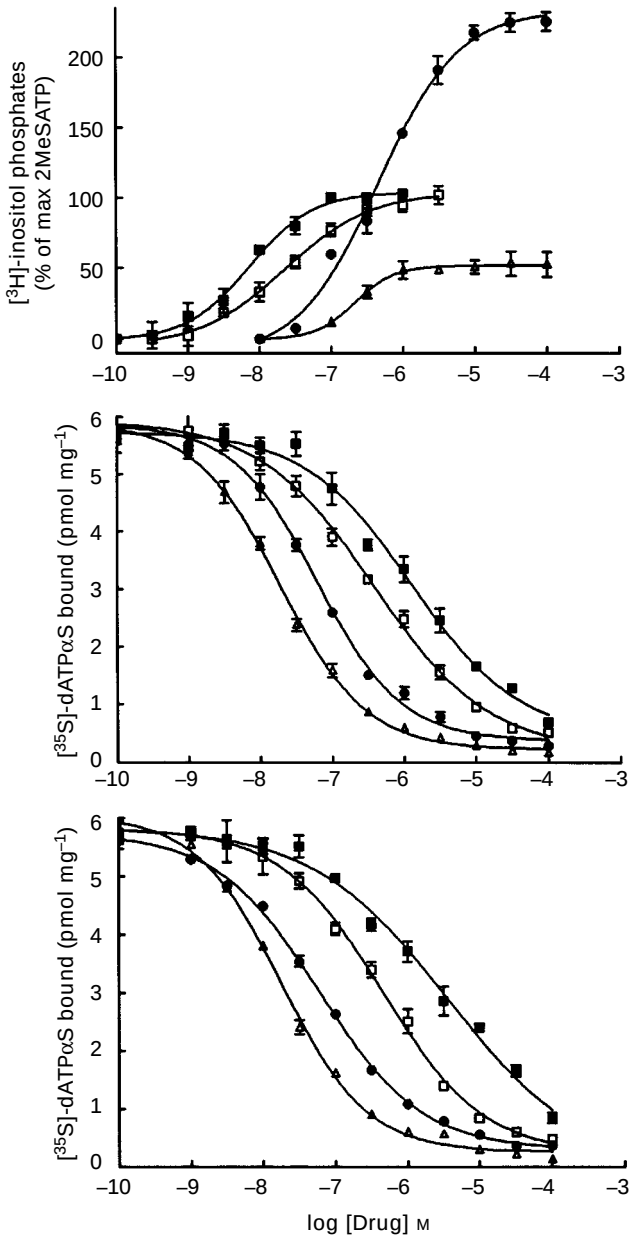

Figure 7 Comparison of functional response with inhibition of radioligand binding in transiently transfected Cos-7 cells. (a) Inositol phosphate accumulation in transiently transfected Cos-7 cells. Results are expressed as the percentage of the maximal response to 2MeSATP (3 fold above basal) and are shown with basal inositol phosphate accumulation subtracted. Activities are shown for 2MeSADP (■), 2MeSATP (□), ATP (●) and dATPαS (△). (b) Inhibition of [^{35}S]-dATPαS binding to membranes from transiently transfected Cos-7 cells. Drugs and symbols are as in (a). (c) Inhibition of [^{35}S]-dATPαS binding to membranes from non-transfected Cos-7 cells (symbols as in (a)).

carried out over a wider range of ligand concentrations were not consistent with binding of [^{35}S]-dATPαS to a single population of sites in membranes from control (Figure 8) or $P2Y_1$ receptor-transfected (data not shown) cells. These data are consistent with the binding of [^{35}S]-dATPαS to a number of sites on Cos-7 membranes which are distinct from the nucleotide binding site of the $P2Y_1$ receptor.

Discussion

Stable expression of the cloned human $P2Y_1$ receptor in 1321N1 human astrocytoma cells confers an inositol phosphate response to a broad range of adenine nucleotides (Schachter *et al.*, 1996). dATPαS exhibited the properties of a partial agonist/competitive antagonist at the expressed $P2Y_1$ receptor, and the K_B value derived in experiments designed to examine the capacity of dATPαS to antagonize competitively the effects of 2MeSATP was in the range of K_B values previously obtained for suramin, PPADS and reactive blue 2 at the $P2Y_1$ receptor (Boyer *et al.*, 1994). Although [^{35}S]-dATPαS bound with relatively high affinity to membranes prepared from 1321N1 and Cos-7 cells stably or transiently expressing the human $P2Y_1$ receptor, the order of potency of adenine nucleotide analogues and P2 receptor antagonists for inhibition of radioligand binding was markedly different from the order of potency for stimulation of inositol phosphate accumulation. Moreover, a similar density of [^{35}S]-dATPαS binding sites and similar pharmacological selectivity for inhibition of binding was observed in nontransfected cells which did not exhibit an inositol phosphate response to $P2Y_1$ receptor-selective agonists.

The relatively high K_B value of dATPαS (644 nM by Schild analysis) suggests that this molecule does not exhibit sufficiently high affinity at $P2Y_1$ receptors to be utilized in a radioligand binding assay for this receptor. [^{35}S]-dATPαS is unlikely to remain $P2Y_1$ receptor-associated during processing of samples by membrane filtration, even in the absence

Table 2 Comparison of EC_{50} values for inositol lipids hydrolysis with IC_{50} values for the inhibition of [^{35}S]-dATPαS binding in transfected and wild type Cos-7 cells

	Wild type Cos-7		*hP2Y$_1$ Cos-7*	
	Inositol phosphates	*Binding*	*Inositol phosphates*	*Binding*
Drug	*EC_{50}* (nM)	*IC_{50}* (nM)	*EC_{50}* (nM)	*IC_{50}* (nM)
Agonists				
2MeSADP	NE	3660±473	5.2±2.2	3910±1320
2MeSATP	NE	451±47	22.8±7.2	377±51
dATPaS	NE	18.0±1.9	215±20	18.9±1.5
ATP	837±93	49.8±9.7	456±87	41.5±10.8
UTP	428±48	170,000±27,000	ND	ND
Antagonists				
Suramin	ND	3040±370	ND	ND
Reactive Blue-2	ND	751±112	ND	ND

NE – not effective; ND – not determined.

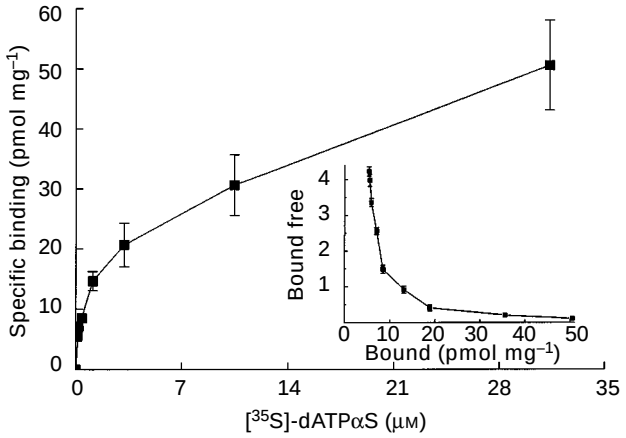

Figure 8 Saturation isotherm for [^{35}S]-dATPαS binding to wild-type Cos-7 membranes. Membranes were incubated with increasing amounts of unlabelled dATPαS in the presence of a fixed concentration of radiolabel, followed by calculation of bound and free concentrations. Nonspecific binding (approximately 5% of total binding) was defined as the amount of [^{35}S]-dATPαS binding remaining in the presence of 10^{-4} M 2MeSATP and this value was subtracted from the total radioligand bound at each concentration of dATPαS before calculation of bound and free concentrations. The inset shows a Scatchard replot of the data.

of binding to other proteins. Moreover, membranes from wild-type Cos-7 and 1321N1 cells bound [^{35}S]-dATPαS with high capacity and in the low nanomolar range of affinities. dATPαS is not a general ligand for P2Y receptors since functional studies indicated that this compound is two orders of magnitude less potent at the $P2Y_2$ receptor and has no agonist or antagonist activity at the $P2Y_4$ or $P2Y_6$ receptors or at an adenylyl cyclase-coupled P2Y receptor in C6 glioma cells.

Adenine nucleotide analogues including [^{35}S]-ATPαS (Keppens *et al*., 1990), [^{35}S]-ADPβS (Cooper *et al*., 1989), [^{35}S]-ATPγS and [α-^{32}P]-ATP (Motte *et al*., 1996), have been used in other attempts to radiolabel P2Y receptors. We have previously questioned the validity of our initial contention (Cooper *et al*., 1989) that [^{35}S]-ADPβS might be utilized to radiolabel the turkey erythrocyte P2Y receptor (Harden *et al*., 1995). Based on a biphasic displacement curve for inhibition of binding by 2MeSATP, Motte *et al*. (1996) have concluded that at least two thirds of the binding sites for [^{35}S]-ATPγS and [α-^{32}P]-ATP on bovine aortic endothelial membranes are not P2Y receptors. In the same study, 2MeSATP did not displace any of the radioligand binding to intact cells, suggesting that cell surface P2Y receptors represent, at most, a minimal percentage of the total cellular adenine nucleotide ligand binding sites. This conclusion is consistent with the extraordinary number of binding sites identified by [^{35}S]-ATPαS binding in guinea-pig and rabbit liver (47 and 71 pmol mg^{-1}, respectively, (Keppens *et al*., 1990)) and by [^{35}S]-dATPαS in membranes from rat brain (39 pmol mg^{-1}, (Simon *et al*., 1995b)) or rabbit gastric glands (16.8 pmol mg^{-1}, (Vallejo *et al*., 1996)).

A member of the G protein-coupled class of receptors with 32 to 36% homology to other P2Y receptors has been cloned from a cDNA library prepared from activated chick T-lymphocytes (Kaplan *et al*., 1993). This orphan receptor, designated 6H1, recently was suggested to be a P2Y receptor (the $P2Y_5$ receptor) based on the fact that [^{35}S]-dATPαS bound to membranes from Cos-7 cells transfected with DNA encoding this receptor (Webb *et al*., 1996). However, no binding data have been obtained for membranes from wild-type cells, and no functional responses of this receptor to dATPαS, ATP or other adenine nucleotide analogues have been found. Our results with [^{35}S]-dATPαS binding indicate that this molecule does not exhibit the properties necessary for use as a specific radioligand for P2Y receptors. Moreover, in membranes from non-transfected Cos-7 cells we observed that [^{35}S]-dATPαS binding was inhibited by adenine nucleotide analogues with similar potencies to those obtained for membranes from 6H1 receptor-transfected Cos-7 cells. These results do not support the contention that the 6H1 receptor is a P2Y receptor subtype.

Kunapuli and coworkers (Akbar *et al*., 1996) have cloned a G protein-coupled receptor that is 24–30% homologous with G protein-coupled P2Y receptors and have proposed this to be a $P2Y_7$ receptor based primarily on the binding of [^{35}S]-dATPαS to membranes from transfected Cos-7 cells. No comparative binding with membranes from wild-type cells was obtained. Moreover, the overall pharmacological selectivity that was observed in [^{35}S]-dATPαS binding assays with membranes from receptor-transfected Cos-7 cells (Akbar *et al*., 1996) was very similar to that observed here with membranes from wild-type Cos-7 cells. A small inositol phosphate response to a single concentration of ATP was found for Cos-7 cells transfected with cDNA for the putative $P2Y_7$ receptor (Akbar *et al*., 1996). Since we consistently observed an inositol phosphate response to ATP in wild type Cos-7 cells, a detailed pharmacological analysis will be necessary to differentiate any response of a heterologously expressed putative P2Y receptor from that of the endogenous nucleotide receptor in Cos-7 cells.

The introduction of a compound that could be used to identify and quantify selectively one or more P2Y receptor subtypes would be of considerable use to the P_2 receptor signalling field. Ideally, such a compound would exhibit nanomolar affinity and selectivity for P_2 receptors as well as be resistant to nucleotidase activity. [^{35}S]-dATPαS is one of a group of radiolabelled purine nucleotide analogues that does not appear to fulfill these criteria. However, this molecule is a partial agonist/competitive antagonist at the $P2Y_1$ receptor. This antagonist activity, together with its relative selectivity for

the $P2Y_1$ receptor, support the idea that derivatives of dATPαS might be synthesized as selective high affinity $P2Y_1$ receptor antagonists.

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