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Second messenger cascade specificity and pharmacological selectivity of the human P_{2Y1}-purinoceptor

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1 The coding sequence of the P_{2Y1}-purinoceptor was cloned from a human genomic library.

2 The open reading frame encodes a protein of 373 amino acids that is 83% identical to the previously cloned chick and turkey P_{2Y1}-purinoceptor and is $\geqslant$95% homologous to the recently cloned rat, mouse, and bovine P_{2Y1}-purinoceptors.

3 The human P_{2Y1}-purinoceptor was stably expressed in 1321N1 human astrocytoma cells using a retroviral vector. Although the P_{2Y1}-purinoceptor agonist, 2MeSATP, had no effect on inositol phosphate accumulation in 1321N1 cells infected with the control virus, this agonist markedly stimulated inositol phosphate accumulation in cells infected with the P_{2Y1}-purinoceptor virus. No effect of 2MeSATP on cyclic AMP accumulation was observed in P_{2Y1}-receptor-expressing 1321N1 cells.

4 The pharmacological selectivity of 18 purinoceptor agonists was established for the expressed human P_{2Y1}-purinoceptor. 2MeSATP was more potent than ATP but less potent than 2MeSADP. ADP also was more potent than ATP. A similar maximal effect was observed with most agonists tested. However, α,β-MeATP had no effect and 3'-NH_2-3'-deoxyATP and A_2P_4 were partial agonists. The order of potency of agonists for activation of the turkey P_{2Y1}-purinoceptor, also stably expressed in 1321N1 cells, was identical to that observed for the human P_{2Y1}-purinoceptor.

5 C6 glioma cells express a P_{2Y}-purinoceptor that inhibits adenylyl cyclase but does not activate phospholipase C. Expression of the human P_{2Y1}-purinoceptor in C6 cells conferred 2MeSATP-stimulated inositol lipid hydrolysis to these cells. The phospholipase C-activating human P_{2Y1}-purinoceptor could be delineated from the endogenous P_{2Y}-purinoceptor of C6 glioma cells by use of the P_2-purinoceptor antagonist, PPADS, which blocks the P_{2Y1}-purinoceptor but does not block the endogenous P_{2Y}-purinoceptor of C6 cells. P_2-purinoceptor agonists also exhibited differential selectivities for activation of these two P_{2Y}-purinoceptors.

Keywords: P_{2Y}-purinoceptor; ATP; cloning; second messenger coupling

Introduction

P_2-purinoceptors for extracellular adenine nucleotides originally were subclassified into the P_{2X}- and P_{2Y}-subtypes on the basis of differential contractile responses of various tissues to a series of hydrolysis resistant analogues of ATP (Burnstock & Kennedy, 1985). Further delineation of putative P_2-purinoceptor subtypes has evolved on the basis of differences in selectivity of various nucleotides for stimulation of certain responses. For example, the existence of P_{2U}-purinoceptors was initially proposed on the basis of the agonist activity of UTP and ATP at this receptor(s) (Dubyak & El-Moatassim, 1993), and the P_{2T}-purinoceptor(s), which is activated by ADP but antagonized by ATP, has been widely studied in platelets (Hourani & Cusack, 1991).

Unambiguous delineation of P_2-purinoceptor subtypes has followed from the cloning and expression of functional protein from nucleotide sequences encoding these receptors. Four subtypes of P_{2X}-purinoceptors have been cloned and shown by functional expression to exhibit the general pharmacological and electrophysiological properties expected of these ligand-gated ion channel receptors (Brake *et al.*, 1994; Valera *et al.*, 1994; Abbracchio & Burnstock, 1994; Chen *et al.*, 1995). Association of pharmacological selectivity with proteins of known structure also has been made for members of a group of P_2-purinoceptors that regulate physiological responses by activation of G-protein-regulated signalling responses (Lustig *et al.*, 1993; Webb *et al.*, 1993; Parr *et al.*, 1994; Filtz *et al.*, 1994; Lazarowski *et al.*, 1995).

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Our laboratory has examined in detail the second messenger signalling properties and pharmacological selectivity of a P_2-purinoceptor on turkey erythrocytes. This receptor, designated the P_{2Y1}-purinoceptor, has been cloned from chick (Webb *et al.*, 1993) and turkey (Filtz *et al.*, 1994) and stably expressed in a null cell line (Filtz *et al.*, 1994). The pharmacological selectivity of the turkey P_{2Y1}-purinoceptor as natively expressed in turkey erythrocytes (Burnstock *et al.*, 1994; Boyer *et al*, 1994; 1995) or heterologously expressed in a mammalian cell line (Filtz *et al.*, 1994) is very similar to the pharmacological profile of responses previously studied in mammalian tissues, e.g. a P_{2Y}-like purinoceptor on guinea-pig taenia coli (Burnstock *et al.*, 1994). However, the pharmacological selectivity of a cloned mammalian P_{2Y}-purinoceptor has not been reported in detail. As such, we have cloned the human P_{2Y1}-purinoceptor and have expressed and extensively characterized the pharmacological and second messenger signalling properties of this receptor.

Methods

Cloning of the human P_{2Y1}-purinoceptor

Using 0.36 μg of rat genomic DNA as template, a 702 bp fragment encoding the rat homologue of the turkey P_{2Y1}-purinoceptor was generated by polymerase chain reaction with degenerate primers (upstream, 5'-TGGATGTT(TC)GTTTT-(TC)CA(TC)ATG-3', and downstream, 5'-GAGATCTA-GATCACAAACTGGTGTCCCCGTT-3') based on the sequence of the turkey P_{2Y1}-purinoceptor, followed by re-amplification with an internal upstream primer (5'-TTCCTC-

AC(CG)TGCAT(AC)AGCGTGCA-3') and the same downstream primer. The fragment was radiolabelled (Pharmacia Radiolabeling Kit) and used to screen a lambda Zap II human brain cDNA library (Stratagene). One positive clone, obtained from 1×10^6 screened, was excised and sequenced by the dideoxy chain termination method (Sequenase II, USB). The clone was 1543 bp in length and contained an open reading frame from nucleotides 1 to 972. This sequence, which was 82% homologous with bases 118 to 1089 of the turkey P_{2Y1}-purinoceptor sequence, was incomplete at the 5' end. A 300 bp probe, corresponding to the 5'-end of this cDNA clone, was generated and used to probe a human genomic library (kindly provided by Dr Beverly Mitchell). Two positive clones containing large (>10 kbp) inserts were obtained. The genomic clones were amplified, and the λDNA was isolated and digested with *Sac*I. A 2000 bp fragment containing the probe sequence was identified in both λ clones by Southern blotting, and each fragment was subcloned and sequenced in both directions. The genomic sequence corresponded exactly to the cDNA sequence but also included 150 bp of additional 5' sequence containing an initiating methionine codon.

Expression of the P_{2Y1}-purinoceptor in 1321N1 cells

The coding sequence of the receptor was amplified with *Pfu* DNA polymerase (Stratagene) using primers containing either an *Eco*RI site (upstream primer) or a *Xho*I site (downstream primer). This fragment was subcloned into the retroviral expression vector pLXSN, and the upstream primer was situated such that 18 bp of 5' untranslated sequence was included in the amplified product. Retrovirus conferring expression of P_{2Y1}-purinoceptor was produced by calcium phosphate transfection of the murine packaging cell line PA317 with pLXSN vectors containing the human or turkey receptor sequences as described by Olsen and colleagues (Comstock *et al.*, 1995). After a 2 day incubation at 32°C in the presence of 5 mM butyrate, the virus-containing supernatant was removed, filtered, and incubated for 2 h with 1321N1 cells in the presence of 8 μg ml^{-1} polybrene. Infected 1321N1 cells were selected for neomycin resistance for two weeks in the presence of 600 μg ml^{-1} G-418. Comparisons of the pharmacological selectivity of human and turkey P_{2Y1}-purinoceptors were made in these neomycin-resistant cell populations. Retroviral infection of C6 rat glioma cells was performed similarly with 800 μg ml^{-1} G418 for selection.

Quantification of inositol lipid hydrolysis

Functional responses to purinoceptor agonists were assessed by measurement of inositol phosphate accumulation as described by Lazarowski & Harden (1994). Briefly, 131N1 cells stably expressing the human P_{2Y1}-purinoceptor were plated at a density of 2×10^5 cells/well in 12 well plates and assayed three days after subculture. The growth medium (DMEM with 5% FBS and 600 μg ml^{-1} G418) was replaced with 0.5 ml of serum-free, inositol-free medium supplemented with 2 μCi ml^{-1} of [^{3}H]-*myo*-inositol (20 Ci mmol^{-1}; American Radiolabeled Chemicals). The culture plates were removed from the incubator 24 h later, the medium was supplemented with 50 μl 250 mM HEPES, pH 7.4, and the plates were placed in a 37°C water bath. Drug challenges were initiated by addition of agonist and 10 μM LiCl in HEPES-buffered, inositol-free DMEM. After a 10 min incubation the medium was aspirated and the assay terminated with 500 μl of cold 5% TCA. The TCA-containing supernatant was extracted three times with ether and inositol phosphates were isolated by chromatography on Dowex AG1-X8.

Quantitation of cyclic AMP accumulation

Cells were prepared essentially as described for the inositol phosphate assay except the medium was replaced with normal DMEM 24 h prior to assay and this was supplemented with 1.0 μCi ml^{-1} [^{3}H]-adenine (25 Ci mmol^{-1}, Amersham) for 2 h before assay. Cells were preincubated with isobutylmethyl xanthine (IBMX, 0.2 mM, final concentration) for 10 min prior to a 10 min incubation with agonists. Drug challenges were terminated by aspiration of the medium and addition of 5% TCA. The extract was applied directly to Dowex and alumina columns for isolation of [^{3}H]-cyclic AMP.

Results

The sequence and pharmacological selectivity have been reported for a P_{2Y}-purinoceptor cloned from chick (Webb *et al.*, 1993) or turkey brain cDNA libraries (Filtz *et al.*, 1994). By use of primers based on the turkey and chick P_{2Y1}-purinoceptor sequences, a sequence encoding a portion of the rat homologue of this receptor was amplified from rat genomic DNA. A probe based on the rat sequence was then used to

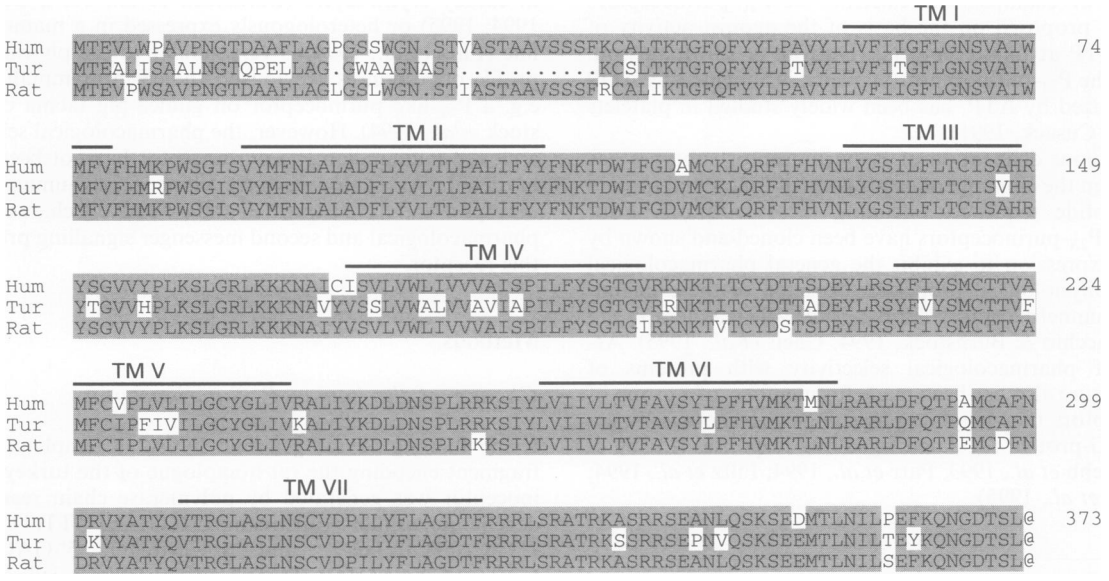

Figure 1 Comparison of the predicted amino acid sequences of the human, turkey and rat, P_{2Y1}-purinoceptor.

isolate a partial cDNA clone of this receptor from a human brain cDNA library, and this partial cDNA subsequently was used to isolated the complete coding sequence of the human homologue of the avian P_{2Y1}-purinoceptor from a human genomic library (Figure 1). An open reading frame of 1119 nucleotides encoding 373 amino acids (calculated Mr = 42,000) was identified. The human P_{2Y1}-purinoceptor sequence is 79% identical at the nucleic acid level and 83% identical at the amino acid level to the turkey P_{2Y1}-purinoceptor sequence (362 amino acids). In addition to low homology in the amino terminal domain, the human sequence contains an 11 amino acid insert near the amino terminus that is absent in the avian sequence. This insert also is present in the sequences of the rat (Tokuyama *et al.*, 1995), mouse (Tokuyama *et al.*, 1995), and bovine (Henderson *et al.*, 1995) homologues of the P_{2Y1} purinoceptor, which have been reported during the completion of this work on the human P_{2Y1}-purinoceptor. Differences in amino acid sequence of the human versus avian P_{2Y1}-purinoceptor also occur elsewhere, particularly in the predicted fourth and fifth transmembrane-spanning domains, although several of these are conservative changes. In contrast, the human P_{2Y1}-purinoceptor sequence is approximately 95% identical at the amino acid level to the rat (Tokuyama *et al.*, 1995), mouse (Tokuyama *et al.*, 1995), and bovine (Henderson *et al.*, 1995) P_{2Y1}-purinoceptors.

The human P_{2Y1}-purinoceptor was stably expressed in 1321N1 human astrocytoma cells after construction of a retroviral vector. The P_{2Y}-purinoceptor agonist, 2MeSATP, had no effect on inositol phosphate accumulation in wild-type 1321N1 cells or in 1321N1 cells infected with an empty retroviral vector (Figure 2). However, 2MeSATP markedly stimulated inositol phosphate accumulation in cells infected with retrovirus containing the coding sequence of the human P_{2Y1}-purinoceptor.

The pharmacological selectively of the stably expressed human P_{2Y1}-purinoceptor was examined by use of 18 nucleotide analogues. The relative potency of these agonists for stimulation of inositol phosphate accumulation was consistent with the order of potency expected for a P_{2Y}-purinoceptor (Figure 3 and Table 1). As initially observed by Burnstock & Kennedy (1985) for P_{2Y}-purinoceptors, the 2-thioether derivative of ATP was more potent than ATP or other ATP analogues. However, 2MeSADP was more potent than 2MeSATP as were other diphosphate analogues compared to their corresponding triphosphate derivatives (Figure 3). A similar maximal effect was observed with most of the agonists tested. However, the P_{2X}-purinoceptor-selective agonist, α,β-methylene ATP, had no effect on inositol phosphate accumulation, and 3'-NH_2-3'-deoxyATP and A_2P_4 exhibited only partial agonist activities (Figure 4). This lack of full agonist effect of these two compounds was not a consequence of their low potency since AppNHp exhibited low potency but was a full agonist. The previously cloned turkey P_{2Y1}-purinoceptor (Filtz *et al.*, 1994) was also stably expressed in 1321N1 cells using the retroviral expression system, and the relative order of potencies for these agonists was directly compared for the two

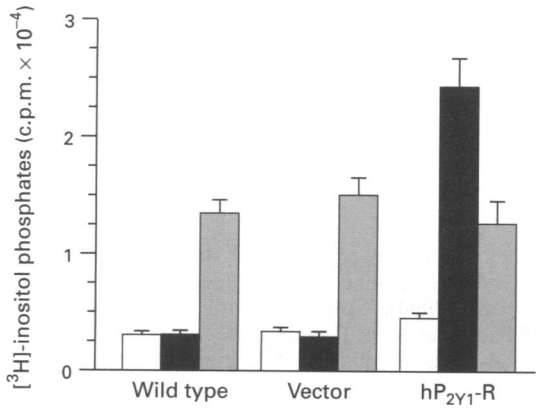

Figure 2 Inositol phosphate responses in 1321N1 cells expressing the human P_{2Y1}-purinoceptor. 1321N1 cells were either mock-infected (wild type), infected with control retroviral vector pLXSN (vector), or infected with this same retroviral vector containing DNA encoding the human P_{2Y1}-purinoceptor (hP_{2Y1}-R). Following selection of infected cells with G-418, all three cell populations were examined for receptor-stimulated inositol phosphate accumulation in the presence of either 1 μM 2MeSATP (solid columns) or 1 mM carbachol (stippled columns); basal (open columns). The data are the mean ± s.e.mean of four experiments performed in triplicate.

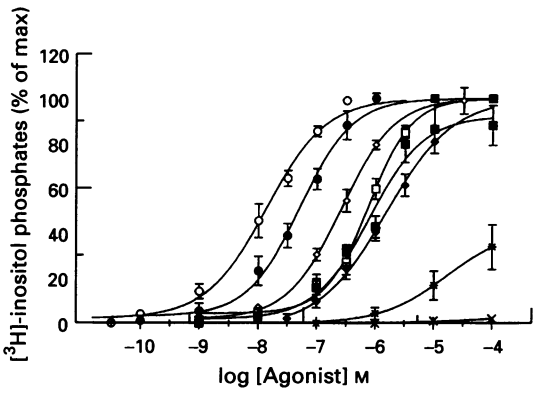

Figure 3 Pharmacological selectivity of the human P_{2Y1}-purinoceptor. Inositol phosphate responses to a series of purinoceptor agonists were examined as described in Methods. The data shown are the combined results of three to five independent experiments for each agonist. Results in each experiment were normalized to the maximal response of 2MeSATP in that experiment to facilitate combination of data from different experiments. Data are shown for 2MeSADP (○), 2MeSATP (●), ADP (◇), ADPβS (□), ATPγS (■), ATP (◆), α,βMeADP (*), and α,βMeATP (×).

Table 1 Agonist potencies at the human and turkey P_{2Y1}-purinoceptors

Agonist	*Human P_{2Y1}*	*Turkey P_{2Y1}*
2MeSADP	13.6 ± 2.1	11.2 ± 2.1
2MeSATP	51.4 ± 6.0	33.2 ± 2.4
ADP	257 ± 14	116 ± 22
ATP	1520 ± 210	876 ± 140
ADPβS	759 ± 74	143 ± 20
ATPγS	795 ± 210	426 ± 81
2ClATP	774 ± 120	384 ± 115
3'-NH_2-3'dATP	(45%) 3640 ± 1100	(61%) 3900 ± 1200
AppNHp	26,300 ± 2700	10,100 ± 1900
AMP	100,000	100,000
UTP	100,000	100,000
α,β MeADP	> 100,000	> 100,000
β,γ MeATP	> 100,000	> 100,000
α,β MeATP	NE	NE
A_2P_2	100,000	100,000
A_2P_4	(49%) 625 ± 135	(67%) 742 ± 267
A_2P_5	> > 100,000	> ; > 100,000
A_2P_6	NE	NE

EC_{50} values are shown (nM) for the stimulation of inositol phosphate accumulation by purinoceptor agonists in 1321N1 cells infected with retrovirus encoding either the human or turkey P_{2Y1}-purinoceptors. Values are the mean ± s.e.mean of three independent determinations performed simultaneously on both cell lines. The efficacies of two partial agonists relative to 2MeSATP are indicated in parentheses preceding the EC_{50} values. NE indicates no effect at 100,000 nM.

P_{2Y1}-purinoceptor homologues. The full agonists tested were approximately two fold more potent at the turkey P_{2Y1}-purinoceptor than at the human homologue of this receptor. Thus, both the order and the ratio of agonist potencies were essentially identical for the two P_{2Y1}-purinoceptor homologues. Similar agonist potencies also were observed whether they were determined in assay incubation times of 1 min or 15 min. This result suggests that hydrolysis of agonists does not influence the EC_{50} values that were observed. 3'NH_2-3'-deoxyATP and A_2P_4 were partial agonists at both the human and avian P_{2Y1}-purinoceptors but in contrast to the results with full agonists, the potency observed for each of these partial agonists was the same whether determined with the human or avian receptor. One interpretation of these results is that a larger degree of receptor reserve exists in 1321N1 cells expressing the turkey P_{2Y1}-purinoceptor than in 1321N1 cells expressing the human P_{2Y1}-purinoceptor.

A P_{2Y}-purinoceptor that inhibits adenylyl cyclase but does not activate phospholipase C, is endogenously expressed on C6 rat glioma cells (Boyer *et al.*, 1993; 1994). To assess whether the cloned human P_{2Y1}-purinoceptor also coupled to adenylyl cyclase, we examined cyclic AMP accumulation in 1321N1 cells expressing this receptor. Although marked P_{2Y}-purinoceptor-promoted inositol lipid hydrolysis was observed, 2MeSATP caused neither stimulation nor inhibition of cyclic AMP accumulation in 1321N1 cells expressing the human P_{2Y1}-purinoceptor (Figure 5). The most parsimonious interpretation of these results is that a P_{2Y}-purinoceptor has been cloned that couples to phospholipase C but not to adenylyl cyclase. Alternatively, the second messenger signalling selectivity of the P_{2Y}-purinoceptor could be specific to the cell type in which the receptor is expressed. To distinguish between these two possibilities, the human P_{2Y1}-purinoceptor was expressed in C6 cells to determine whether expression in this cell line would result in a different second messenger signalling phenotype. Infection of C6 cells with human P_{2Y1}-purinoceptor retrovirus conferred an inositol phosphate response to analogues of ATP that was not observed in wild type or in vector-infected cells (Figure 6). In contrast, 2MeSATP-promoted inhibition of cyclic AMP accumulation was essentially indistinguishable between wild type C6 cells and C6 cells expressing the human P_{2Y1}-purinoceptor (not shown).

P_2-purinoceptor agonists and antagonists also were utilized to distinguish between the human P_{2Y1}-purinoceptor stably expressed in C6 cells and the adenylyl cyclase-linked P_{2Y}-purinoceptor that is native to these cells. We previously have reported that whereas the P_2-purinoceptor antagonist, PPADS, is a competitive antagonist at the phospholipase C-linked P_{2Y}-purinoceptor of turkey erythrocytes, this drug has no effect on the adenylyl cyclase-linked P_{2Y}-purinoceptor of C6 glioma cells (Boyer *et al.*, 1994). Consistent with these previous results, PPADS had no effect of P_{2Y}-purinoceptor-promoted inhibition of cyclic AMP accumulation in human P_{2Y1}-purinoceptor-expressing C6 cells (Figure 7a). In contrast, PPADS antagonized 2MeSATP-stimulated inositiol phosphate accumulation under the same conditions. Thus, 30 μM PPADS resulted in a 10 fold shift to the right of the concentration-effect curve for 2MeSATP (Figure 7b). PPADS did not affect muscarinic receptor-stimulated inositol phosphate accumulation in these cells (data not shown), illustrating that PPADS is selective for the phospholipase C-linked P_{2Y}-purinoceptor over the adenylyl cyclase-linked P_{2Y}-purinoceptor endogenous to the C6 cell.

In addition to selective blockade by PPADS of 2MeSATP-stimulated inositol lipid hydrolysis in C6 cells expressing the

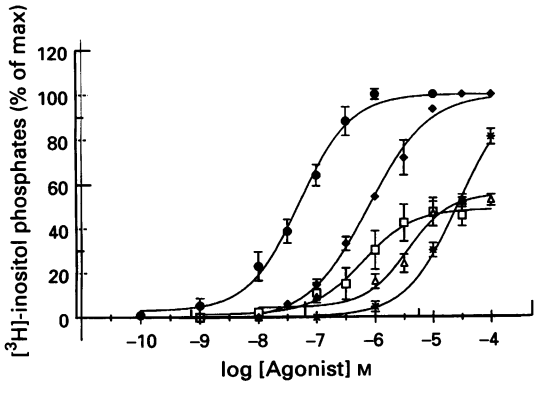

Figure 4 Demonstration of agents with partial agonist activity. Inositol phosphate responses to partial and full agonists were assessed in 1321N1 cells stably expressing the human P_{2Y1}-purinoceptor. The data are normalized as in Figure 3. Data are shown for 2MeSATP (●), 2ClATP (◆), A_2P_4 (□), 3'-NH_2-3'-dATP (△) and AppNHp (*).

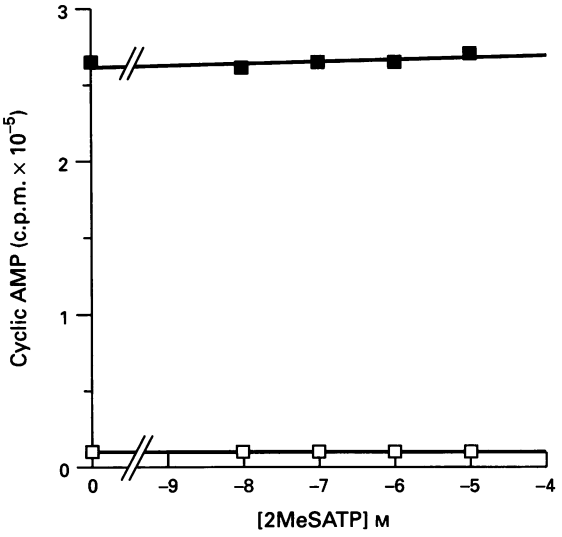

Figure 5 Effect of the human P_{2Y1}-purinoceptor on cyclic AMP accumulation. Cyclic AMP accumulation was measured in 1321N1 cells expressing the human P_{2Y1}-purinoceptor in the presence of various concentrations of 2MeSATP and in the presence (■) or absence (□) of 20 μM isoprenaline and 10 μM forskolin. The data are shown from a single experiment, and are representative of results from three similar experiments.

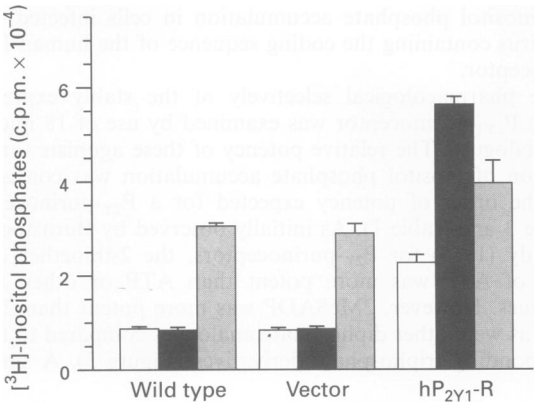

Figure 6 Inositol phosphate accumulation in C6 cells stably expressing the human P_{2Y1}-purinoceptor. Inositol phosphate accumulation was measured in C6 glioma cells stably expressing the human P_{2Y1}-purinoceptor. Assays were in the presence of vehicle (open columns), 1 μM 2MeSATP (solid columns), or 10 μM AlF_4^- (stippled columns). The data are the means ± s.e.mean from four experiments.

human P_{2Y1}-purinoceptor, the inositol phosphate and cyclic AMP responses also were activated differently by certain agonists (Figure 8a and b). For example, the order of potencies of four agonists for stimulation of inositol lipid hydrolysis was similar whether determined with 1321N1 or C6 cells expressing the human P_{2Y1}-purinoceptor (compare Table 1 with Table 2). These four drugs exhibited a different order of potency and, in the case of 3'-NH_2-3'-deoxyATP, a different efficacy, for P_{2Y}-purinoceptor-promoted inhibition of adenylyl cyclase in C6 cells. These data strongly support the contention that the cloned human P_{2Y1}-purinoceptor is functionally, and therefore, structurally distinct from the P_{2Y}-purinoceptor subtype endogenous to C6 rat glioma cells.

Discussion

Pharmacological characterization of P_2-purinoceptors has been compromised by the presence in many tissues of multiple and undefined P_2-purinoceptors and by high levels of ectonucleotidase activity in these tissues. Molecular cloning and functional expression of the proteins that comprise the large family of P_2-purinoceptors provides a means of circumventing these potential problems and associating drug selectivities with receptor proteins of defined structure. Based on the original report of Webb and coworkers (Webb *et al.*, 1993) we previously cloned, expressed, and determined the pharmacological selectivity of the turkey homologue of the P_{2Y1}-purinoceptor (Filtz *et al.*, 1994). This avian receptor sequence now has been used to clone the first human homologue of a seven-transmembrane spanning receptor that exhibits the pharmacological selectivity of the classically defined P_{2Y}-purinoceptor.

The pharmacological selectivity of the human P_{2Y1}-purinoceptor when expressed in a null cell line is remarkably similar to that proposed by Burnstock & Kennedy (1985) in

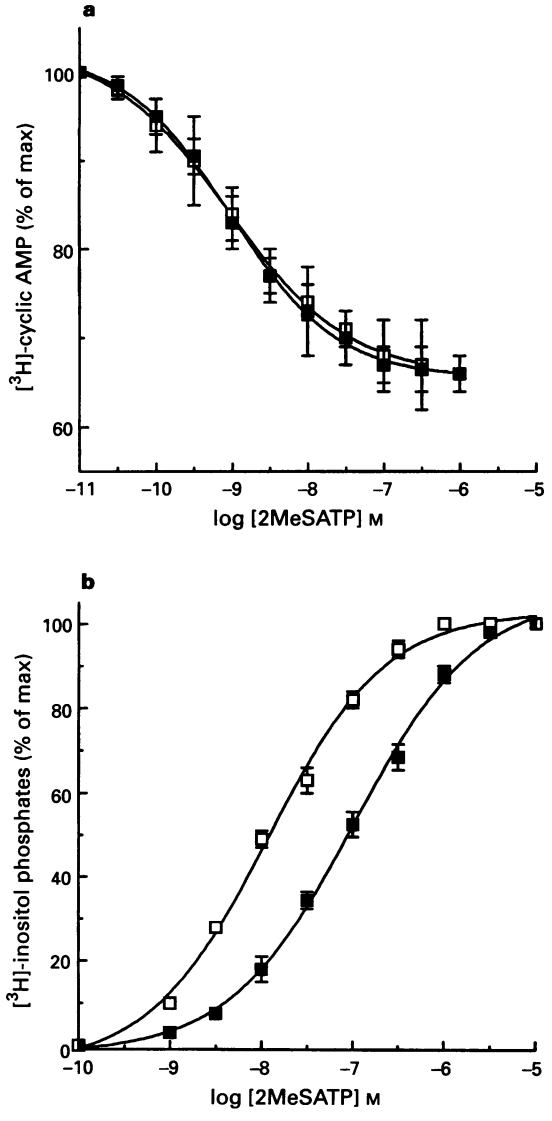

Figure 7 Effect of a P_2-purinoceptor antagonist on P_2-purinoceptor-promoted responses in C6 cells expressing the human P_{2Y1}-purinoceptor. The cyclic AMP (a) and inositol phosphate (b) responses to 2MeSATP were examined in the presence (■) and absence (□) of 30 μM PPADS in C6 glioma cells stably expressing the human P_{2Y1}-purinoceptor. The EC_{50} for 2MeSATP for inhibition of cyclic AMP accumulation was 1 ± 0.2 nM in the absence or in the presence of PPADS. The EC_{50} of 2MeSATP for stimulation of inositol phosphate accumulation was 13 ± 2 nM in the absence of PPADS and 110 ± 6 nM in its presence.

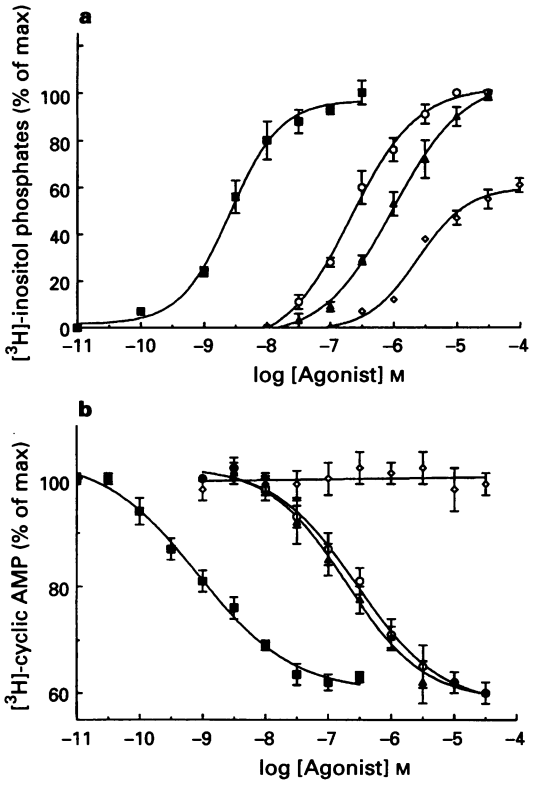

Figure 8 Relative agonist potencies for the stimulation of inositol phosphate and cyclic AMP responses in C6 glioma cells expressing the human P_{2Y1}-purinoceptor. The relative potencies of 2MeSADP (■), ADP (○), ADPβS (▲), and 3'-NH_2-3'-deoxyATP (◇) were determined for stimulation of inositol phosphate accumulation (a) and inhibition of cyclic AMP accumulation (b) in C6 glioma cells stably expressing the human P_{2Y1} receptor. The data are presented as percentage of maximal response to 1 μM 2MeSADP (a) or percentage of isoprenaline-stimulated cyclic AMP accumulation (b).

Table 2 Agonist potencies for second messenger effects in C6 cells expressing the human P_{2Y1}-purinoceptor

Agonist	*Inositol phosphates*	*Cyclic AMP*
2MeSADP	2.6 ± 0.33	0.76 ± 0.15
ADP	210 ± 32	290 ± 30
ADPβS	971 ± 112	161 ± 44
3'NH_2-3'dATP	2380 ± 580	NE

EC_{50} values of four agonists were determined for two second messenger responses in C6 rat glioma cells expressing the human P_{2Y1}-purinoceptor. Values (shown in nanomolar ± s.e.mean) are averages of three experiments performed in triplicate.

their original description of a P_{2Y}-purinoceptor. 2MeSATP was much more potent than ATP, and α,βMeATP was without effect. The original classification of P_{2Y}-purinoceptors did not specifically consider the relative potency of ADP and analogues of this diphosphate. However, our data with the avian (Burnstock *et al.*, 1994; Filtz *et al.*, 1994) and now the human P_{2Y1}-purinoceptor indicate that this receptor is also readily activated by ADP and by ADP analogues at concentrations typically lower than those necessary for activation by the corresponding ATP analogues. A receptor that is activated by ADP and referred to as a P_{2T}-purinoceptor has been widely studied in platelets (Hourani & Cusack, 1991). Although the P_{2Y1}-purinoceptor shares with the platelet P_{2T}-purinoceptor the capacity to be activated by ADP, these receptors can be distinguished readily by the action of ATP. ATP is a competitive antagonist of the platelet purinoceptor but is a full agonist at the human P_{2Y1}-purinoceptor.

The pharmacological selectivity previously determined for a broad range of agonists for activation of the avian P_{2Y}-purinoceptor closely matched the selectivity of these agonists for activation of a P_{2Y}-like receptor on guinea-pig taenia coli (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). Nonetheless, the true relevance of the avian receptor to P_{2Y}-purinoceptors in mammalian tissues has been unclear. Data obtained with the cloned and expressed human P_{2Y1}-purinoceptor now place the avian receptor in clearer context. The relative order of potency of a large number of agonists at the human receptor matches the order of potency of the same agonists at the avian receptor. Thus, the turkey P_{2Y1}-purinoceptor is apparently an accurate pharmacological model for a human P_{2Y}-purinoceptor. Delineation of the specific role of P_{2Y1}-purinoceptors in various of the physiological responses that have been attributed to P_{2Y}-purinoceptors, including the aforementioned response observed in guinea-pig taenia coli, will be important to establish.

Not only was the pharmacological selectivity of the human receptor the same as that of the avian P_{2Y1}-purinoceptor, but an identical specificity of second messenger signalling was observed. This selectivity was established by stable expression of the human receptor in two different cell lines. A cell line, 1321N1 human astrocytoma cells, that does not express a measurable phospholipase C response to extracellular nucleotides has been widely used by our laboratory and others to express stably P_2-purinoceptors. As we previously observed with the avian P_{2Y1}-purinoceptor (Filtz *et al.*, 1994), stimulation of inositol lipid hydrolysis was observed in 1321N1 cells stably expressing the human P_{2Y1}-purinoceptor. In contrast, the potent P_{2Y}-purinoceptor agonist 2MeSATP neither inhibited nor activated adenylyl cyclase in these cells. Lack of effect of the expressed human P_{2Y1}-purinoceptor on cyclic AMP accumulation is an important observation in face of the existence of a P_{2Y}-purinoceptor on C6 rat glioma cells that inhibits adenylyl cyclase but has no effect on inositol lipid hydrolysis or Ca^{2+} mobilization. This specificity of coupling of P_{2Y}-purinoceptors to second messenger signalling cascades is not an artifact of the existence of signalling proteins in C6 glioma cells that are not present in 1321N1 cells or *vice versa*, since the human P_{2Y1}-purinoceptor retained fidelity of coupling to the inositol lipid signalling pathway when expressed in C6 glioma cells. Pharmacological differences between the phospholipase C-linked P_{2Y1}-purinoceptor and the yet-to-be-cloned adenylyl cyclase-linked P_{2Y}-purinoceptor were maintained in C6 cells expressing both of these receptors. For example, we previously reported that the P_2-purinoceptor antagonist, PPADS, competitively antagonizes the avian P_{2Y1}-purinoceptor, but has no effect on the adenylyl cyclase-linked P_{2Y}-purinoceptor endogenously expressed in C6 cells (Boyer *et al.*, 1993; 1994). In C6 cells engineered to express both of these receptors, the inositol phosphate response to 2MeSATP was blocked by PPADS, whilst the cyclic AMP response was unaffected. Differences in agonist potencies for inositol phosphate and cyclic AMP responses identified from our previous studies of the avian and C6 cell receptors also were observed in C6 cells expressing both receptors. These data strongly support our previous contention that a novel P_{2Y}-like purinoceptor exists on C6 glioma cells that differs in pharmacological and biochemical properties from the previously cloned phospholipase C-linked P_{2Y1}-purinoceptor. Cloning of nucleotide sequence encoding this G_i-linked P_2-purinoceptor is an important goal, particularly since this would provide the first receptor sequence for what may prove to be a larger class of G_i-linked P_2-purinoceptors.

Henderson *et al.* (1995) recently reported the cloning and expression of a cDNA that apparently encodes the bovine homologue of the P_{2Y1}-purinoceptor. However, the functional receptor expressed from this cDNA exhibited a different pharmacological selectivity with three agonists from that previously reported from our laboratory for the cloned expressed turkey P_{2Y1}-purinoceptor. ADP and 2MeSATP were equipotent (EC_{50} = 30 nM) and ATP was considerably less potent and less efficacious for activation of the bovine receptor (Henderson *et al.*, 1995). The 86% sequence similarity between the bovine and avian sequences led Henderson *et al.* (1995) to conclude tentatively that the bovine and avian receptors are species homologues and that differences in apparent pharmacological selectivity were probably explained by the different cell types (Jurkat versus 1321N1 cells) used for stable expression and the different second messenger responses (Ca^{2+} mobilization versus inositol phosphate accumulation, Filtz *et al.*, 1994) used in the two studies to monitor receptor activation. The extensive comparison reported in the current work of pharmacological selectivities of the turkey and human receptors expressed in the same cell type and assayed under identical conditions supports the conclusion that the avian and human receptors are species homologues that exhibit no apparent differences either in their second messenger signalling cascade specificity or in their pharmacological selectivities. These relative agonist potencies also should be an accurate reflection of relative affinities for the human and turkey P_{2Y1}-purinoceptor since extensive analyses have failed to reveal any evidence for agonist hydrolysis during the assays that underlie the determination of these drug potencies.

In summary, the nucleotide sequence encoding the human P_{2Y1}-purinoceptor has been cloned and stably expressed. Therefore, we are now in a position to establish the extent to which the P_{2Y1}-purinoceptor accounts for P_{2Y}-purinoceptor-mediated regulation of physiological responses in mammalian tissues. The availability of a single human P_{2Y}-purinoceptor of defined structure in a preparation that allows simple tests of receptor agonist and antagonist activities should be useful in identifying increasingly specific P_{2Y}-purinoceptor agonists and antagonists.

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