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Fidelity in functional coupling of the rat $P2Y_1$ receptor to phospholipase C

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1 The rat homologue of the $P2Y_1$ receptor has been heterologously expressed in 1321N1 human astrocytoma cells and in C6 rat glioma cells.

2 As has been shown previously for the turkey and human $P2Y_1$ receptors, the rat $P2Y_1$ receptor expressed in either cell type responded to 2MeSATP with increases in inositol phosphate accumulation that were competitively blocked by the antagonist PPADS. Neither of the wild type cell lines exhibited inositol phosphate responses to $P2Y_1$ receptor agonists.

3 Expression of the rat $P2Y_1$ receptor did not confer a capacity of 2MeSATP to inhibit adenylyl cyclase activity in 1321N1 cells. Moreover, the inhibition of adenylyl cyclase mediated by an endogenous P2Y receptor of C6 glioma cells was not enhanced by expression of the rat $P2Y_1$ receptor. The P2Y receptor-mediated inhibition of adenylyl cyclase in C6 glioma cells expressing both the endogenous P2Y receptor and the rat $P2Y_1$ receptor remained unaffected by PPADS.

4 Since the P2Y receptor responsible for inhibition of adenylyl cyclase in C6 glioma cells does not share the pharmacological or functional properties of the $P2Y_1$ receptor, even when both receptors originate from the same species and are simultaneously expressed in the same cell line, it is concluded that the $P2Y_1$ receptor is distinct from an endogenous P2Y receptor in C6 cells that couples to inhibition of adenylyl cyclase.

Keywords: $P2Y_1$ receptor; purine nucleotide; signalling; inositol lipid signalling; cyclic AMP

Introduction

The family of G protein-coupled P2Y receptors mediates a broad array of physiological responses to extracellular nucleotides (Dubyak & El-Moatassim, 1993). Mammalian receptors have been identified that respond to either adenine ($P2Y_1$) or uridine nucleotides ($P2Y_4$, $P2Y_6$), or to both ($P2Y_2$) (Fredholm *et al*., 1997). All of the cloned P2Y receptors have been shown to activate phospholipase C and mobilize calcium when expressed in 1321N1 human astrocytoma cells (Filtz *et al*., 1994; Parr *et al*., 1994; Communi *et al*., 1995; 1996; Chang *et al*., 1995; Nguyen *et al*., 1995; Schachter *et al*., 1996).

At least two additional receptors for adenine nucleotides have been proposed to exist but have not been cloned. A receptor (the P2T receptor) that mediates adenosine 5′-diphosphate (ADP)-stimulated platelet aggregation has been thought (Hourani & Hall, 1994) to differ from the adenine nucleotide-sensitive $P2Y_1$ receptor, since it inhibits adenylyl cyclase and ATP and ATP analogues act as antagonists of the P2T receptor, while functioning as agonists for mammalian $P2Y_1$ receptors (Filtz *et al*., 1994; Schachter *et al*., 1996). However, Leon and coworkers have recently proposed that the P2T and $P2Y_1$ receptors are in fact the same signalling protein (Leon *et al*., 1997). Another adenine nucleotide-sensitive receptor has been proposed to be endogenously expressed by C6 rat glioma cells (Pianet *et al*., 1989; Boyer *et al*., 1993), and a receptor with similar properties has been shown to exist on rat brain microvascular endothelial cells (Vigne *et al*., 1994; Feolde *et al*., 1995). This receptor (which will be tentatively referred to as the P2Y-C6 receptor) exhibits an agonist selectivity that is similar, but not identical, to the $P2Y_1$ receptor (Boyer *et al*., 1993). However, these two receptors are more clearly distinguished on the basis of their differential functional coupling and antagonist selectivities. The $P2Y_1$ receptor selectively stimulates inositol phosphate accumulation and agonist activation of this response is competitively blocked by pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (Boyer *et al*., 1994) and adenosine-3′,5′-biphosphate (Boyer *et al*., 1996). In contrast, the P2Y-C6 receptor couples to the inhibition of adenylyl cyclase (Boyer *et al*., 1993; 1994; 1995) and is insensitive to these antagonists (Boyer *et al*., 1994; 1996).

Since functional assessments of the $P2Y_1$ receptor have been carried out in turkey erythrocytes and in 1321N1 human astrocytoma cells, while the P2Y-C6 receptor has been studied in C6 glioma cells and possibly in rat microvascular endothelial cells, we have previously considered the possibility that these two receptors might be identical and that the apparent selectivity in functional coupling of the $P2Y_1$ receptor is influenced by the cell line in which it is expressed. Thus, the human $P2Y_1$ receptor was expressed in rat C6 glioma cells where it conferred a PPADS-sensitive, adenine nucleotide-stimulated inositol phosphate response without alteration of the endogenous P2Y-receptor-mediated adenylyl cyclase response (Schachter *et al*., 1996). We concluded from this study that the $P2Y_1$ receptor couples selectively to phospholipase C and is distinct from the endogenous P2Y receptor of C6 cells that couples to inhibition of adenylyl cyclase. This conclusion recently was questioned due to the fact that the heterologous $P2Y_1$ receptor expressed in rat cells was of human origin rather than rat (Webb *et al*., 1996). Moreover, Webb *et al*. proposed that the P2Y receptor coupling to the inhibition of adenylyl cyclase in C6 rat glioma and in B10 rat microvascular endothelial cells is, in fact, the rat homologue of the $P2Y_1$ receptor and that the selectivity of functional coupling of the rat $P2Y_1$ receptor is determined by the cell line in which the receptor is expressed. They also suggested that sequence differences between the rat receptor and the human or turkey $P2Y_1$ receptors may explain the lack of sensitivity of the rat receptor to PPADS.

To examine more directly the pharmacological and functional selectivity of the rat $P2Y_1$ receptor we have expressed this receptor in both 1321N1 human astrocytoma cells, which do not express endogenous P2Y receptors, and in C6 rat glioma cells, which express an endogenous P2Y receptor that couples to the inhibition of adenylyl cyclase but does not

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couple to the activation of inositol phosphate accumulation. The results indicate that the rat $P2Y_1$ receptor is pharmacologically and functionally identical to previously studied human and avian $P2Y_1$ receptor orthologues, and that $P2Y_1$ receptors, irrespective of species of origin, selectively couple to activation of phospholipase C rather than interacting with Gi to attenuate adenylyl cyclase activity.

Methods

Amplification and expression of the rat $P2Y_1$ receptor

The rat $P2Y_1$ receptor was amplified with Pfu polymerase from 1 μg of rat genomic DNA by use of primers (5′GAG AGA ATT CCT GAG TTG GAA AGA AGA GGA-3′ for the sense strand and 5′-GAG ACT CGA GTG GAT CTC CTG CCT TCA CAA-3′ for the antisense strand) designed to introduce 5′-EcoR1 and 3′-Xho1 restriction sites. The amplified product was ligated into the retroviral expression vector pLXSN and sequenced by dideoxynucleotide chain termination. Retrovirus encoding the rat $P2Y_1$ receptor was produced in PA317 cells as described by Comstock *et al* (1997) and was used to infect 1321N1 and C6 cells. Infected cells were selected with G418 for two weeks before the functional assays.

Inositol phosphate accumulation

Cells were plated in 24 well plates at 1×10^5 cells per well and cultured for three days in DMEM containing 5% foetal bovine serum. Cells were labelled for 24 h with 0.4 μCi of [^{3}H]-*myo*-inositol (20 Ci mmol^{-1}, American Radiolabeled Chemicals) in 200 μl of inositol-free, serum free DMEM. Agonists were dissolved in 250 mM HEPES, pH 7.3, and 50 μl of a five fold concentrated stock solution containing 50 mM LiCl was added to each well. Incubations proceeded for 15 min at 37°C and were terminated by rapidly aspirating the medium and adding 750 μl of boiling water containing 10 mM EDTA, pH 8.0. After 20 min, the cell lysate was removed from the culture plates and applied to Dowex AG1-X8 columns for isolation of inositol phosphates (Berridge *et al*., 1983).

Cyclic AMP accumulation

Accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) was carried out under similar conditions to the inositol phosphate assay with the following modifications. Before the assay, cells were labelled for 2 to 3 h with 1 μCi [^{3}H]-adenine (20 Ci mmol^{-1}, American Radiolabeled Chemicals) in 200 μl of DMEM. Assays for stimulation of cyclic AMP accumulation were initiated by addition of 50 μl of 250 mM HEPES, pH 7.5, containing 1 mM isobutyl methyl xanthine (IBMX) and five fold concentrated nucleotide agonist. Assays for inhibition of cyclic AMP accumulation also included forskolin at 30 μM final concentration. Incubations proceeded for 15 min at 37°C and were terminated by rapidly aspirating the medium and adding 750 μl of 5% trichloroacetic acid. [^{3}H]-cyclic AMP was isolated by Dowex and alumina chromatography (Salomon *et al*., 1974).

Materials

Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Reagents for cycle sequencing were purchased as a kit from Perkin Elmer (Foster City, CA). Cell culture reagents were from Gibco BRL (Grand Island, NY) and chromatography resins were from Biorad (Hercules, CA). 2-Methylthioadenosine triphosphate (2MeSATP) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) were obtained from Research Biochemicals Inc. (Natick, MA). We are grateful to Dr John Olsen (Department of Medicine, University of North Carolina) for his kind gifts of PA317 cells and the pLXSN vector.

Results

Wild type or vector-transfected 1321N1 or C6 cells did not exhibit an inositol phosphate response to 2MeSATP or other adenine nucleotide analogues (data not shown). In contrast, 2MeSATP markedly elevated inositol phosphate levels in both 1321N1 and C6 cells expressing the rat $P2Y_1$ receptor (Figures 1a and b). The potency of 2MeSATP at the rat $P2Y_1$ receptor (EC_{50} approximately 30 nM in either cell population) was similar to the potency observed previously at the expressed human and turkey $P2Y_1$ receptors. Inclusion of the antagonist PPADS (30 μM) resulted in an approximately ten fold rightward shift of the concentration-effect curve for 2MeSATP for stimulation of inositol phosphate accumulation in both cell types. This concentration of PPADS did not alter the inositol phosphate response to the muscarinic agonist carbachol in 1321N1 cells (data not shown), demonstrating that PPADS does not act nonspecifically on the phospholipase C signalling pathway.

Examination of adenylyl cyclase responses in wild type and in rat $P2Y_1$ receptor-expressing C6 cells did not reveal differences in the capacity of 2MeSATP to decrease forskolin-stimulated cyclic AMP accumulation (Figure 2). In the presence of maximally effective concentrations of 2MeSATP cyclic AMP accumulation was nearly identical for both cell types,

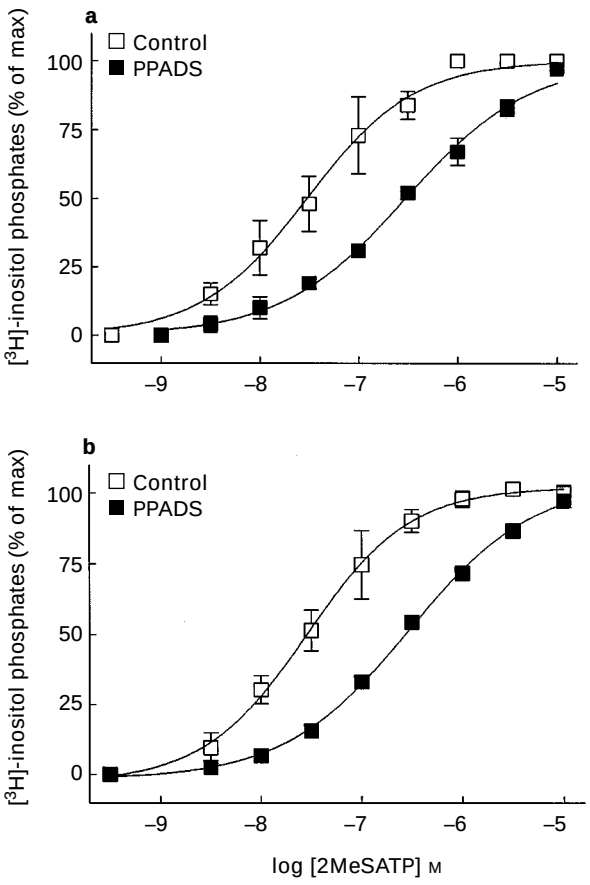

Figure 1 Effects of PPADS on 2MeSATP-stimulated inositol phosphate accumulation in cells expressing the rat $P2Y_1$ receptor. (a) Inositol phosphate responses to 2MeSATP in 1321N1-r$P2Y_1$ cells were assessed in the absence (control) and presence of PPADS as described in Methods. Assays were initiated by simultaneous addition of 2MeSATP, without or with 30 μM PPADS (final concentration), and 10 mM LiCl. Data shown are the average (vertical lines show s.e.mean) of three experiments performed in triplicate and normalized to the maximal response from each experiment (7,000 – 12,000 c.p.m. above a basal value of 2,300 c.p.m.) (b) Same as in (a) but performed with C6-r$P2Y_1$ cells stimulated for 10 min (maximal responses were 30,000 – 50,000 c.p.m. above a basal activity of 6,400 c.p.m.).

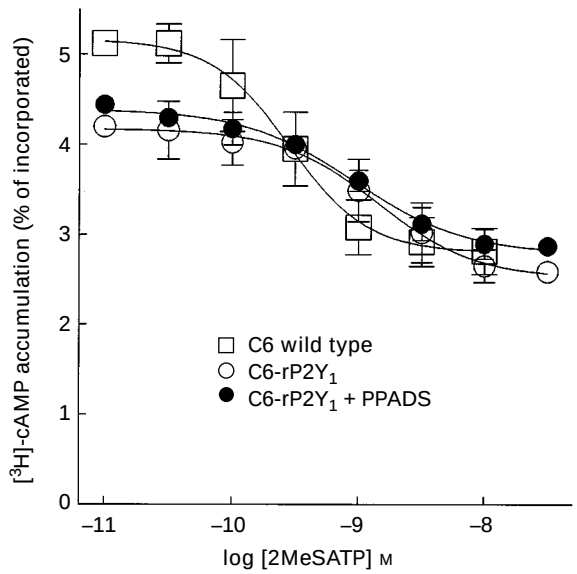

Figure 2 Lack of effect of rP2Y$_1$ receptor expression or PPADS on 2MeSATP-stimulated inhibition of cyclic AMP accumulation in rat C6 glioma cells. Cyclic AMP accumulation was assessed in wild type and in rP2Y$_1$ receptor expressing C6 cells with 30 μM forskolin and 200 μM IBMX. Simultaneous addition of 2MeSATP reduced the stimulation of cyclic AMP accumulation in the absence (open symbols) or presence (solid symbols) of 30 μM PPADS. Data are expressed as % conversion of incorporated label (360,000$\pm$ 12,000 c.p.m. for wild type 180,000$\pm$20,000 for rP2Y$_1$ expressing cells) to cyclic AMP.

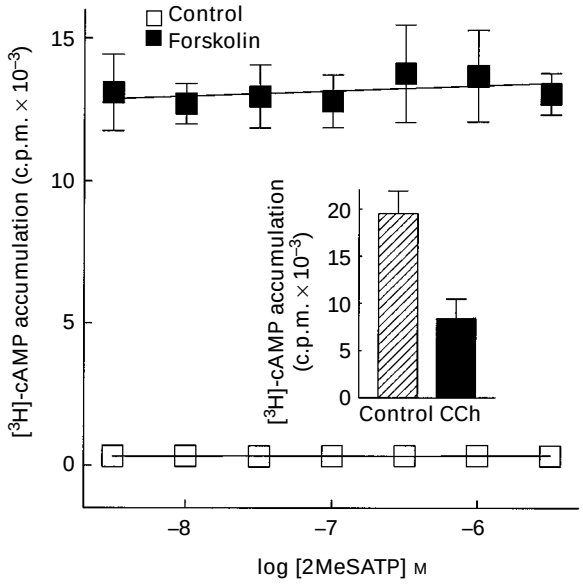

Figure 3 Lack of effect of 2MeSATP on cyclic AMP accumulation in 1321N1-rP2Y$_1$ cells. The same cell line used for the data shown in Figure 1a was examined for cyclic AMP accumulation in the absence (control) or presence of 30 μM forskolin. Various concentrations of 2MeSATP were added simultaneously with forskolin and IBMX. Data in the inset are from 1321N1 cells in which the M_2-muscarinic receptor has been expressed. Cyclic AMP accumulation was stimulated by 30 μM forskolin in the absence (control) or presence of 1 μM carbachol (CCh).

although the magnitude of cyclic AMP accumulation stimulated by forskolin alone was somewhat less in C6-rP2Y$_1$ cells than in wild type C6 cells. The reason for this apparent reduction in forskolin-stimulated cyclic AMP accumulation in C6-rP2Y$_1$ cells was not investigated. The addition of PPADS at 30 μM did not affect either the stimulation of cyclic AMP accumulation by 30 mM forskolin, or the inhibition of cyclic AMP accumulation by 2MeSATP. These data are consistent with our previous observations that PPADS is a competitive antagonist of the phospholipase C-activating P2Y$_1$ receptor, but is not an antagonist at the endogenous P2Y receptor of C6 glioma cells that inhibits adenylyl cyclase. Furthermore, the data indicate that expression of the rat P2Y$_1$ receptor in the rat C6 cell line does not alter the cyclic AMP response to 2MeSATP.

Cyclic AMP accumulation was also examined in human 1321N1 cells expressing the rat P2Y$_1$ receptor. 2MeSATP neither stimulated nor inhibited cyclic AMP accumulation in these cells (Figure 3). However, when the Gi-coupled M_2 muscarinic receptor was expressed in 1321N1 cells a robust inhibition of adenylyl cyclase was observed in response to carbachol (Figure 3). Thus, the rat P2Y$_1$ receptor markedly activates phospholipase C (Figure 1a) under conditions in which no functional coupling of this receptor to adenylyl cyclase can be observed.

Discussion

Examination of second messenger coupling properties of the cloned turkey and human P2Y$_1$ receptors demonstrated that both of these species orthologues activate phospholipase C (PLC), but do not inhibit adenylyl cyclase activity when heterologously expressed in 1321N1 human astrocytoma cells (Filtz *et al*., 1994; Schachter *et al*., 1996). In contrast, a P2Y receptor that is endogenously expressed on rat C6 glioma cells and apparently on rat B10 microvascular endothelial cells inhibits adenylyl cyclase but does not activate phospholipase C (Pianet *et al*., 1989; Boyer *et al*., 1993; Feolde *et al*., 1995; Webb *et al*., 1996). Based on data obtained from radioligand binding studies with [^{35}S]-dATPαS and from RT-PCR of rat cell extracts, Webb and coworkers (1996) concluded that the P2Y receptor promoting inhibition of adenylyl cyclase in the rat microvascular endothelial and glioma cell lines is the rat homologue of the P2Y$_1$ receptor, and that the second messenger coupling specificity and antagonist sensitivity of the P2Y$_1$ receptor depends on either the species of origin of the receptor, or the cell type in which it is expressed.

The conclusions previously made by Webb *et al*. regarding the P2Y$_1$ receptor are open to interpretation. First, the use of [^{35}S]-dATPαS as a ligand to label specifically the P2Y$_1$ receptor has been questioned (Schachter & Harden, 1997). Second, the use of RT-PCR to identify species of expressed receptors is problematic. For example, Webb *et al*. (1996) did not rule out that small amounts of residual genomic DNA were amplified from B10 cells by illustrating that amplification of the rat P2Y$_1$ receptor sequence was reverse-transcriptase dependent. Moreover, even under conditions where receptor sequence is unambiguously demonstrated to arise from reverse-transcribed mRNA, amplification of a sequence from RT-PCR does not prove that this sequence is responsible for the functional responses to nucleotides observed in these cells. The significance of the finding that degenerate oligonucleotide sequences amplified only the rat P2Y$_1$ sequence in extracts from B10 endothelial cells or C6 glioma cells is unclear, since a Gi-linked P2Y receptor would be expected to have low sequence homology with the PLC-coupled P2Y$_1$ receptor. Direct evidence to support the contention that the rat P2Y$_1$ receptor can couple to the inhibition of adenylyl cyclase would require that the amplified sequence be expressed in a cell line where this functional response to adenine nucleotides was then observed as a consequence of receptor expression. Our results suggest that this does not occur. That is, expression of the rat P2Y$_1$ receptor in either 1321N1 or C6 glioma cells resulted in conference of 2MeSATP-promoted inositol phosphate accumulation that was antagonized by PPADS. However, expression of the rat P2Y$_1$ receptor neither conferred a capacity of 2MeSATP to modulate adenylyl cyclase activity in 1321N1 cells, nor did it alter the capacity of 2MeSATP to inhibit adenylyl

cyclase activity in C6 cells. The inhibition of adenylyl cyclase in C6 cells expressing the rat $P2Y_1$ receptor was unaffected by PPADS. Thus, these data demonstrate that the rat $P2Y_1$ receptor, like its human and avian homologues, couples selectively to phospholipase C regardless of the cell line in which it is expressed.

The conclusions by Webb *et al.* (1996), that the receptor coupled to the inhibition of adenylyl cyclase was identical to the rat $P2Y_1$ receptor and that the functional coupling of this receptor is determined by the cell type in which it is expressed, have prompted Leon *et al.* (1997) to speculate that the platelet P2T receptor is also identical to the $P2Y_1$ receptor. While it is quite possible that platelets express the $P2Y_1$ receptor and that this receptor may contribute to the physiological responses of platelets to ADP, it is clear that the functional coupling response in platelets ascribed to the P2T receptor (inhibition of adenylyl cyclase) is distinct from that of the $P2Y_1$ receptor. Whether ATP, an antagonist at the P2T receptor, is a true agonist at the $P2Y_1$ receptor is a point of debate (see Leon *et al.*, 1997), but clearly establishing this difference would further distinguish these two receptors.

With the objective of developing compounds that selectively target individual P2Y subtypes, it is important to establish not only whether a given sequence is a functional P2Y receptor but also whether a receptor in one tissue, cell line or species is the same or different from the receptor from another source. The cloned $P2Y_1$ receptor and the yet to be cloned endogenous P2Y receptor of C6 glioma cells exhibit similar but not identical agonist selectivities, and have been clearly distinguished through the use of the antagonists PPADS and adenosine 3′,5′ bisphosphate (Boyer *et al.*, 1994; 1996; Schachter *et al.*, 1996). Since the P2Y receptor of C6 cells is both pharmacologically and functionally distinguished from the rat $P2Y_1$ receptor when expressed in the same cell line, it is concluded that these two entities are distinct receptor subtypes. However, this conclusion does not rule out the possibility that when overexpressed at very high levels the $P2Y_1$ receptor might also couple to Gi and adenylyl cyclase, as has been shown to be the case for many Gq/PLC- and Gi/adenylyl cyclase-linked receptors.

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