British Journal of Pharmacology (2001) 132, 318–326

www.nature.com/bjp

Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase

[1]Ai-Dong Qi, [2]Charles Kennedy, [1]T. Kendall Harden & *,[1]Robert A. Nicholas

[1]Department of Pharmacology, Mary Ellen Jones Building, CB#7365, University of North Carolina School of Medicine at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7365, U.S.A. and [2]Department of Physiology and Pharmacology, Strathclyde Institute of Biomedical Research, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR

1 The human $P2Y_{11}$ ($hP2Y_{11}$) receptor was stably expressed in two cell lines, 1321N1 human astrocytoma cells (1321N1-$hP2Y_{11}$) and Chinese hamster ovary cells (CHO-$hP2Y_{11}$), and its coupling to phospholipase C and adenylyl cyclase was assessed.

2 In 1321N1-$hP2Y_{11}$ cells, ATP promoted inositol phosphate (IP) accumulation with low μM potency ($EC_{50} = 8.5 \pm 0.1$ μM), whereas it was 15 fold less potent (130 ± 10 μM) in evoking cyclic AMP production.

3 In CHO-$hP2Y_{11}$ cells, ATP promoted IP accumulation with slightly higher potency ($EC_{50} = 3.6 \pm 1.3$ μM) than in 1321N1-$hP2Y_{11}$ cells, but it was still 15 fold less potent in promoting cyclic AMP accumulation ($EC_{50} = 62.4 \pm 15.6$ μM) than for IP accumulation. Comparable differences in potencies for promoting the two second messenger responses were observed with other adenosine nucleotide analogues.

4 In 1321N1-$hP2Y_{11}$ and CHO-$hP2Y_{11}$ cells, down regulation of PKC by chronic treatment with phorbol ester decreased ATP-promoted cyclic AMP accumulation by 60–80% ($P < 0.001$) with no change in its potency. Likewise, chelation of intracellular Ca^{2+} decreased ATP-promoted cyclic AMP accumulation by ~45% in 1321N1-$hP2Y_{11}$ cells, whereas chelation had no effect on either the efficacy or potency of ATP in CHO-$hP2Y_{11}$ cells.

5 We conclude that coupling of $hP2Y_{11}$ receptors to adenylyl cyclase in these cell lines is much weaker than coupling to phospholipase C, and that activation of PKC and intracellular Ca^{2+} mobilization as consequences of inositol lipid hydrolysis potentiates the capacity of ATP to increase cyclic AMP accumulation in both 1321N1-$hP2Y_{11}$ and CHO-$hP2Y_{11}$ cells.

British Journal of Pharmacology (2001) **132,** 318–326

Keywords: ATP; cyclic AMP; inositol phosphates; P2Y receptors; $P2Y_{11}$ receptor; protein kinase C; Ca^{2+} mobilization

Abbreviations: 1321N1-hD1, 1321N1 cells expressing the human D1 dopamine receptor; 1321N1-$hP2Y_{11}$, 1321N1 cells expressing the human $P2Y_{11}$ receptor; ATPγS, adenosine 5′-triphosphate-γ-thiophosphate; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester; CHO-$hP2Y_{11}$, CHO-K1 cells expressing the human $P2Y_{11}$ receptor; CHO-K1 cells, Chinese hamster ovary cells; DMEM, Dulbecco's Modified Eagle's Medium; hD1 receptor, human D1 dopamine receptor; $hP2Y_{11}$ receptor, human $P2Y_{11}$ receptor; IBMX, 3-isobutyl-1-methylxanthine; IPs, inositol phosphates; PGE_2, prostaglandin E_2; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate

Introduction

P2Y receptors are heptahelical, G protein-coupled receptors activated by extracellular nucleotides. These receptors are expressed in almost all cells and tissues, where they regulate a wide range of physiological processes. To date, five subtypes of P2Y receptors have been cloned in humans ($hP2Y_{1,2,4,6,11}$) (North & Barnard, 1997; Harden, 1998; King *et al*., 1998). The unambiguous association of a P2Y receptor subtype with a specific physiological effect has proven difficult to establish, in part due to the lack of subtype-selective agonists and antagonists. Thus, the pharmacological selectivities of these receptors have been defined by expressing individual subtypes of cloned P2Y receptors in null cell lines and determining the rank order of potencies of the natural agonists ATP, ADP, UTP and UDP, and with nucleotide analogues.

*Author for correspondence; E-mail: nicholas@med.unc.edu

The $hP2Y_1$ receptor is highly selective for ADP and ATP and their 2-methylthio derivatives (Schachter *et al*., 1996; Leon *et al*., 1997; Palmer *et al*., 1998). The triphosphate-preferring $hP2Y_2$ receptor is activated by both UTP and ATP with essentially equal potency, whilst ADP and UDP have little or no effect (Lustig *et al*., 1993; Nicholas *et al*., 1996). UTP is a potent full agonist at the $hP2Y_4$ receptor, whereas ADP and UDP have little or no effect (Nguyen *et al*., 1995; Communi *et al*., 1996a; Nicholas *et al*., 1996). Recently, we demonstrated that ATP is a competitive antagonist at the $hP2Y_4$ receptor, but is a potent full agonist at the rat $P2Y_4$ receptor (Kennedy *et al*., 2000). The $hP2Y_6$ receptor is a pyrimidine-preferring receptor, with UDP more potent than UTP. ADP is a low potency agonist whereas ATP has no effect (Nicholas *et al*., 1996; Communi *et al*., 1996b; Li *et al*., 1998). Finally, the $hP2Y_{11}$ receptor is highly selective for ATP (Communi *et al*., 1997; 1999).

All hP2Y receptor subtypes cloned to date are linked to activation of phospholipase C, generation of inositol phosphates (IPs), activation of protein kinase C (PKC) and release of intracellular Ca^{2+} stores. In addition to coupling to phospholipase C, the $hP2Y_{11}$ receptor also couples to adenylyl cyclase, resulting in increased cyclic AMP synthesis (Communi *et al*., 1997; 1999). ATP was reported to activate adenylyl cyclase with similar (Communi *et al*., 1997) or greater potency (Communi *et al*., 1999) compared to activation of phospholipase C. However, these second messenger assays were carried out in different cell lines expressing the $hP2Y_{11}$ receptor, i.e., 1321N1 human astrocytoma cells for IP accumulation and CHO-K1 cells for cyclic AMP accumulation. Given that agonist potencies are greatly influenced by the level of receptor reserve (Kenakin, 1997; Palmer *et al*., 1998), the efficiency at which the $hP2Y_{11}$ receptor activates phospholipase C compared to adenylyl cyclase remains unestablished. Thus, the aim of this study was to determine the efficiency with which the $hP2Y_{11}$ receptor couples to these two second messenger pathways in the same cell line and to determine the effects of intracellular Ca^{2+} mobilization and phospholipase C activation on activation of adenylyl cyclase. A preliminary account of these results has been published (Kennedy *et al*., 1999).

Methods

PCR amplification of the coding sequence of the $hP2Y_{11}$ receptor

PCR primers complementary to the published sequence of the $hP2Y_{11}$ receptor (Communi *et al*., 1997) were used to amplify the coding sequence from 0.24 μg of human genomic DNA with *Amplitaq* DNA polymerase. The primers contained at their 5′ ends either an *Eco*RI restriction site (5′-AGAGAATTCCACCATGG ATCGAGGTGCCAAGTCCTGCCCT-3′; upstream primer) or a *Xho*I restriction site (5′-GAGCTCGAGTCATTGGCTCAGCTCACGG-3′; downstream primer). In addition, the upstream primer also contained a consensus Kozak translation initiation consensus sequence (CACCATGG; Kozak, 1986) preceding the start ATG codon, all nine coding bases of exon 1 and the first 18 bases of exon 2, whilst the downstream primer contained the final 16 coding bases of exon 2 and the stop codon. The amplification conditions were 94°C for 3 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 70 s; and 7 min at 72°C. The amplified product was purified, digested with *Eco*RI and *Xho*I, and ligated into the similarly digested retroviral expression vector, pLXSN. Individual clones encoding the receptor were sequenced using the Amplicycle Sequencing Kit, and the sequence obtained was identical to that reported by Communi *et al*. (1997), except for the presence of a C in place of a T at position 240. This difference was found in clones originating from separate amplification reactions; however, it did not alter the protein sequence.

Expression of $hP2Y_{11}$ and hD1 receptors in 1321N1 and CHO-K1 cells

Recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 packaging cells with the pLXSN vector (Miller & Rosman, 1989; GenBank accession no. M28248) containing $hP2Y_{11}$ cDNA (Comstock *et al*., 1997). 1321N1 human astrocytoma cells and CHO-K1 cells were grown in monolayer culture at 37°C in 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (10% for CHO-K1 cells), 100 units ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B. Cells were infected with retrovirus harbouring the $hP2Y_{11}$ coding sequence or with control retrovirus. CHO-K1 cells were pretreated with the glycosylation inhibitor tunicamycin (0.3 μg ml^{-1}) for 19 h prior to infection to inhibit the production of endogenous factors that suppress infection of hamster cells (Miller & Miller, 1992; 1993). Geneticin-resistant cells were selected for 2 (1321N1 cells) to 4 (CHO-K1 cells) weeks with medium containing 1 mg ml^{-1} G-418 and were then maintained in medium containing 0.4 mg ml^{-1} G-418.

The human D1-dopamine receptor (GenBank accession no. X55760) was amplified by PCR from human genomic DNA under similar conditions as above with the following primers: up primer, 5′-AGAGAATTCACCATGCACCATG-3′ and down primer, 5′-GAGCTCGAGTCAGGTTGGGTGCTGACCGT-3′. Primers incorporated either an *Eco*RI (upstream primer) or *Xho*I (downstream primer) restriction site to facilitate cloning. The amplified receptor sequence was cloned into the *Eco*RI and *Xho*I sites of pLXSN and sequenced. Retroviral particles were prepared as described above and used to generate a stable population of expressing 1321N1 cells. Radioligand binding analysis with [^{3}H]-SCH22799 indicated that the hD1 receptor was expressed at $\sim$200 fmol mg^{-1} (data not shown).

IP and cyclic AMP formation

1321N1 and CHO-K1 cells stably expressing the $hP2Y_{11}$ receptor (1321N1-$hP2Y_{11}$ and CHO-$hP2Y_{11}$ cells, respectively) were seeded in 24-well plates at either 1×10^5 or 5×10^4 cells per well, respectively, and assayed 3 days later when confluent. Inositol lipids were radiolabelled by incubation of the cells for 22 h with 200 μl inositol-free, serum-free DMEM high glucose and 0.4 μCi *myo*-[^{3}H]-inositol in a humidified CO_2 incubator. No changes of medium were made subsequent to the addition of [^{3}H]-inositol. Agonists were added in 50 μl of a 5 fold concentrated solution in 50 mM LiCl, 250 mM HEPES, pH 7.4. Following a 5 min incubation at 37°C, the medium was aspirated and the assay terminated by adding 0.75 ml of boiling 10 mM EDTA, pH 8.0. [^{3}H]-IPs were resolved by Dowex AG1-X8 columns as described previously (Lazarowski *et al*., 1995).

To monitor cyclic AMP accumulation, the medium was replaced 2 h before the assay with 200 μl serum-free DMEM containing 0.8 μCi of [^{3}H]-adenine. Control experiments, in which cells in 200 μl serum-free DMEM high glucose for 22 h were labelled by adding 0.8 μCi [^{3}H]-adenine in 10 μl directly to the medium 2 h before the assay, indicated that changing the medium 2 h before the assay had no effect on nucleotide-promoted cyclic AMP accumulation. No changes in medium were made subsequent to the addition of [^{3}H]-adenine. Twenty minutes before the assay, HEPES buffer, pH 7.4, was added to 50 mM, followed 10 min later by addition of 200 μM IBMX (final concentration) in 50 mM HEPES buffer,

pH 7.4, to inhibit the hydrolysis of cyclic AMP by phosphodiesterases. Agonists were added in 50 μl of a 6 fold concentrated solution in Hank's balanced salt solution (without Ca^{2+}, Mg^{2+}). Where indicated, cells were treated with the phorbol ester PMA (1 μM) for either 20 h to down-regulate PKC or for 10 min to activate PKC prior to challenge with agonists. For chelation of intracellular [Ca^{2+}], cells were treated for 10 min with 50 μM BAPTA-AM prior to challenge with agonists. Control experiments confirmed that this concentration of BAPTA-AM was sufficient to prevent agonist-promoted increases in intracellular [Ca^{2+}]. Following a 10 min incubation at 37°C, the assay was terminated by aspirating the medium and adding 1 ml ice-cold trichloroactetic acid. [^{3}H]-cyclic AMP was isolated on Dowex and alumina columns as described previously (Harden *et al*., 1982).

Statistics

Data in the text are expressed as the mean ± s.d. for EC_{50} values. Concentration-response curves were fitted to the data by logistic (Hill equation), non-linear regression analysis (GraphPad Prism, San Diego, U.S.A.). Data were compared as appropriate by Student's paired *t*-test or by one-way analysis of variance and Tukey's comparison, with $P<0.05$ considered to be statistically significant.

Materials

AmpliTaq DNA polymerase and the Amplicycle Sequencing Kit were obtained from Perkin-Elmer (Norfolk, CT, U.S.A.). All tissue culture reagents and Hank's balanced salt solution were supplied by the Lineberger Comprehensive Cancer Center tissue culture facilities (University of North Carolina at Chapel Hill, NC, U.S.A.). ATP was purchased from Pharmacia (Piscataway, NJ, U.S.A.), ADP was from Roche Biochemicals (Indianapolis, IN, U.S.A.), 2MeSATP, 2MeSADP, ATPγS, ADPβS and forskolin were from RBI (Natick, MA, U.S.A.), PMA and BAPTA-AM were from Calbiochem (La Jolla, CA, U.S.A.) and PGE_1, IBMX, and dopamine were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of ADP and 2MeSADP (10 mM) were incubated with 50 U ml^{-1} hexokinase (Roche Biochemicals, Indianapolis, IN, U.S.A.) in DMEM high glucose medium for 2 h before use to eliminate triphosphate contamination. In addition, assay solutions contained 5 U hexokinase ml^{-1}. Similarly, stock solutions (10 mM in DMEM high glucose) of the nucleotidase-resistant nucleotide analogues, ATPγS and ADPβS, were treated with 3 U ml^{-1} apyrase (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 30 min before use.

Results

Properties of the hP2Y$_{11}$ receptor expressed in 1321N1 cells

1321N1 cells infected with viruses produced from pLXSN vector alone showed no responses to ATP (data not shown). ATP increased IP accumulation in a concentration-dependent manner ($EC_{50}=8.5\pm0.1$ μM) in 1321N1 cells expressing the hP2Y$_{11}$ receptor (Figure 1). At higher concentrations, ADP also promoted IP accumulation, but only to levels ~35% of those achieved with maximal concentrations of ATP (basal subtracted). ATP also promoted cyclic AMP accumulation, with an EC_{50} of 130 ± 10 μM (Figure 1). Thus, in 1321N1-hP2Y$_{11}$ cells, ATP promotes IP accumulation with 15 fold greater potency than it promotes cyclic AMP accumulation. In contrast to its ability to promote IP accumulation, ADP did not increase cyclic AMP levels in these cells. Concentration-response curves for ATP-promoted cyclic AMP accumulation (Figures 1–4) were steep. The reason(s) for this deviation from law of mass interaction at a single site has not been pursued.

Effects of ATP in CHO-K1 cells expressing the hP2Y$_{11}$ receptor

To ensure that cell-specific differences do not account for the differential sensitivity of ATP in promoting the two second messenger responses, we also expressed the hP2Y$_{11}$ receptor in CHO-K1 cells. CHO-K1 cells express a P2Y$_2$ receptor that responds to ATP (Iredale & Hill, 1993), but expression of the hP2Y$_{11}$ receptor in these cells gave rise to ATP-promoted increases in IP accumulation 20 fold higher than those in vector-infected cells (Figure 2A). This allowed us to address the issue of differential coupling of the hP2Y$_{11}$ receptor in an additional cell line.

In CHO-hP2Y$_{11}$ cells, ATP increased IP accumulation in a concentration-dependent manner and with a potency slightly greater than in 1321N1-hP2Y$_{11}$ cells ($EC_{50}=3.6\pm1.3$ μM) (Figure 2B; Table 1). ATP also evoked cyclic AMP synthesis with a potency greater than in 1321N1 cells ($EC_{50}=62\pm16$ μM), but still 15 fold less than the EC_{50} for inositol lipid hydrolysis. Thus, similar differences in coupling of the hP2Y$_{11}$ receptor to adenylyl cyclase and phospholipase C were observed in two cell lines, strongly suggesting that these differences are an inherent property of the receptor. ADP promoted inositol lipid hydrolysis ($EC_{50}=50.1\pm20.6$ μM) to

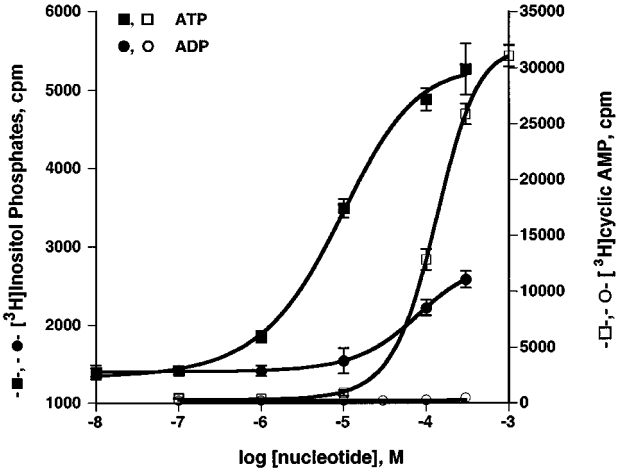

Figure 1 Concentration-response curves of ATP and ADP for increasing IP and cyclic AMP levels in 1321N1-hP2Y$_{11}$ cells. Each point is the mean response from three experiments. Vertical lines indicate s.d. mean.

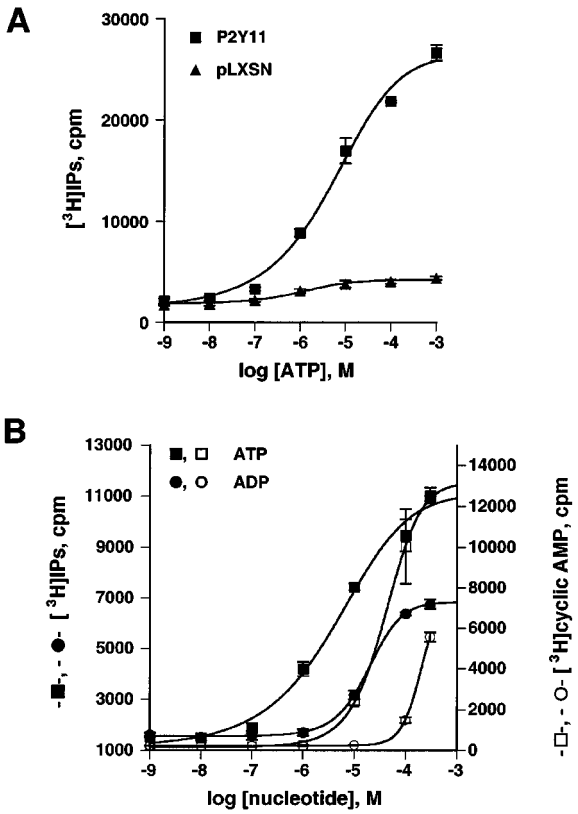

Figure 2 Concentration-response curves of ATP and ADP for increasing IP and cyclic AMP levels in CHO-hP2Y$_{11}$ cells. (A) Concentration-response curves for ATP-promoted IP production in CHO-K1 cells infected with empty retrovirus (pLXSN) or with retrovirus harbouring hP2Y$_{11}$ receptor cDNA (P2Y$_{11}$). (B) Concentration-response curves of ATP and ADP for increasing IP and cyclic AMP levels in CHO-hP2Y$_{11}$ cells. Each point is the mean response from five experiments. Vertical lines indicate s.d. mean.

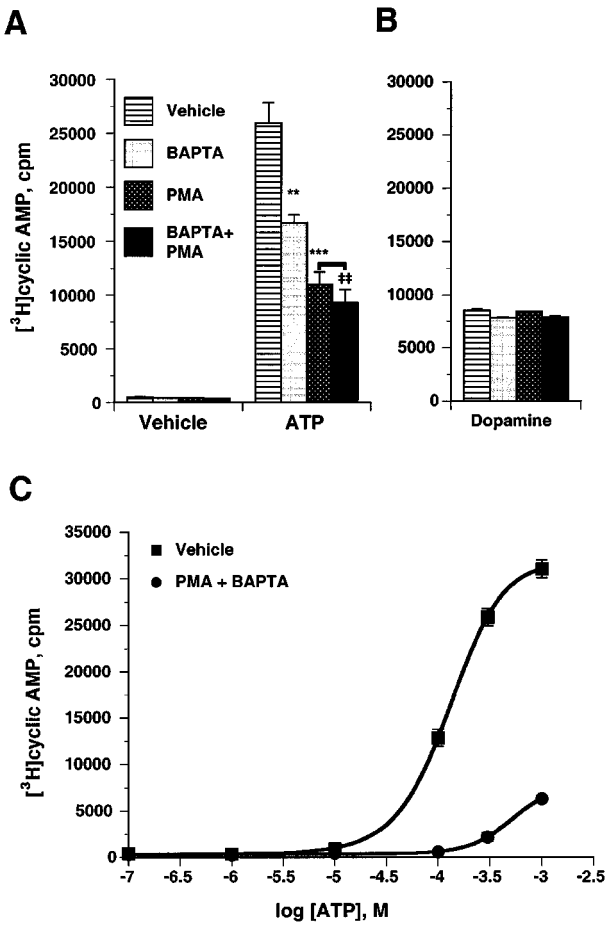

Figure 3 Effects of Ca^{2+} chelation and PKC down-regulation on ATP-promoted cyclic AMP accumulation in 1321N1-hP2Y$_{11}$ cells. (A) Cells treated with BAPTA-AM (50 μM) for 10 min, PMA (1 μM) for 20 h, or a combination of both were challenged with buffer or 300 μM ATP and cyclic AMP accumulation was measured as described in Methods. (B) 1321N1 cells expressing the hD1 dopamine receptor were treated as described in (A), challenged with 100 μM dopamine for 10 min, and cyclic AMP accumulation was measured. (C) Concentration-response curves of ATP for promotion of cyclic AMP accumulation in vehicle- or PMA- (1 μM for 20 h) plus BAPTA-AM- (50 μM for 10 min) treated 1321N1-hP2Y$_{11}$ cells. Data shown are the mean$\pm$s.d. of triplicate assays from a representative experiment repeated three times. $^{**}P\leqslant0.01$ and $^{***}P\leqslant0.001$ relative to control response. $\ddagger\ddagger P\leqslant0.01$ relative to PMA treatment alone.

levels $\sim$50% of that of the maximal levels promoted by ATP (Figure 2B). In contrast to 1321N1-hP2Y$_{11}$ cells, ADP increased cyclic AMP levels in CHO-hP2Y$_{11}$ cells, although the concentration-response curve did not reach a clear maximum.

Coupling efficiency of the hP2Y$_{11}$ receptor activated with other adenine nucleotides

To determine whether other hP2Y$_{11}$ agonists also exhibited differences in potency for promotion of IP and cyclic AMP accumulation, concentration-response curves of several adenine nucleotide analogues, including ATPγS, ADPβS, 2MeSATP, and 2MeSADP, were generated. All of these nucleotide analogues were considerably more potent (7–20 fold) for promotion of IP accumulation than cyclic AMP accumulation (Table 1). The rank order of potency of these agonists for stimulation of IP accumulation (ATPγS > 2MeSATP > ATP $\sim$ ADPβS > 2MeSADP > ADP) is similar to that previously reported for the hP2Y$_{11}$ receptor (ATPγS > ATP > ADPβS > 2MeSATP; Communi *et al.*, 1999) with the exception of 2MeSATP, which had a higher potency relative to the other agonists in the present experiments.

Effects of phosphodiesterase inhibitors on hP2Y$_{11}$ receptor signalling

These data differ from those reported by Communi *et al.* (1997; 1999), in which ATP was either equipotent or even more potent for stimulating cyclic AMP accumulation compared with IP production. One possible explanation of this discrepancy is that in the present study IBMX was used to inhibit cyclic AMP breakdown by phosphodiesterase, but rolipram was employed by Communi *et al.* (1997). To test the possibility that IBMX, an antagonist at adenosine receptors (Bruns *et al.*, 1986; Ukena *et al.*, 1986; Coffin & Spealman, 1989), also acts as an antagonist at the hP2Y$_{11}$ receptor, we included 200 μM IBMX in the incubation medium during IP assays. However, its inclusion had no effect on the ability of

ATP to evoke IP synthesis (data not shown), indicating that IBMX is not an antagonist at the hP2Y$_{11}$ receptor and its use does not underlie the discrepancy between the present study and those of Communi *et al.* (1997; 1999).

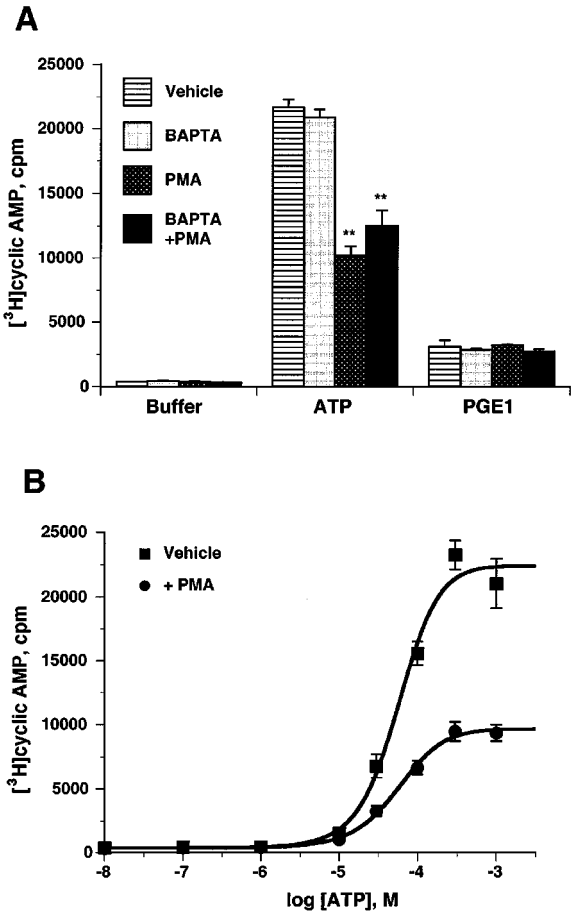

Figure 4 Effects of Ca^{2+} chelation and PKC down-regulation on ATP-promoted cyclic AMP accumulation in CHO-hP2Y$_{11}$ cells. (A) Cells treated with BAPTA-AM (50 μM) for 10 min, PMA (1 μM) for 20 h, or a combination of both were challenged with buffer, 300 μM ATP, or 10 μM PGE$_1$ for 10 min and cyclic AMP accumulation was measured as described in Methods. (B) Concentration-response curves of ATP for promotion of cyclic AMP accumulation in untreated or PMA- (1 μM for 20 h) and BAPTA-AM-treated (50 μM for 10 min) CHO-hP2Y$_{11}$ cells. Data shown are the mean$\pm$s.d. of triplicate assays from a representative experiment repeated three times. $^{**}P \leqslant 0.01$ relative to control responses.

Influence of PKC activation and intracellular Ca^{2+} mobilization on cyclic AMP accumulation in 1321N1-hP2Y$_{11}$ and CHO-hP2Y$_{11}$ cells

Because the hP2Y$_{11}$ receptor couples more efficiently to phospholipase C than to adenylyl cyclase, inositol lipid hydrolysis is near maximal at concentrations of ATP that only minimally increase cyclic AMP accumulation. Thus, the downstream signalling effects of ATP-promoted inositol lipid hydrolysis (i.e., intracellular Ca^{2+} mobilization and PKC activation) potentially influence cyclic AMP accumulation. Therefore, we investigated the effects of both intracellular Ca^{2+} mobilization and PKC activation on the capacity of ATP to promote cyclic AMP accumulation in both 1321N1-hP2Y$_{11}$ and CHO-hP2Y$_{11}$ cells. Cells were treated with BAPTA-AM (50 μM) for 10 min to chelate intracellular Ca^{2+}, with the phorbol ester PMA (1 μM) for 20 h to down regulate PKC, or with both agents, and cyclic AMP accumulation was measured following challenge of the cells with 300 μM ATP. In 1321N1-hP2Y$_{11}$ cells, intracellular Ca^{2+} chelation decreased ATP-promoted cyclic AMP accumulation by $41\pm5\%$ ($P<0.01$), whereas downregulation of PKC resulted in a $57\pm1\%$ decrease ($P<0.001$; Figure 3A). Combination of both treatments caused a slight but significant further decrease in cyclic AMP accumulation ($66\pm2\%$, $P<0.01$) compared to PMA treatment alone. In contrast, intracellular Ca^{2+} chelation had no effect on ATP-promoted cyclic AMP accumulation in CHO-hP2Y$_{11}$ cells, whereas downregulation of PKC resulted in a $58\pm4\%$ decrease ($P<0.01$) in ATP-promoted cyclic AMP accumulation (Figure 4A). The effect of both treatments was not significantly different from PMA treatment alone.

To determine whether coupling of the hP2Y$_{11}$ receptor to inositol lipid hydrolysis is responsible for the potentiation of cyclic AMP accumulation in these cells, we measured the effects of intracellular Ca^{2+} chelation or down regulation of PKC on other Gs-coupled receptors. Thus, we generated 1321N1 cells stably expressing the human D1 dopamine receptor, which couples exclusively to Gs/adenylyl cyclase, and determined the effects of intracellular Ca^{2+} chelation and down regulation of PKC on dopamine-promoted increases in cyclic AMP accumulation. In contrast to ATP-promoted

Table 1 EC_{50} values and relative efficacies of adenine nucleotides and nucleotide analogues for promotion of IP production and cyclic AMP accumulation in CHO-hP2Y$_{11}$ cells

	IP production		*Cyclic AMP accumulation*		
Nucleotide	*EC_{50} (μM)*	*R.E. (%)*[a]	*EC_{50} (μM)*	*R.E. (%)*	*Ratio*[b]
ATP	3.6 ± 1.3	100	62.4 ± 15.6	100	17.3
ADP	50.1 ± 20.6	60 ± 4	NM[c]	NM	
ATPγS	1.2 ± 0.4	95 ± 4	23.2 ± 6.5	84 ± 7	19.3
ADPβS	3.7 ± 0.7	72 ± 1	25.9 ± 0.4	78 ± 6	7
2MeSATP	2.4 ± 0.4	99 ± 8	26.5 ± 3.7	99 ± 6	11
2MeSADP	14.6 ± 3.2	61 ± 6	NM	NM	

Results are presented as mean$\pm$s.d. of 3–5 experiments. [a]R.E., relative efficacy, normalized by dividing the maximal response for each nucleotide by the maximal response to 300 μM ATP and multiplying by 100. [b]Ratio of EC_{50} for cyclic AMP accumulation to EC_{50} for IP accumulation. [c]NM, not maximal, indicates the response did not reach the maximum at the highest concentrations of agonist.

cyclic AMP accumulation in 1321N1-hP2Y$_{11}$ cells, PMA/BAPTA-AM treatment of 1321N1-hD1 cells had no effect on dopamine-promoted cyclic AMP accumulation (Figure 3B). In CHO-K1 cells, an endogenously expressed Gs-coupled prostaglandin receptor was utilized to test the effects of intracellular Ca^{2+} chelation and down regulation of PKC on cyclic AMP accumulation. PGE_2-promoted cyclic AMP accumulation also was not affected in PMA/BAPTA-AM-treated cells (Figure 4A). These data indicate the potentiation of ATP-promoted cyclic AMP accumulation is due to the coupling of the hP2Y$_{11}$ receptor to inositol lipid hydrolysis.

To characterize further the influence of PKC and intracellular [Ca^{2+}] on ATP-promoted cyclic AMP accumulation, we compared the concentration-response curves of ATP in vehicle versus PMA- and BAPTA-AM-treated 1321N1-hP2Y$_{11}$ and CHO-hP2Y$_{11}$ cells. In 1321N1-hP2Y$_{11}$ cells, the response to 1 mM ATP was decreased by nearly 80% in PMA/BAPTA-AM-treated cells, but the concentration-response curve of treated cells did not reach a maximum and an EC_{50} could not be calculated (Figure 3C). In CHO-hP2Y$_{11}$ cells, the maximal response to ATP was decreased by $\sim$60% in PMA-treated cells, whereas the EC_{50} values were not significantly different from one another (Figure 4B).

We also investigated the effect of short term activation of PKC on cyclic AMP accumulation in both cell lines. Consistent with the effect of PKC downregulation, which decreased cyclic AMP accumulation, activation of PKC by short-term addition of PMA (1 μM, 10 min) significantly potentiated ATP-, PGE_1-, and forskolin-promoted increases in cyclic AMP accumulation in CHO-hP2Y$_{11}$ cells (Figure 5A). To verify that receptor-stimulated intracellular Ca^{2+} mobilization and PKC activation could increase adenylyl cyclase activity, cyclic AMP accumulation was measured in 1321N1-hD1 cells treated with dopamine alone or with carbachol, which activates an endogenous M3 muscarinic receptor coupled to Gq/phospholipase C (Figure 5B). M3 receptor activation alone had no affect on cyclic AMP levels, whereas M3 receptor activation increased dopamine-stimulated cyclic AMP accumulation by nearly 2 fold ($P<0.001$), consistent with the potentiating effects of PKC activation and intracellular Ca^{2+} mobilization on adenylyl cyclase activity.

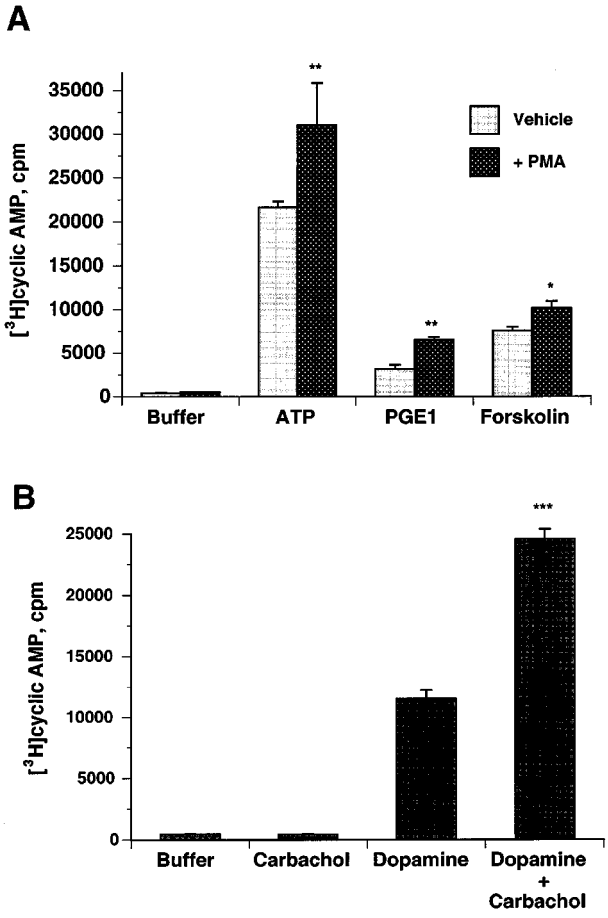

Figure 5 Effects of acute PKC activation and intracellular Ca^{2+} mobilization on cyclic AMP accumulation in CHO-hP2Y$_{11}$ cells and 1321N1-hD1 cells. (A) CHO-hP2Y$_{11}$ cells were treated with either vehicle or PMA (1 μM) for 10 min, and cyclic AMP accumulation was measured following challenge with buffer, 300 μM ATP, 10 μM PGE_1 or 30 μM forskolin for an additional 10 min. $^*P\leqslant0.05$, $^{**}P\leqslant0.01$ relative to untreated responses. (B) 1321N1-hD1 cells were challenged with either vehicle or dopamine (100 μM) for 10 min in the presence or absence of carbachol (300 μM) and cyclic AMP accumulation was measured. Data shown are the mean$\pm$s.d. of triplicate assays from a representative experiment repeated three times. $^{***}P\leqslant0.001$ relative to dopamine alone.

Discussion

We show here that the hP2Y$_{11}$ receptor, when exogenously expressed in either 1321N1 or CHO-K1 cells, couples to both phospholipase C and adenylyl cyclase with marked differences in efficiency. That is, ATP promotes IP accumulation in both cell lines with 15 fold greater potency than it promotes cyclic AMP accumulation. In addition, other adenine nucleotides promoted both second messenger responses in CHO-hP2Y$_{11}$ cells with similar differences (7–20 fold) in potency. The marked differences in EC_{50} values for agonist-promoted IP and cyclic AMP accumulation of the hP2Y$_{11}$ receptor contrasts with another dual-coupled P2Y receptor, an avian p2y receptor that simultaneously activates phospholipase C and inhibits adenylyl cyclase (Boyer *et al.*, 1997; 2000). This avian p2y receptor couples with similar efficiency to both inhibition of adenylyl cyclase and activation of phospholipase C.

The data reported here are different from those of Communi and colleagues, who have reported that ATP has either similar (Communi *et al.*, 1997) or even greater (Communi *et al.*, 1999) potency at the hP2Y$_{11}$ receptor for promotion of cyclic AMP accumulation relative to inositol lipid hydrolysis. However, in those studies IP accumulation was measured in transfected 1321N1 cells, whereas cyclic AMP accumulation was measured in transfected CHO-K1 cells. It was reported in Communi *et al.* (1999) that when both IP and cyclic AMP accumulation were measured in the same transfected 1321N1 cell line, ATP promoted cyclic AMP accumulation with much lower potency than it promoted inositol phosphate accumulation. Thus, the differences in potencies for promoting the two second messenger pathways in the same cell line reported in Communi *et al.* (1999) are similar to the data presented here, although no data were shown and this observation was not investigated

further. Moreover, the authors reported that CHO-K1 cells transfected with the $hP2Y_{11}$ receptor did not show significant enhancement of ATP-promoted IP accumulation over the response observed in untransfected cells, making them unsuitable to characterize the coupling to inositol lipid hydrolysis in this cell line. Thus, no direct comparison of EC_{50} values for agonist-promoted second messenger responses could be made. The lack of a significant response in transfected CHO-K1 cells contrasts with the data reported here, in which ATP application to CHO-K1 cells expressing the $hP2Y_{11}$ receptor raised IP levels to 20 fold higher than those in vector-infected cells. The reasons for these differences are not clear, but may be due to the different expression systems or differences in the parental cell lines used.

The EC_{50} of ATP for promoting IP accumulation in 1321N1-$hP2Y_{11}$ cells in this study was 4–8 fold greater compared to previous studies (Communi *et al*., 1997; 1999). Similarly, ADP was inactive in the earlier studies, but increased IP levels here. This suggests greater receptor reserve of the $hP2Y_{11}$ receptor or an increase in its coupling to $G_{q/11}$ in the population of cells we used. ADP had no effect on cyclic AMP levels in 1321N1-$hP2Y_{11}$ cells, but this was probably due to the much lower coupling efficiency of the $hP2Y_{11}$ receptor to adenylyl cyclase in these cells and the lower potency and efficacy of ADP compared with ATP. Likewise, the ability of ADP to promote cyclic AMP accumulation in CHO-K1 cells infected with $hP2Y_{11}$ receptor, together with the greater potency of ATP for promotion of inositol lipid hydrolysis in CHO-$hP2Y_{11}$ versus 1321N1-$hP2Y_{11}$ cells, is consistent with a higher level of expression of the $hP2Y_{11}$ receptor in CHO-K1 cells than in 1321N1 cells. Higher levels of expression of the $hP2Y_{11}$ receptor in CHO-K1 cells than in 1321N1 cells may explain the greater potency of ATP for promotion of cyclic AMP accumulation than for IP accumulation reported in earlier studies (Communi *et al*., 1999). The influence of receptor density on coupling efficiency of the $hP2Y_{11}$ receptor is unknown, but possibly could be addressed with an inducible expression system.

It is possible that the activity of ADP in our experiments was due to either contamination of ADP stocks with ATP or that ADP was enzymatically converted to ATP by ecto-nucleoside diphosphokinase activity from UTP or CTP released by the cells into the medium. However, we believe this possibility to be unlikely, since ADP stocks were treated with hexokinase and hexokinase also was included in the assay medium. We have shown previously that these precautions are sufficient to eliminate ATP contamination in ADP stocks and to prevent bioconversion in the assay medium (Nicholas *et al*., 1996). Furthermore, we have shown recently that ADP is a partial agonist for promoting Ca^{2+} mobilization in 1321N1-$hP2Y_{11}$ cells under conditions that minimize nucleotide metabolism and bioconversion (Qi *et al*., manuscript in preparation). Thus, ADP appears to be a partial agonist at the $hP2Y_{11}$ receptor.

We also show here that concomitant stimulation of inositol lipid hydrolysis, which increases intracellular Ca^{2+} mobilization and activates PKC, markedly potentiates the maximal levels of cyclic AMP accumulation without changing agonist potency. In 1321N1-$hP2Y_{11}$ cells, both chelation of intracellular Ca^{2+} and downregulation of PKC markedly inhibit ATP-promoted adenylyl cyclase activity, whereas in CHO-$hP2Y_{11}$ cells, ATP-promoted adenylyl cyclase activity is refractory to $[Ca^{2+}]$ but is decreased when PKC is down-regulated. This effect is specific to the $hP2Y_{11}$ receptor; cyclic AMP accumulation promoted by Gs-coupled receptors that do not stimulate inositol lipid hydrolysis, such as hD1 dopamine or PGE_2 receptors, is not affected by BAPTA-AM or chronic PMA treatment. In addition, cyclic AMP accumulation promoted by these Gs-coupled receptors was potentiated following acute treatment of cells with PMA or following co-activation of an endogenous Gq-coupled muscarinic receptor. The most parsimonius explanation of these results is that receptor-promoted increases in intracellular Ca^{2+} mobilization and/or PKC activation potentiates adenylyl cyclase activity in 1321N1 and CHO-K1 cells. Several studies have documented cross-talk between Gs- and Gq-coupled receptors (Ho *et al*., 1988; Donaldson *et al*., 1988; Alexander *et al*., 1992; Klinger *et al*., 1998). However, these studies focused on the consequences of activation of two distinct G protein-coupled receptors, one coupled solely to phospholipase C and the other coupled solely to activation of adenylyl cyclase. In contrast, with the $hP2Y_{11}$ receptor cross-talk between signalling pathways occurs following activation of a single receptor.

The differences in sensitivity to PKC down-regulation or Ca^{2+} chelation in 1321N1 and CHO-K1 cells are likely due to the different isoforms of adenylyl cyclase expressed in these cells. For example, adenylyl cyclase types II, III, V, or VII are stimulated by PKC, whereas types I, III and VIII are stimulated by Ca^{2+} (Tang & Hurley, 1998; Cooper *et al*., 1995) Thus, the type(s) of adenylyl cyclase expressed in a cell line or tissue can have marked influence on the maximal levels of $hP2Y_{11}$-promoted cyclic AMP accumulation. A recent study in HL-60 cells (Suh *et al*., 2000), which endogenously express the $P2Y_{11}$ receptor, showed that acute treatment with PMA potentiated dATP-, forskolin- and cholera toxin-mediated increases in cyclic AMP accumulation, suggesting that these cells express an isoform of adenylyl cyclase that is potentiated by PKC. Thus, potentiation of adenylyl cyclase in HL-60 cells by PKC is consistent with our data in both CHO-K1 and 1321N1 cells.

There are relatively few examples of receptors that couple to the activation of both phospholipase C and adenylyl cyclase. These include receptors for the pituitary adenylyl cyclase-activating polypeptide (PACAP; Spengler *et al*., 1993; Pisegna & Wank, 1996), luteinizing hormone (LH; Gudermann *et al*., 1992), calcitonin (CT; Houssami *et al*., 1994), thyrotropin (TSH; Van Sande *et al*., 1990), parathyroid hormone (PTH; Abou-Samra *et al*., 1992) and three tachykinins (substance P, substance K, and neuromedin K; Nakajima *et al*., 1992). Interestingly, all of these receptors are class 1b receptors, which recognize peptide hormones as their cognate ligands (Bockaert & Pin, 1999). Although the $hP2Y_{11}$ receptor is activated by small nucleotides, sequence analysis indicates that P2Y receptors are more closely related to peptide receptors than they are to receptors for small molecules such as biogenic amines and adenosine (Lustig *et al*., 1993). Thus, dual coupling to phospholipase C and adenylyl cyclase may be confined to a small subset of class 1b receptors.

These dual-coupled receptors also show different efficiencies in their coupling to cyclic AMP and IP accumulation. In contrast to the $P2Y_{11}$ receptor, the PACAP, LH, CT, and

TSH receptors couple more efficiently (6–40 fold) to cyclic AMP accumulation than to inositol lipid hydrolysis (Spengler *et al*., 1993; Pisegna & Wank, 1996; Gudermann *et al*., 1992; Offermans *et al*., 1996). However, the tachykinin receptors showed similar coupling efficiencies as the $hP2Y_{11}$ receptor, in which stimulation of inositol lipid hydrolysis occurred at agonist concentrations 10 fold lower than for stimulation of cyclic AMP accumulation (Nakajima *et al*., 1992). To date, no studies have shown whether these differences have any functional relevance for the physiological activities of the receptors.

In conclusion, the $hP2Y_{11}$ receptor is coupled to both phospholipase C and adenylyl cyclase, but is more efficiently coupled to phospholipase C. In addition, the capacity of ATP to promote cyclic AMP accumulation in 1321N1-$hP2Y_{11}$ and CHO-$hP2Y_{11}$ cells is markedly potentiated by mobilization of intracellular Ca^{2+} and/or activation of PKC as a consequence of coupling to Gq/phospholipase C.

This work was supported by grants from The Wellcome Trust and The Caledonian Research Foundation (C. Kennedy), by United States Public Health Service Grant GM38213 (T.K. Harden and R.A. Nicholas), and by an American Heart Association Grant-in-Aid 9950675N (R.A. Nicholas). During the course of this study, R.A. Nicholas was an Established Investigator of the American Heart Association.

References

ABOU-SAMRA, A.B., JUPPNER, H., FORCE, T., FREEMAN, M.W., KONG, X.F., SCHIPANI, E., URENA, P., RICHARDS, J., BONVENTRE, J.V., POTTS, J.T. JR., KRONENBERG, H.M. & SEGRE, G.V. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 2732–2736.

ALEXANDER, S.P.H., CURTIS, A.R., HILL, S.J. & KENDALL, D.A. (1992). Activation of a metabotropic excitatory amino acid receptor potentiates A2b adenosine receptor-stimulated cyclic AMP accumulation. *Neurosci. Lett.,* **146,** 231–233.

BOCKAERT, J. & PIN, J.P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.,* **18,** 1723–1729.

BOYER, J.L., DELANEY, S.M., VILLANUEVA, D. & HARDEN, T.K. (2000). A molecularly identified P2Y receptor simultaneously activates phospholipase C and inhibits adenylyl cyclase and is nonselectively activated by all nucleoside triphosphates. *Mol. Pharmacol.,* **57,** 805–810.

BOYER, J.L., WALDO, G.L. & HARDEN, T.K. (1997). Molecular cloning and expression of an avian G protein-coupled p2y receptor. *Mol. Pharmacol.,* **52,** 928–934.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterisation of A_2 adenosine receptor labelled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.,* **29,** 331–346.

COFFIN, V.L. & SPEALMAN, R.D. (1989). Psychomotor-stimulant effects of 3-isobutyl-1-methylxanthine: comparison with caffeine and 7-(2-chloroethyl) theophylline. *Eur. J. Pharmacol.,* **170,** 35–40.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.-M. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.,* **272,** 31969–31973.

COMMUNI, D., MOTTE, S., BOEYNAEMS, J.-M. & PIROTTON, S. (1996a). Pharmacological characterization of the human $P2Y_4$ receptor. *Eur. J. Pharmacol.,* **317,** 383–389.

COMMUNI, D., PARMENTIER, M. & BOEYNAEMS, J.-M. (1996b). Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem. Biophys. Res. Commun.,* **222,** 303–308.

COMMUNI, D., ROBAYE, B. & BOEYNAEMS, J.-M. (1999). Pharmacological characterization of the human $P2Y_{11}$ receptor. *Br. J. Pharmacol.,* **128,** 1199–1206.

COMSTOCK, K.E., WATSON, N.F. & OLSEN, J.C. (1997). Design of retroviral expression vectors. In *Methods in Molecular Biology, vol 62: Recombinant Gene Expression Protocols*. ed. Tuan, R. pp. 207–222. Humana Press Inc.: Totawa, NJ.

COOPER, D.M.F., MONS, N. & KARPEN, J.W. (1995). Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature,* **374,** 421–424.

DONALDSON, J., HILL, S.J. & BROWN, A.W. (1988). Kinetic studies on the mechanism by which histamine H1 receptors potentiate cyclic AMP accumulation in guinea pig cerebral cortical slices. *Mol. Pharmacol.,* **33,** 626–633.

GUDERMANN, T., BIRNBAUMER, M. & BIRNBAUMER, L. (1992). Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2+} mobilization. *J. Biol. Chem.,* **267,** 4479–4488.

HARDEN, T.K. (1998). The G-Protein-coupled P2Y receptors. In *Cardiovascular Biology of Purines*. ed. Burnstock, G., Dobson, J.G., Liang, B.T. & Linden, J. pp. 181–205. London, Boston: Kluwer Academic Publishers.

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

HO, A.K., CHIK, C.L. & KLEIN, D.C. (1988). Effects of protein kinase inhibitor (1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) on protein kinase C activity and adrenergic stimulation of cAMP and cGMP in rat pinealocytes. *Biochem. Pharmacol.,* **37,** 1015–1020.

HOUSSAMI, S., FINDLAY, D.M., BRADY, C.L., MYERS, D.E., MARTIN, T.J. & SEXTON, P.M. (1994). Isoforms of the rat calcitonin receptor: consequences for ligand binding and signal transduction. *Endocrinology,* **135,** 183–190.

IREDALE, P.A. & HILL, S.J. (1993). Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells. *Br. J. Pharmacol.,* **110,** 1305–1310.

KENAKIN, T. (1997). *Pharmacologic analysis of drug-receptor interaction*. 3rd edition, Lippencott-Raven Publishers: Philadelphia, PA.

KENNEDY, C., QI, A.-D., HEROLD, C.L., NICHOLAS, R.A. & HARDEN, T.K. (2000). ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol. Pharmacol.,* **57,** 926–931.

KENNEDY, C., QI, A., NICHOLAS, R.A. & HARDEN, T.K. (1999). Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase. *Br. J. Pharmacol.,* **126,** 22P.

KING, B.F., TOWNSEND-NICHOLSON, A. & BURNSTOCK, G. (1998). Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol. Sci.,* **19,** 506–514.

KLINGER, C., ANCELLIN, N., BARRAULT, M.-B., MOREL, A. & CORMAN, B. (1998). Potentiation of receptor-mediated cAMP production:role in the cross-talk between vasopressin V1a and V2 receptor transduction pathways. *Biochem. J.,* **330,** 1023–1028.

KOZAK, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell,* **44,** 283–292.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, J.M., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the cloned human P2U-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.,* **116,** 1619 – 1627.

LEON, C., HECHLER, B., VIAL, C., LERAY, C., CAZENAVE, J.P. & GACHET, C. (1997). The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett.,* **403,** 26 – 30.

LI, Q., OLESKY, M., PALMER, R.K., HARDEN, T.K. & NICHOLAS, R.A. (1998). Evidence that the $P2Y_6$ receptor is the nearest mammalian homologue of the avian p2y3 receptor. *Mol. Pharmacol.,* **54,** 541 – 546.

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

MILLER, D.G. & MILLER, A.D. (1992). Tunicamycin treatment of CHO cells abrogates multiple blocks to retrovirus infection, one of which is due to a secreted inhibitor. *J. Virol.,* **66,** 78 – 84.

MILLER, D.G. & MILLER, A.D. (1993). Inhibitors of retrovirus infection are secreted by several hamster cell lines and are also present in hamster sera. *J. Virol.,* **67,** 5346 – 5352.

MILLER, A.D. & ROSMAN, G.J. (1989). Improved retroviral vectors for gene transfer and expression. *BioTechniques,* **7,** 980 – 990.

NAKAJIMA, Y., TSUCHIDA, K., NEGISHI, M., ITO, S. & NAKANISHI, S. (1992). Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected chinese hamster ovary cells. *J. Biol. Chem.,* **267,** 2437 – 2442.

NICHOLAS, R.A., WATT, W.C., LAZAROWSKI, E.R., LI, Q. & HARDEN, T.K. (1996). Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: Identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol. Pharmacol.,* **50,** 224 – 229.

NGUYEN, T., ERB, L., WEISMAN, G.A., MARCHESE, A., HENG, H.H., GARRAD, R.C., GEORGE, S.R., TURNER, J.T. & O'DOWD, B.F. (1995). Cloning, expression, and chromosomal localization of the human uridine nucleotide receptor gene. *J. Biol. Chem.,* **270,** 30845 – 30848.

NORTH, R.A. & BARNARD, E.A. (1997). Nucleotide receptors. *Curr. Opin. Neurobiol.,* **7,** 346 – 357.

OFFERMANNS, S., IIDA-KLEIN, A., SEGRE, G.V. & SIMON, M.I. (1996). G alpha q family members couple parathyroid hormone (PTH)/PTH-related peptide and calcitonin receptors to phospholipase C in COS-7 cells. *Mol. Endocrinol.,* **10,** 566 – 574.

PALMER, R.K., BOYER, J.L., SCHACHTER, J.B., NICHOLAS, R.A. & HARDEN, T.K. (1998). Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol. Pharmacol.,* **54,** 1118 – 1123.

PISEGNA, J.R. & WANK, S.A. (1996). Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase. *C. J. Biol. Chem.,* **271,** 17267 – 17274.

SCHACHTER, J.B., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human $P2Y_1$-purinergic receptor. *Br. J. Pharmacol.,* **118,** 167 – 173.

SPENGLER, D., WAEBER, C., PANTALONI, C., HOLSBOER, F., BOCKAERT, J., SEEBURG, P.H. & JOURNOT, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature,* **365,** 170 – 173.

SUH, B.-C., KIM, T.-D., LEE, I.-S. & KIM, K.-T. (2000). Differential regulation of $P2Y_{11}$ receptor-mediated signalling to phospholipase C and adenylyl cyclase by protein kinase C in HL-60 promyelocytes. *Br. J. Pharmacol.,* **131,** 489 – 497.

TANG, W.-J. & HURLEY, J.H. (1998). Catalytic mechanism and regulation of mammalian adenylyl cyclases. *Mol. Pharmacol.,* **54,** 231 – 240.

UKENA, D., JACOBSON, K.A., PADGETT, W.L., AYALA, C., SHAMIM, M.T., KIRK, K.L., OLSSON, R.O. & DALY, J.W. (1986). Species differences in structure activity relationships of adenosine agonists and xanthine antagonists at brain A_1 adenosine receptors. *FEBS Lett.,* **209,** 122 – 128.

VAN SANDE, J., RASPE, E., PERRET, J., LEJEUNE, C., MAENHAUT, C., VASSART, G. & DUMONT, J.E. (1990). Thyrotropin activates both the cyclic AMP and the PIP_2 cascades in CHO cells expressing the human cDNA of TSH receptor. *Mol. Cell Endocrinol.,* **74,** R1 – R6.

(Received July 3, 2000
Revised October 9, 2000
Accepted October 23, 2000)