0026-895X/00/040805-06$3.00/0

MOLECULAR PHARMACOLOGY, **57:**805–810 (2000).

A Molecularly Identified P2Y Receptor Simultaneously Activates Phospholipase C and Inhibits Adenylyl Cyclase and Is Nonselectively Activated by All Nucleoside Triphosphates

JOSÉ L. BOYER, SUZANNE M. DELANEY, DEMETRIO VILLANUEVA, and T. KENDALL HARDEN

Department of Pharmacology, The School of Medicine, University of North Carolina, Chapel Hill, North Carolina

Received November 24, 1999; accepted December 20, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

We recently cloned and expressed a novel P2Y receptor (tp2y receptor) from a turkey cDNA library. Expression of this receptor in 1321N1 human astrocytoma cells confers nucleotide-dependent stimulation of phospholipase C activity; however, as we demonstrate here, it also confers nucleotide-dependent inhibition of adenylyl cyclase. Both the phospholipase C and adenylyl cyclase responses were promoted by receptor agonists over a similar range of concentrations. Moreover, not only did UTP and ATP activate the avian receptor but ITP, GTP, xanthosine 5′-triphosphate, and CTP were also agonists, with EC_{50} values ranging between 0.1 and 1 μM. Similar potencies, rank-order, and selectivity of nucleotide agonists were also demonstrated for intracellular Ca^{2+} mobilization measured during a 30-s stimulation under constant superfusion conditions. This observation indicates that receptor activation by nucleoside 5′-triphosphates is not produced by interconversion of these nucleotides into ATP or UTP. Pretreatment of cells with pertussis toxin completely abolished the inhibitory effect of nucleotide agonists on adenylyl cyclase, whereas the activation of phospholipase C was only partially inhibited. These results demonstrate that the avian P2Y receptor is a nucleoside triphosphate receptor of broad agonist selectivity that interacts with both pertussis toxin-insensitive and -sensitive G proteins to activate phospholipase C and to inhibit adenylyl cyclase. This is the first cloned P2Y receptor that is clearly Gi/adenylyl cyclase-linked.

Physiological responses to extracellular nucleotides are mediated through a large group of ionotropic P2X receptors and metabotropic P2Y receptors (Harden et al., 1995; Fredholm et al., 1997). Seven mammalian P2X receptors and five mammalian P2Y receptors have been cloned to date (Fredholm et al., 1997; King et al., 1998), and both pharmacological and signaling data suggest the existence of additional receptors. For example, a receptor(s) exists on platelets and C6 glioma cells that couples through Gi to inhibit adenylyl cyclase rather than through Gq to activate phospholipase C as occurs with the five P2Y receptors that have been cloned to date (Boyer et al., 1993, 1995; Daniel et al., 1998; Fagura et al., 1998).

Cellular ATP is released as an extracellular signaling molecule in many if not all tissues. However, three members of the P2Y receptor family are activated by uridine nucleotides, and evidence for release of UTP also is beginning to accumulate (Enomoto et al., 1994; Anderson and Parkinson, 1997; Harden et al., 1997; Lazarowski et al., 1997; Connolly et al., 1998; Lazarowski and Harden, 1999). Burnstock and coworkers recently reported that a *Xenopus laevis* P2Y receptor is not only activated by ATP and UTP, but also responds to relatively high concentrations of ITP, CTP, and GTP (Bogdanov et al., 1997). This observation suggests that nucleotides in addition to ATP and UTP may function as extracellular signaling molecules. For example, cell damage and other mechanisms may result in release of all intracellular nucleotides, perhaps in the ratio of their intracellular concentrations.

We recently reported the molecular cloning of an avian P2Y receptor that activates phospholipase C and is regulated with a general agonist selectivity that closely matches that of the human $P2Y_2$ receptor (Boyer et al., 1997). Further study of this receptor has uncovered two novel properties. First, the avian receptor is potently and essentially nonselectively activated by all nucleoside triphosphates. Moreover, pharmacological analyses revealed that activation occurs at very low (nanomolar) concentrations of these nucleotides, suggesting that this activity has physiological relevance. Second, in addition to coupling through the Gq pathway to activate phospholipase C, activation of this receptor results in pertussis toxin-sensitive inhibition of adenylyl cyclase. Thus, this re-

This work was supported by United States Public Health Service Grants HL54889 to J.L.B. and GM38213 to T.K.H. S.M.D. is the recipient of a postdoctoral fellowship from the Medical Research Council of Canada.

ABBREVIATIONS: XTP, xanthosine 5′-triphosphate; IBMX, 3-isobutyl-1-methylxanthine; Ap4A, diadenosine tetraphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid.

ceptor is the first P2Y receptor to be cloned that is clearly Gi/adenylyl cyclase-linked. Its apparent equally effective coupling to two different classes of G proteins also distinguishes it from previously cloned P2Y receptors.

Experimental Procedures

Materials. ATP, UTP, CTP, GTP, dATP, dUTP, dGTP, and dCTP were obtained from Pharmacia (Piscataway, NJ). ITP, xanthosine 5′-triphosphate (XTP), isoproterenol, 3-isobutyl-1-methylxanthine (IBMX), potato apyrase, and diadenosine tetraphosphate (AP_4A) were obtained from Sigma Chemical Co. (St. Louis, MO). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was purchased from Research Biochemicals International (Natick, MA). Hexokinase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). Pertussis toxin was obtained from List Biological Laboratories Inc. (Campbell, CA). The sources of all other reagents have been reported previously (Boyer et al., 1996a, 1997).

Cell Culture. 1321N1 Human astrocytoma cells and NIH-3T3 mouse fibroblast cells stably expressing the tp2y receptor were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (1321N1 cells) or 10% bovine calf serum (NIH-3T3 cells) and 600 μg/ml G-418. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Intracellular Calcium Measurement. 1321N1 Cells stably expressing the avian p2y receptor were grown on glass coverslips for 48 h to a cell density of approximately 40% confluence. Intracellular calcium was measured as described previously by Palmer et al. (1998). In brief, coverslips containing Fura-2-loaded (5 μM) cells were mounted on a RC-20 flow-through chamber (36-μl volume; Warner Instruments Corp., Hamden, CT) and superfused continuously at 1.0 ml/min with Hanks' balanced saline solution alone or with the indicated concentration of nucleotide. The flow-through chamber was secured to the stage of a Nikon inverted fluorescence microscope. The cells were exposed to alternating excitation wavelengths of 340 and 380 nm, and a Cohu high-performance charge-coupled device camera monitored fluorescence emission at 510 nm. The 340/380-nm fluorescence emission was determined and converted to intracellular Ca^{2+} concentration using the equation of Grynkiewicz et al. (1985). Data were recorded and processed using an InCyt Im2 imaging system (Intracellular Imaging Inc., Cincinnati, OH).

Phosphoinositide Hydrolysis Assays. 1321N1 Human astrocytoma and NIH-3T3 cells were seeded in 48-well plates and assayed 3 to 4 days after subculture. Twenty-four hours before the assay, the inositol lipid pool of 1321N1 cells was radiolabeled by incubation in 200 μl of serum-free, inositol-free Dulbecco's modified Eagle's medium containing 0.4 μCi of *myo*-[^{3}H]inositol. NIH-3T3 cells were labeled under the same conditions in the presence of 0.5% dialyzed serum. Before the assay, the cell medium was supplemented with 40 mM HEPES, pH 7.4, and 10 mM LiCl (final concentration) and placed in a 37°C water bath. Ten minutes after LiCl addition, cells were challenged with receptor agonists for an additional 10 min. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μl of ice-cold 50 mM formic acid. After 15 min at 4°C, samples were neutralized with 150 μl of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by ion exchange chromatography on Dowex AG 1-X8 columns as described previously (Boyer et al., 1996a).

cAMP Accumulation Assays. Human astrocytoma cells were seeded in 48-well plates and assayed 3 to 4 days after subculture. The intracellular ATP pool was labeled by incubation with 1 μCi/ml [^{3}H]adenine for 2 h. Before the assay, cells were supplemented with 40 mM HEPES, pH 7.4, and 200 μM IBMX (final concentrations in the assay) and placed in a 37°C water bath. Ten minutes after IBMX addition, cells were stimulated with the simultaneous addition of 10 μM isoproterenol and various concentrations of nucleotide agonists. The reactions were stopped after 10 min by aspiration of the drug-containing medium and the addition of 1 ml of ice-cold 5% trichloroacetic acid. [^{3}H]cAMP accumulation was determined by Dowex and alumina chromatography as described previously (Harden et al., 1982).

Results

We reported previously that the avian P2Y receptor is activated by ATP, UTP, and Ap_4A (Boyer et al., 1997). We have extended the pharmacological analysis of this receptor to include all naturally occurring nucleoside triphosphates (Fig. 1 and Table 1). Surprisingly, not only did ATP and UTP potently stimulate inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing the avian receptor (Fig. 1), but ITP, GTP, XTP, and CTP also were very potent agonists. The 2′-deoxy nucleoside triphosphates dATP, dUTP, dGTP, and dCTP were also full agonists at the tp2y receptor with lower affinities than the corresponding oxy-nucleotides (Table 1). ITP, GTP, XTP, and CTP also exhibited potencies similar to those of ATP and UTP in NIH-3T3 fibroblasts stably expressing the avian receptor (data not shown), indicating that the observed response to all nucleoside triphosphates is not a cell-specific phenomenon. In contrast to the potent agonist activity of naturally occurring nucleoside triphosphates, γ-thiol derivatives of ATP and GTP were weak agonists (data not shown). As we reported previously (Boyer et al., 1997), nucleoside diphosphates also were weak agonists at the avian receptor.

1321N1 Human astrocytoma cells express ectoenzymes that both interconvert and hydrolyze nucleotides, which can lead to misleading conclusions about the apparent agonist selectivity of expressed recombinant P2Y receptors (Lazarowski et al., 1997). Thus, one interpretation of the agonist activity of ITP, GTP, XTP, and CTP is that they either are converted to ATP or UTP or promote receptor stimulation by preventing the hydrolysis of accumulated ATP and/or UTP

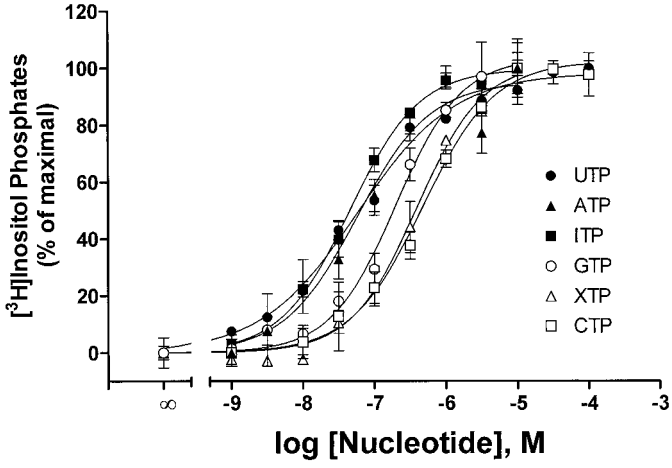

Fig. 1. Effect of nucleoside 5′-triphosphates on tp2y receptor-induced activation of phospholipase C. [^{3}H]Inositol-labeled 1321N1 human astrocytoma cells stably expressing the tp2y receptor were incubated with the indicated concentrations of UTP (●), ATP (▲), ITP (■), GTP (○), XTP (△), and CTP (□) as indicated in *Experimental Procedures*. The [^{3}H]inositol phosphate response was normalized to the response obtained with a maximally effective concentration of UTP in the same experiment (100%). Data shown are the mean ± S.E. of triplicate assays from a representative experiment repeated at least three times.

released constitutively or by mechanical stimulation. As such, potential activation of the avian P2Y receptor by a mechanism not related to direct agonist activity of these nucleotides was addressed by the study of Ca^{2+} transients measured using Fura-2 imaging in monolayers of cells continuously superfused with medium. We have shown previously that this superfusion-based assay system circumvents the potential problems associated with either accumulation of released nucleotide or nucleotide metabolism (Palmer et al., 1998). As illustrated in Fig. 2, ITP, GTP, XTP, and CTP were potent agonists for mobilization of Ca^{2+} in avian P2Y receptor-expressing 1321N1 cells superfused with medium. Indeed, ATP and ITP exhibited very similar EC_{50} values irrespective of whether determined by accumulation of inositol phosphates or by Ca^{2+} mobilization (Fig. 3). We conclude that the effects of ITP, GTP, XTP, and CTP are entirely explained by direct agonist activity of these molecules at the avian P2Y receptor.

The emphasis thus far has been on the phospholipase C-activating properties of the avian receptor, and its unique agonist selectivity for promotion of inositol lipid hydrolysis and mobilization of intracellular Ca^{2+} distinguishes it from other previously cloned P2Y receptors. We have previously stably expressed the human $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors in 1321N1 cells. Although activation of all four of these receptors markedly elevates intracellular inositol phosphates and Ca^{2+} levels, no effect of the cognate agonists of these receptors on cAMP levels was observed. However, expression of the Gi/adenylyl cyclase-linked M2 muscarinic receptor in 1321N1 cells conferred capacity to respond to carbachol with a decrease in intracellular cAMP levels (Schachter et al., 1997), confirming our earlier conclusions that these cells express the component proteins necessary to observe Gi-promoted inhibition of adenylyl cyclase. Communi et al. (1997) have recently cloned the human $P2Y_{11}$ receptor and reported that it activates both phospholipase C and adenylyl cyclase. We have confirmed their results after stably expressing the $P2Y_{11}$ receptor in 1321N1 cells, although the concentrations of agonist sufficient to elicit the inositol phosphate response were approximately 30-fold lower than that necessary to stimulate adenylyl cyclase (Kennedy et al., 1999b). Thus, 1321N1 cells express an adenylyl cyclase that is responsive to both inhibitory and activating G protein-coupled receptors. The capacity of the avian receptor to inhibit or augment intracellular cAMP levels was also examined in 1321N1 cells expressing the tp2y receptor. Surprisingly, we observed that ATP caused a marked decrease in isoproterenol-stimulated (Fig. 4) or forskolin-stimulated (data not shown) cAMP levels. In light of the ATP-promoted inhibition of cAMP accumulation in avian P2Y receptor-expressing cells, a series of concentration effect curves were carried out with other nucleoside triphosphates and diphosphates. As was shown for the inositol phosphate response (Fig. 1 and Table 1), all nucleotides were potent agonists for promotion of decreases in cAMP levels (Fig. 4). Indeed, the EC_{50} values for most of the agonists tested were 1.5- to 20-fold lower than the corresponding EC_{50} values determined in assays of inositol phosphate accumulation (Table 1). Although most of the studies of the cAMP response were carried out in 1321N1 cells stably expressing the avian P2Y receptor, similar results were obtained in NIH-3T3 fibroblasts expressing this receptor (data not shown). Thus, the capacity of the avian receptor to inhibit adenylyl cyclase is not a cell-specific phenomenon.

TABLE 1
Agonist selectivity of the tp2y receptor

	Activation of Phospholipase C	Inhibition of Adenylyl Cyclase
	EC_{50}, nM	
Agonists		
UTP	81 ± 19	13 ± 7
ITP	41 ± 6	25 ± 15
ATP	101 ± 20	15 ± 4
GTP	248 ± 70	17 ± 6
XTP	253 ± 70	172 ± 81
CTP	598 ± 215	182 ± 34
Ap_4A	1,310 ± 560	63 ± 26
ADP	71,820 ± 24,410	8,500 ± 3,400
UDP	142,000 ± 46,000	127,276 ± 115,901
dUTP	573 ± 247	253 ± 111
dATP	502 ± 24	289 ± 104
dGTP	13,160 ± 9,073	67 ± 22
dCTP	6,140 ± 2,350	77 ± 24
Antagonists		
A3P5P	NE	NE
PPADS	NE	NE
Suramin	NE	NE

NE, no effect at 100 μM; A3P5P, adenosine 3′,5′-bisphosphate.

The inhibition of adenylyl cyclase apparently occurs through a member of the Gi family of G proteins, because pretreatment of cells with a low concentration of pertussis toxin completely prevented the capacity of ATP (or UTP) to inhibit cAMP accumulation (Fig. 5A). Pretreatment of cells with pertussis toxin also partially inhibited the capacity of UTP (or ATP) to stimulate inositol phosphate accumulation (Fig. 5B), suggesting that activation of phospholipase C by the avian receptor may occur through both Gq- and Gi-mediated effects. No effect of pertussis toxin-treatment was observed on the EC_{50} values of nucleotides for activation of phospholipase C (Fig. 5B) or on basal and isoproterenol-stimulated cAMP levels (data not shown).

We also examined the effects of P2 receptor antagonists adenosine 3′5′-bisphosphate ($P2Y_1$ antagonist), PPADS and suramin (nonselective P2 receptor antagonists) on nucleotide-activated tp2y receptor stimulation of inositol phosphates and inhibition of cAMP accumulation. As indicated In Table 1, none of these compounds antagonized the activation of tp2y receptors.

Discussion

The human P2Y receptors include a receptor that is preferentially activated by ADP ($P2Y_1$ receptor), a receptor that is activated by both ATP and UTP ($P2Y_2$ receptor), a receptor that is selectively activated by UTP ($P2Y_4$ receptor), a receptor that is selectively activated by UDP ($P2Y_6$ receptor), and a receptor that is activated selectively by ATP ($P2Y_{11}$ receptor) (King et al., 1998). In contrast to the adenine and uridine selectivity of these receptors, we conclude from the data presented here that an avian P2Y receptor we have recently cloned (Boyer et al., 1997) is nonselectively activated by all nucleoside 5′-triphosphates. This receptor also exhibits signaling properties that distinguish it from the other P2Y receptors that have been cloned to date. That is, activation of the avian receptor promotes Gi-dependent inhibition of adenylyl cyclase; this activity, which is not observed with any of the aforementioned mammalian P2Y receptors, exists con-

comitantly with similarly robust activation of phospholipase C. Inhibition of adenylyl cyclase through the avian receptor apparently is not a trivial consequence of its overexpression. For example, the EC_{50} values for agonists promoting the cAMP response were slightly to considerably lower than the corresponding EC_{50} values for activating phospholipase C.

Observation of activation of a P2Y receptor by low concentrations of all nucleoside triphosphates is both surprising and unprecedented. Although primary data were not presented, Bogdanov et al. (1997) reported that CTP, GTP, and ITP at relatively high concentrations all activated an *X. laevis* receptor; the relative potencies of these three molecules relative to ATP and UTP were not presented. This *X. laevis* receptor has a very long carboxyl terminus that distinguishes it from the cloned avian receptor and the five cloned mammalian receptors (Bogdanov et al., 1997; King et al., 1998). However, if the carboxyl-terminal domain is excluded from the comparison, the avian P2Y receptor studied here is more similar (60% identical) to the amphibian receptor and the human $P2Y_4$ receptor (57% identical) than to the other mammalian P2Y receptors. Although we have demonstrated that the human $P2Y_4$ receptor is specifically activated by UTP but not by ATP and other nucleoside triphosphates, this agonist selectivity is not strictly shared across other mammalian species homologues of the $P2Y_4$ receptor. Thus, Webb et al. (1998) and Bogdanov et al. (1998) recently reported that the rat $P2Y_4$ receptor is equipotently activated by UTP and ATP; Bogdanov et al. (1998) also reported potent agonist effects of ITP at the rat $P2Y_4$ receptor. Our group has also addressed the agonist selectivity of the rat $P2Y_4$ receptor in experiments using Fura-2 quantification of Ca^{2+} responses in cells superfused with medium as described above (see Fig. 2). Although UTP and ATP were the most potent agonists, activity was observed with essentially all nucleoside triphosphates with the general order of potency of UTP > ATP > Ap_4A > ITP > GTP > CTP > XTP (Kennedy et al., 1999a). Thus, the rat $P2Y_4$ receptor exhibits much broader nucleoside triphosphate selectivity than the human $P2Y_4$ receptor and, at least at the level of our preliminary analyses, may resemble the avian P2Y receptor studied here in its capacity to be activated by all nucleoside triphosphates. We cannot conclude from the available information whether the avian receptor (and perhaps the *X. laevis* P2Y receptor) should be considered species homologues of the mammalian

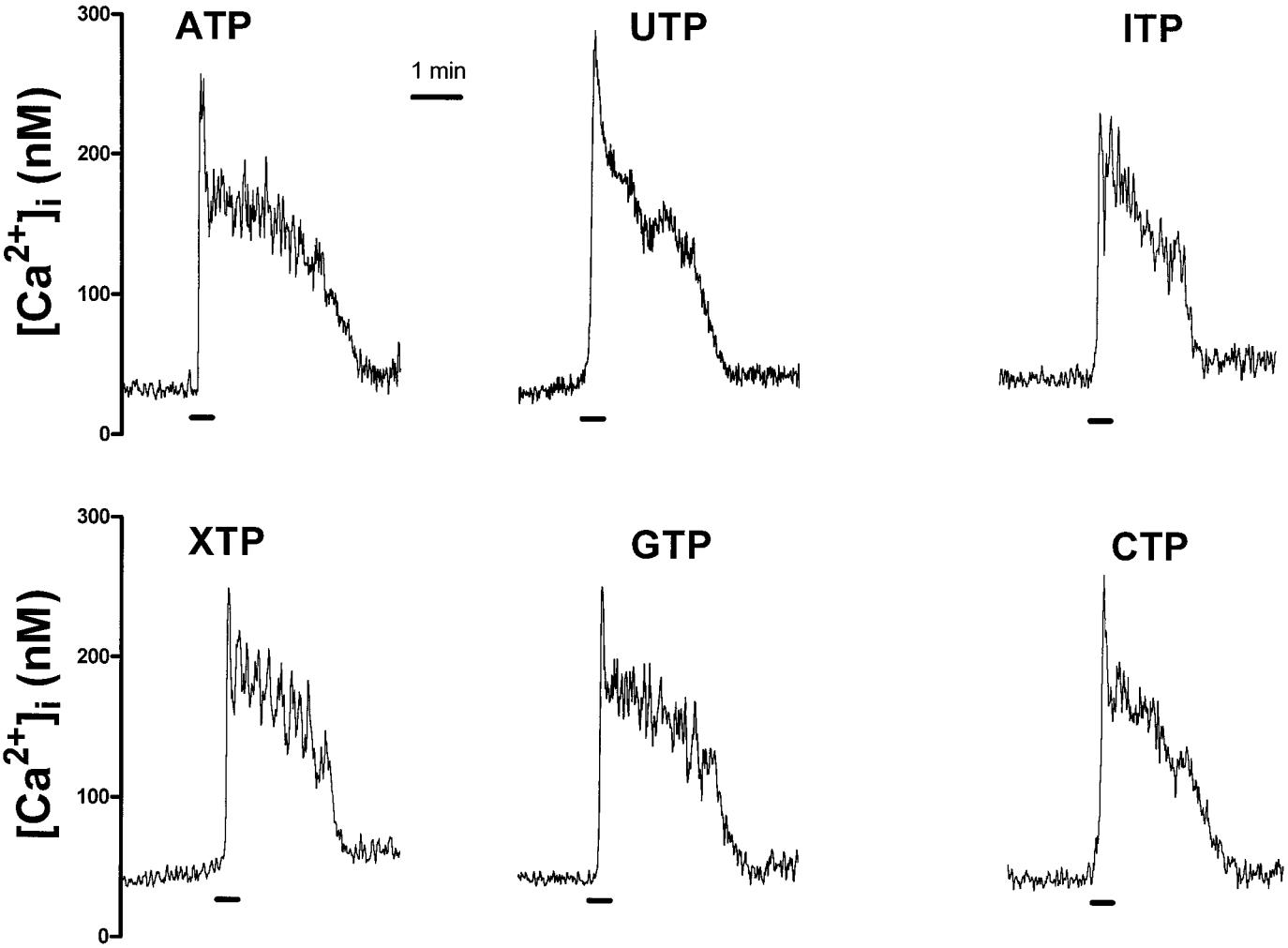

Fig. 2. Nucleotide-induced calcium responses of tp2y receptor-expressing 1321N1 human astrocytoma cells. Fura 2-loaded 1321N1 human astrocytoma cells stably expressing the tp2y receptor grown to approximately 40% confluence were continuously superfused with Hanks' buffered saline solution. After an equilibration period of 30 min, the superfusion solution was supplemented with 1 μM (final concentration) of the indicated nucleotides for 30 s. Traces shown are from a single cell in a field of approximately 10 cells, all of which responded to the nucleotide. Similar results were obtained in three identical experiments using different coverslips.

$P2Y_4$ receptors. However, assuming that the *X. laevis* receptor in further studies turns out to be potently activated by all nucleoside triphosphates and our preliminary studies of the rat receptor are confirmed, these three receptors indeed exhibit strong similarity based on both their 55 to 60% sequence identity and their similar agonist selectivities. Our studies to date screening various mammalian cDNA libraries with probes made from the avian P2Y receptor have identified no molecular species more homologous to the avian receptor than a $P2Y_4$ receptor. Thus, the remarkable difference in agonist selectivity of the human $P2Y_4$ receptor from the selectivity of other species homologs of this receptor may turn out to be even greater than that already noted by Webb et al. (1998) and Bogdanov et al. (1998).

The observation that cell surface receptors exist that are activated by all nucleoside triphosphates suggests that molecules other than ATP or UTP may serve extracellular signaling roles through P2Y receptors. Recent studies indicate that mechanical stimulation of cells results in the release of pharmacologically relevant concentrations of UTP (Lazarowski et al., 1997). Comparison of extracellular UTP to ATP concentrations across approximately 10 different cell types indicated that the ratio of these nucleotides is relatively similar irrespective of the cell type studied (Lazarowski and Harden, 1999). One potential explanation of this result is that UTP and ATP are released by a mechanism that accesses an intracellular pool of UTP and ATP at their intracellular concentrations. If this is the case, it is similarly possible that this mechanism simply releases all nucleoside triphosphates in the ratio of their intracellular concentrations. Thus, it will be important to quantify the extracellular concentrations of nucleotides in addition to ATP and UTP.

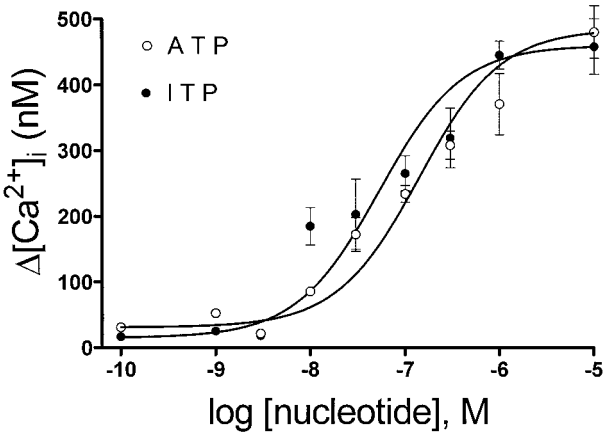

Fig. 3. Concentration dependence of nucleotide-promoted Ca^{2+} mobilization in tp2y receptor expressing 1321N1 cells. Single coverslips were exposed to one concentration of ATP (○) or ITP (●) in Hanks' buffered saline solution for 30 s. Data points correspond to the average ± S.E. of the peak intracellular Ca^{2+} concentrations recorded from 15 to 20 cells. Each nucleotide concentration was tested in at least two different coverslips.

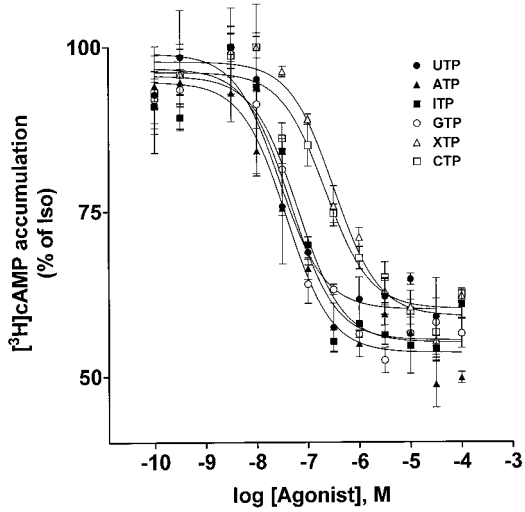

Fig. 4. Inhibition of cAMP accumulation by nucleoside 5′-triphosphates in tp2y receptor-expressing 1321N1 human astrocytoma cells. The capacity of the indicated concentrations of UTP (●), ATP (▲), ITP (■), GTP (○), XTP (△), and CTP (□) to inhibit isoproterenol-stimulated cAMP accumulation was determined as described in *Experimental Procedures*. The maximal isoproterenol response (100%) was 15,789 ± 234 cpm. Data shown are the mean ± S.E. of triplicate assays from a representative experiment repeated at least three times.

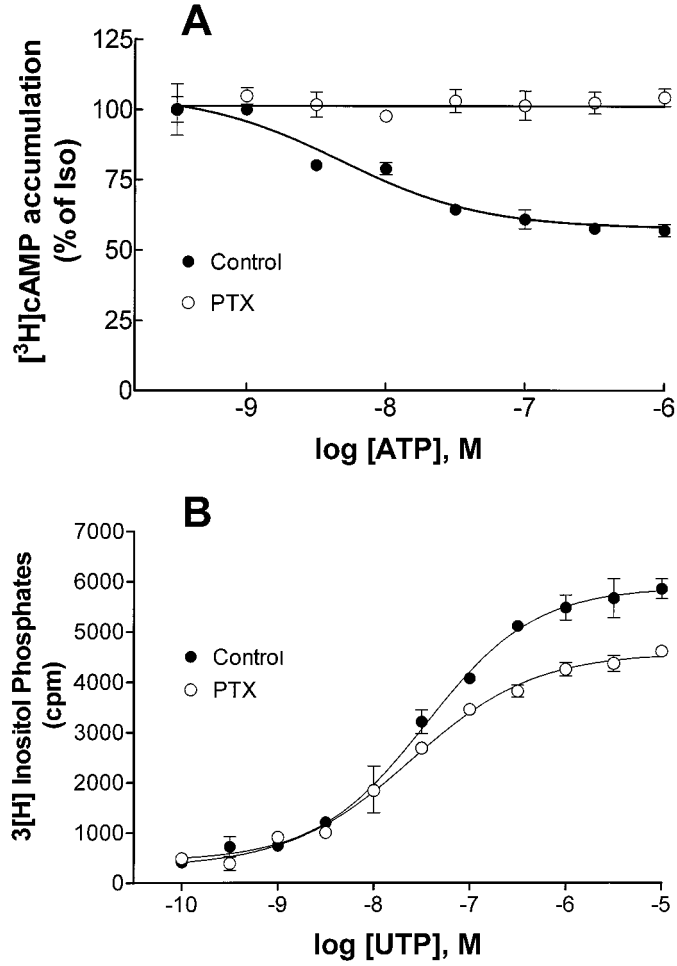

Fig. 5. Effect of pertussis toxin treatment on nucleotide-induced inhibition of adenylyl cyclase and activation of phospholipase C in 1321N1 human astrocytoma cells expressing the tp2y receptor. Cells were incubated overnight in the absence (●) or in the presence (○) of pertussis toxin (30 ng/ml). A, capacity of the indicated concentrations of ATP to inhibit isoproterenol-stimulated cAMP accumulation was determined as indicated in *Experimental Procedures*. The data are the mean ± S.E. of triplicate determinations and the results are representative of those obtained in three separate experiments. B, [^{3}H]inositol-labeled cells were incubated overnight in the absence (●) or in the presence (○) of pertussis toxin (30 ng/ml). The capacity of the indicated concentrations of UTP to stimulate the hydrolysis of inositol lipids was determined as indicated in *Experimental Procedures*. The data shown are the mean ± S.E. of triplicate assays from an experiment repeated at least three times.

The receptor for ADP on platelets was the first P2Y receptor to be studied biochemically and one of the first to be associated with a physiological response to extracellular nucleotides (Gaarder et al., 1961). This receptor also was one of the first receptors to be shown to negatively couple to adenylyl cyclase (Cooper and Rodbell, 1979; Mellwig and Jakobs, 1980). We have studied extensively a P2Y receptor on C6 rat glioma cells that negatively couples to adenylyl cyclase without interacting with Gq/regulated phospholipase C (Boyer et al., 1993, 1994, 1995, 1996b; Schachter et al., 1997). Although the pharmacological selectivity for agonists of the Gi-linked receptor of platelets (Hourani and Cusack, 1991) and C6 glioma cells resembles that of the $P2Y_1$ receptor, potent antagonists of the $P2Y_1$ receptor do not antagonize the Gi-linked P2Y receptor in either preparation (Daniel et al., 1998; Fagura et al., 1998). Whether a single or multiple P2Y receptors exist with adenylyl cyclase-inhibiting properties is yet to be determined. Because this receptor(s) has not been cloned, its molecular relationship to the previously cloned Gq/phospholipase C-coupled P2Y receptors remains to be established.

The demonstration that the avian P2Y receptor studied here is well coupled to adenylyl cyclase is notable in light of the absence of molecular information on Gi-linked P2Y receptors. Thus, we observed that a receptor with sequence identity to the $P2Y_4$ receptor of almost 60% effected a signaling response heretofore not recognized in a cloned P2Y receptor. Studies with a broad group of G protein-coupled receptors indicate that it is the third cytoplasmic loop that is usually, but not exclusively, involved in G protein coupling. The sequence of the third cytoplasmic loop of the avian P2Y receptor does not remarkably distinguish it from the $P2Y_4$ or other P2Y receptors. However, chimeric and/or mutated constructs may help define the minimum sequence in the avian receptor that, for example, confers efficient coupling of the $P2Y_4$ receptor to Gi and adenylyl cyclase, or that resolves Gi from Gq coupling in the avian receptor.

Acknowledgments

We are indebted to Jesus Mateo, Mary Adams, Gary Waldo, Eduardo Lazarowski, and Rob Nicholas for helpful discussions and suggestions.

References

Anderson CM and Parkinson FE (1997) Potential signalling roles for UTP and UDP: Sources, regulation and release of uracil nucleotides. *Trends Pharmacol Sci* **18:** 387–392.

Bogdanov YD, Dale L, King BF, Whittock N and Burnstock G (1997) Early expression of a novel nucleotide receptor in the neural plate of *Xenopus* embryos. *J Biol Chem* **272:**12583–12590.

Bogdanov YD, Wildman SS, Clements MP, King BF and Burnstock G (1998) Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br J Pharmacol* **124:**428–430.

Boyer JL, Lazarowski ER, Chen X-H and Harden TK (1993) Identification of a P_{2Y}-purinergic receptor that inhibits adenylyl cyclase. *J Pharmacol Exp Ther* **267:**1140–1146.

Boyer JL, O'Tuel JW, Fischer B, Jacobson KA and Harden TK (1995) 2-Thioether derivatives of adenosine nucleotides are exceptionally potent agonists at adenylyl cyclase-linked P_{2Y}-purinergic receptors. *Br J Pharmacol* **116:**2611–2616.

Boyer JL, Romero T, Schachter JB and Harden TK (1996a) Identification of competitive antagonists of the $P2Y_1$-receptor. *Mol Pharmacol* **50:**1323–1329.

Boyer JL, Siddiqi S, Fischer B, Jacobson KA and Harden TK (1996b) Identification of potent P_{2Y}-purinoceptor agonists that are derivatives of adenosine 5′-monophosphate. *Br J Pharmacol* **118:**1956–1964.

Boyer JL, Waldo GL and Harden TK (1997) Molecular cloning and expression of an avian G-protein coupled P2Y receptor. *Mol Pharmacol* **52:**928–934.

Boyer JL, Zohn I, Jacobson KA and Harden TK (1994) Differential effects of putative P_2-purinergic receptor antagonists on adenylyl cyclase- and phospholipase C-coupled P_{2Y}-purinergic receptors. *Br J Pharmacol* **113:**614–620.

Communi D, Govaerts C, Parmentier M and Boeynaems J-M (1997) Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J Biol Chem* **272:**31969–31973.

Connolly GP, Demaine C and Duley JA (1998) Ecto-nucleotidases in isolated intact rat vagi, nodose ganglia, and superior cervical ganglia. *Adv Exp Med Biol* **431:** 769–776.

Cooper DMF and Rodbell M (1979) ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. *Nature (Lond)* **282:**517–518.

Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273:**2024–2029.

Enomoto K, Furuya K, Yamagishi S, Oka T and Maeno T (1994) The increase in the intracellular Ca2+ concentration induced by mechanical stimulation is propagated via release of pyrophosphorylated nucleotides in mammary epithelial cells. *Pflugers Arch* **427:**533–542.

Fagura MS, Dainty IA, McKay GD, Kirk IP, Humphries RG, Robertson MJ, Dougall IG and Leff P (1998) $P2Y_1$-receptors in human platelets which are pharmacologically distinct from P2Y(ADP)-receptors. *Br J Pharmacol* **124:**157–164.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U and Williams M (1997) Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* **18:**79–82.

Gaarder A, Jonsen A, Laland S, Hellem AJ and Owen P (1961) Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature (Lond)* **192:**531–532.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.

Harden TK, Boyer JL and Nicholas RA (1995) P_2-purinergic receptors: Subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* **35:** 541–579.

Harden TK, Lazarowski ER and Boucher RC (1997) Release, metabolism and interconversion of adenine and uridine nucleotides: Implications for G protein-coupled P2 receptor agonist selectivity. *Trends Pharmacol Sci* **18:**43–46.

Harden TK, Scheer AG and Smith MM (1982) Differential modification of the interaction of cardiac muscarinic cholinergic and β-adrenergic receptors with a guanine nucleotide binding component(s). *Mol Pharmacol* **21:**570–580.

Hourani SMO and Cusack NJ (1991) Pharmacological receptors on blood platelets. *Pharmacol Rev* **43:**243–298.

Kennedy C, Herold CL, Qi A, Nicholas RA and Harden TK (1999a) Differing pharmacological properties of the human and rat $P2Y_4$ receptors. *Br J Pharmacol* **126:**21P.

Kennedy C, Qi A, Nicholas RA and Harden TK (1999b) Differential coupling of the human $P2Y_{11}$ receptor to phospholipase C and adenylyl cyclase. *Br J Pharmacol* **126:**22P.

King BF, Townsend-Nicholson A and Burnstock G (1998) Metabotropic receptors for ATP and UTP: Exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol Sci* **19:**506–514.

Lazarowski ER and Harden TK (1999) Quantitation of extracellular UTP using a sensitive enzymatic assay. *Br J Pharmacol* **127:**1272–1278.

Lazarowski ER, Homolya L, Boucher RC and Harden TK (1997) Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem* **272:**24348–24354.

Mellwig KP and Jakobs KH (1980) Inhibition of platelet adenylate cyclase by ADP. *Thromb Res* **18:**7–17.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA and Harden TK (1998) Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol Pharmacol* **54:**1118–1123.

Schachter JB, Boyer JL, Li Q, Nicholas RA and Harden TK (1997) Fidelity in functional coupling of the rat $P2Y_1$ receptor to phospholipase C. *Br J Pharmacol* **122:**1021–1024.

Webb TE, Henderson DJ, Roberts JA and Barnard EA (1998) Molecular cloning and characterization of the rat $P2Y_4$ receptor. *J Neurochem* **71:**1348–1357.

Send reprint requests to: Dr. José L. Boyer, Department of Pharmacology, CB # 7365, Mary Ellen Jones Bldg., University of North Carolina, Chapel Hill, NC 7599-7365. E-mail: boyerl@med.unc.edu