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Evidence that the p2y3 Receptor Is the Avian Homologue of the Mammalian $P2Y_6$ Receptor

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ABSTRACT

A P2Y receptor with 65% identity to mammalian $P2Y_6$ receptors, termed the p2y3 receptor, was recently cloned from a chick brain cDNA library and was proposed to represent a novel P2Y receptor subtype [*Mol Pharmacol* **50:**258–265 (1996)]. We cloned the turkey homologue of the chick p2y3 receptor, which shares high sequence identity (97.6%) with the chick receptor, and we stably expressed this receptor and the rat $P2Y_6$ receptor in 1321N1 human astrocytoma cells. The capacities of uridine and adenine nucleotides to promote inositol phosphate accumulation and intracellular Ca^{2+} mobilization were determined for both receptors. UDP and 5-bromo-UDP were the most potent agonists and UTP was a less potent full agonist at both receptors. In contrast, adenine nucleotides and nucleotide derivatives were relatively more potent at the turkey p2y3 receptor than at the rat $P2Y_6$ receptor. To determine whether the avian p2y3 receptor defined a new subtype of mammalian P2Y receptor or was a species homologue of the mammalian $P2Y_6$ receptor, we screened two different human genomic libraries and a Southern blot with a p2y3 receptor probe, under low-stringency conditions that allowed the clear identification of the human $P2Y_6$ receptor gene. Our data indicated that the human genome does not contain a receptor that is more homologous to the avian p2y3 receptor than the $P2Y_6$ receptor. Taken together, these data further define the pharmacological selectivities of these UDP-selective receptors and strongly suggest that the avian p2y3 receptor is a species homologue of the mammalian $P2Y_6$ receptor.

P2Y receptors are G protein-coupled receptors that mediate a wide variety of physiological effects in response to extracellular adenine and uridine nucleotides (Harden *et al.,* 1995; Filtz *et al.,* 1997). Although the existence of multiple subtypes of P2Y receptors was evident from early pharmacological studies, the application of molecular cloning and expression of P2Y receptors has provided a better understanding of this family of nucleotide receptors. Eleven different G protein-coupled receptors (termed $P2Y_1$ through $P2Y_{11}$) have been claimed to be members of the P2Y receptor family, but only five of these receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) have been cloned from mammalian species and shown unambiguously to mediate nucleotide-promoted second messenger responses. The $P2Y_1$ receptor is activated specifically by adenine nucleotides, with ADP being more potent than ATP (Henderson *et al.,* 1995; Schachter *et al.,* 1996), although recent studies have suggested that ATP and ATP derivatives are antagonists at the $P2Y_1$ receptor (Leon *et al.,* 1997; Hechler *et al.,* 1998). The $P2Y_2$ receptor is activated equipotently by ATP and UTP (Parr *et al.,* 1994), the $P2Y_4$ receptor is activated only by UTP (Nguyen *et al.,* 1995; Communi *et al.,* 1996; Lazarowski *et al.,* 1997), the $P2Y_6$ receptor is activated selectively by UDP (Nicholas *et al.,* 1996), and the $P2Y_{11}$ receptor is activated primarily by ATP (Communi *et al.,* 1997). All of these receptors activate PLC. In addition to coupling to PLC, the $P2Y_{11}$ receptor has been reported to activate adenylyl cyclase (Communi *et al.,* 1997).

The cloning from a chick brain cDNA library and characterization of an additional P2Y receptor, termed the p2y3 receptor,[1] was reported recently (Webb *et al.,* 1996). The p2y3 receptor is 65% identical to the rat $P2Y_6$ receptor and 38, 42, and 41% identical to human $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors, respectively. The nucleotide selectivity and second messenger signaling properties of the p2y3 receptor have not been completely defined. In studies measuring the amplitudes of Ca^{2+}-activated Cl^- currents in p2y3 receptor-expressing *Xenopus laevis* oocytes, ADP (100 μM) elicited the largest current, followed by ATP-γ-thiol, UTP, ATP, and UDP. In contrast, UDP was the most potent agonist, followed by UTP, ADP, ATP-γ-thiol, and ATP, in measurements of intracellular Ca^{2+} mobilization in p2y3 receptor-expressing Jurkat

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[1] In accordance with the recommendations of the International Union of Pharmacology Nomenclature Committee (Vanhoutte *et al.,* 1996), we have used the designation p2y3 for this P2Y receptor because it is not from a mammalian species and no mammalian homologue has been identified.

ABBREVIATIONS: PLC, phospholipase C-β; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; AR, adrenergic receptor.

cells. Although it is likely that the mobilization of intracellular Ca^{2+} was a consequence of activation of PLC and production of inositol-1,4,5-trisphosphate, no direct analysis of inositol lipid hydrolysis was reported.

Webb *et al.* (1996) proposed that the chick p2y3 receptor is a unique subtype of P2Y receptor. However, given the similarities in both sequence identity and apparent nucleoside diphosphate selectivity of the chick p2y3 receptor and the rat $P2Y_6$ receptor, it is conceivable that the p2y3 receptor is the avian homologue of the previously reported mammalian $P2Y_6$ receptor. To distinguish between these two possibilities and to directly compare the signaling properties of these two receptors, we have expressed both the turkey p2y3 receptor and the rat $P2Y_6$ receptor in 1321N1 cells and have determined their agonist selectivities and second messenger signaling properties under identical conditions. In addition, we have screened at low stringency two human genomic libraries and a Southern blot with a turkey p2y3 probe, to identify a possible human homologue. Our results indicate that mammalian $P2Y_6$ and avian p2y3 receptors both activate PLC and that the receptors have similar but not identical agonist selectivities. Moreover, no receptor apparently exists in the human genome with greater homology to the avian p2y3 receptor than the human $P2Y_6$ receptor. We conclude from these data that the chick p2y3 receptor is the avian homologue of the mammalian $P2Y_6$ receptor.

Experimental Procedures

Materials. ATP, ADP, UTP, and UDP were purchased from Pharmacia (Piscataway, NJ). The purity of these nucleotides was estimated to be >99% by HPLC analysis. 2-Methylthio-ATP, 2-methylthio-ADP, and ADP-β-thiol were purchased from Research Biochemicals (Natick, MA). 5-Bromo-UTP was from Sigma Chemical Co. (St. Louis, MO), and 5-bromo-UDP was synthesized by incubating 5-bromo-UTP with hexokinase and glucose (Lazarowski *et al.,* 1996). *myo*-[^{3}H]Inositol was from American Radiolabeled Chemicals (St. Louis, MO). [α-^{32}P]dATP was from New England Nuclear. Hexokinase was from Sigma. All tissue culture reagents were from the tissue culture facility at the Lineberger Comprehensive Cancer Center (University of North Carolina).

Cloning and expression of the turkey p2y3 receptor. Because of the similarity between chick and turkey genes, the turkey p2y3 receptor was amplified from turkey genomic DNA by PCR with primers based on the published chick p2y3 receptor sequence (Filtz *et al.,* 1994; Webb *et al.,* 1996). The upstream and downstream oligonucleotides were 5′-GAGACTCGAGCCACC**ATG**AGCATGGCCAACTTCACGGG-3′ (complementary to bases 1–23 of the chick p2y3 receptor coding sequence, with the ATG codon in bold type) and 5′-GAGAGGATCCCATCCCCATCTCCGCACCATG-3′ (complementary to bases 60–81 downstream of the stop codon of the chick p2y3 receptor coding sequence), respectively. The entire coding sequence of the turkey p2y3 receptor was amplified from 1 μg of turkey genomic DNA with *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA), using the following amplification conditions: 94° for 30 sec, 58° for 30 sec, and 72° for 1 min for 35 cycles. The amplified p2y3 receptor gene was subcloned into the *Hpa*I site of pLXSN, and clones harboring the insert in the correct orientation were identified by restriction mapping. Recombinant viral particles were produced in PA317 cells and used to infect 1321N1 cells as described previously (Comstock *et al.,* 1997). 1321N1 cells expressing the rat $P2Y_6$ receptor were produced in a similar manner (Nicholas *et al.,* 1996), except that a single clone was expanded from the cell population and used in activity assays. Cells were selected with 1 mg/ml G418 in Dulbecco's modified Eagle medium and were maintained in the same medium with 400 μg/ml G418.

Measurement of [^{3}H]inositol phosphate accumulation. Cells were plated in 24-well plates at 5×10^4 cells/well and were assayed after 3 days in culture. Inositol lipids were radiolabeled by overnight incubation with 0.4 μCi of *myo*-[^{3}H]inositol/well, in 200 μl of inositol-free Dulbecco's modified Eagle medium. No changes of medium were made after the addition of [^{3}H]inositol. Drug challenges were initiated by addition of 50 μl of 5-fold concentrated agonists in 50 mM LiCl. After a 5-min incubation at 37°, the reactions were stopped by aspiration of the medium and addition of 0.5 ml of boiling 10 mM EDTA, pH 8.0. [^{3}H]Inositol phosphates were isolated by chromatography on Dowex AG1-X8 resin, as described previously (Filtz *et al.,* 1994; Lazarowski *et al.,* 1995). All nucleoside diphosphates were treated with hexokinase, as described (Nicholas *et al.,* 1996), to remove contaminating nucleoside triphosphates.

Measurement of intracellular Ca^{2+} levels. 1321N1 cells expressing either the turkey p2y3 receptor or the rat $P2Y_6$ receptor were plated at low density on glass coverslips and assayed after 2–3 days in culture. On the day of the assay, the cells were incubated for 30 min with 0.5 μM fura-2/acetoxymethyl ester, washed, placed in a sealed recording chamber, and continuously superfused with Hanks' buffered saline solution at a constant flow rate of 1.4 ml/min (Palmer *et al.,* 1994). To initiate responses, cells were superfused for 30 sec with UTP and UDP (freshly purified by HPLC). Changes in intracellular Ca^{2+} levels were quantified by recording the fluorescence of 8–12 cells at 510 nm, after excitation at 340 and 380 nm. No more than 25 cells were in the visual field in any single experiment. The fluorescence ratio values from individual cells were averaged for each concentration and plotted versus time, and the area under the curve was determined by using Prism (GraphPad) software.

Screening of human genomic libraries with a turkey p2y3 probe. Two genomic libraries were screened with a turkey p2y3 receptor probe, in an attempt to identify a human homologue of the p2y3 receptor. One of the libraries was purchased from Stratagene (a λ-fix library of placental DNA with insert sizes of 9–23 kilobases), and the other was constructed by Dr. John Lowe (University of Michigan, Ann Arbor, MI) (a λ-fix library of DNA isolated from peripheral blood lymphocytes, with insert sizes of 9–15 kilobases). For each screening, 1×10^6 phage were plated and transferred in duplicate to nylon filters. The filters were hybridized at 42° in 4.5× SSC (1× = 150 mM NaCl, 15 mM sodium citrate), 50% formamide, 1× Denhardt's solution (1× = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), and 0.1% SDS, with a ^{32}P-labeled probe encompassing the entire coding sequence of the turkey p2y3 receptor. The filters were washed three times, for 15 min each, in 0.5× SSC/0.1% SDS at 50° and were apposed to film for 2–4 days. All plaques displaying hybridization on both sets of filters were isolated and plaque-purified. The identity of each positively hybridizing plaque was determined by a combination of high-stringency screening (wash conditions of 0.1× SSC/0.1% SDS at 70°) and PCR with P2Y receptor-specific primers.

For Southern blotting, genomic DNA isolated from HeLa cells was digested with the appropriate restriction enzymes, separated on 0.7% agarose gels, and transferred to nylon membranes after denaturation and renaturation of the DNA. The blots were hybridized with either a p2y3 receptor probe or a rat $P2Y_6$ receptor probe also encompassing the entire coding sequence. The blot hybridized with the p2y3 receptor probe was washed under low-stringency conditions, whereas the blot hybridized with the rat $P2Y_6$ receptor probe was washed under high-stringency conditions.

Results

Direct comparison of the agonist selectivities of rat $P2Y_6$ and turkey p2y3 receptors expressed in 1321N1 cells. The turkey homologue of the chick p2y3 receptor was

amplified from genomic DNA using primers based on the chick sequence. The amino acid sequence of the turkey p2y3 receptor, like many other turkey signaling proteins, was nearly identical (97.6%) to that of its chick homologue, with eight amino acid differences between the two (GenBank accession number AF069555). Because the pharmacological selectivities and second messenger signaling properties of the chick p2y3 receptor and the rat $P2Y_6$ receptor have not been directly compared, the turkey p2y3 and rat $P2Y_6$ receptors were expressed in 1321N1 cells and assayed under identical conditions, as described in Experimental Procedures. As predicted from the results of Webb *et al.* (1996), who measured mobilization of intracellular Ca^{2+} in Jurkat cells, both UTP and UDP stimulated inositol phosphate accumulation in 1321N1 cells stably expressing the turkey p2y3 receptor (see below). Therefore, the avian p2y3 receptor, like the mammalian $P2Y_6$ receptor, activates PLC.

We assessed the capacities of a variety of nucleotides to activate both the turkey p2y3 receptor and the rat $P2Y_6$ receptor, to compare the agonist selectivities of these receptors (Figs. 1– 3, Table 1). UDP was the most potent agonist at both the rat $P2Y_6$ receptor and the turkey p2y3 receptor in promoting inositol phosphate accumulation (Fig. 1). The potent effects of UDP were not the result of the presence of contaminating UTP, because UDP (and other nucleoside diphosphates) was treated with hexokinase and glucose before the assays and during the incubations with the cells (Nicholas *et al.,* 1996). 5-Bromo-UDP was equipotent with UDP and exhibited nearly identical potencies at both p2y3 and $P2Y_6$ receptors (Fig. 1). UTP was apparently a less potent full agonist, with nearly identical EC_{50} values for the turkey p2y3 and rat $P2Y_6$ receptors. However, the agonist effects of UTP in this type of experiment are difficult to assess, because some or all of this effect could be the result of degradation of UTP to UDP, which is a potent agonist at both receptors.

The capacity of UTP and UDP to stimulate intracellular Ca^{2+} mobilization was assessed under conditions that minimize or eliminate nucleotide breakdown. Assays were carried out with HPLC-purified nucleotides using continuously superfused cells present at low density. UTP was a full agonist at both receptors in measurements of Ca^{2+} mobilization (Fig. 2). The EC_{50} values of UDP and UTP determined for promotion of intracellular Ca^{2+} mobilization for the p2y3 receptor were 24 nM and 1.3 μM, respectively, and those for the $P2Y_6$ receptor were 13 nM and 1.0 μM, respectively. These EC_{50} values were approximately 2-fold lower than the corresponding EC_{50} values determined for promotion of inositol phosphate accumulation (Table 1). The fact that the relative potencies of UTP and UDP determined with the two sets of assay conditions were the same suggests that significant amounts of UDP did not accumulate in the medium of 1321N1 cells during the 5-min inositol phosphate assays. Moreover, these data indicate that the selectivities of the turkey p2y3 and rat $P2Y_6$ receptors for uridine nucleotides are essentially identical.

In contrast to the similar effects of uridine nucleotides at the turkey p2y3 and rat $P2Y_6$ receptors, adenine nucleotides were more potent at the p2y3 receptor than at the $P2Y_6$ receptor (Fig. 3, Table 1). For example, whereas ADP was a relatively potent full agonist at the p2y3 receptor, it was nearly 20-fold less potent at the rat $P2Y_6$ receptor. Similarly, ATP was a low-potency full agonist at the turkey p2y3 receptor but had little effect at the rat $P2Y_6$ receptor. 2-Methylthio-ADP was nearly equipotent with ADP at both receptors, whereas ADP-β-thiol was 15-fold less potent at the rat $P2Y_6$ receptor than at the turkey p2y3 receptor (Fig. 3, Table 1).

Attempts to clone a human homologue of the avian p2y3 receptor. Unambiguous confirmation that the p2y3 receptor is a unique P2Y receptor subtype would follow from the cloning of its mammalian homologue. Therefore, we devised a strategy to identify a potential human homologue of the turkey p2y3 receptor, based on low-stringency screening of human genomic libraries with a p2y3 receptor probe. Genomic libraries were chosen for this purpose because several genome equivalents could be sampled in a single screening, no prior knowledge of tissue distribution was required,

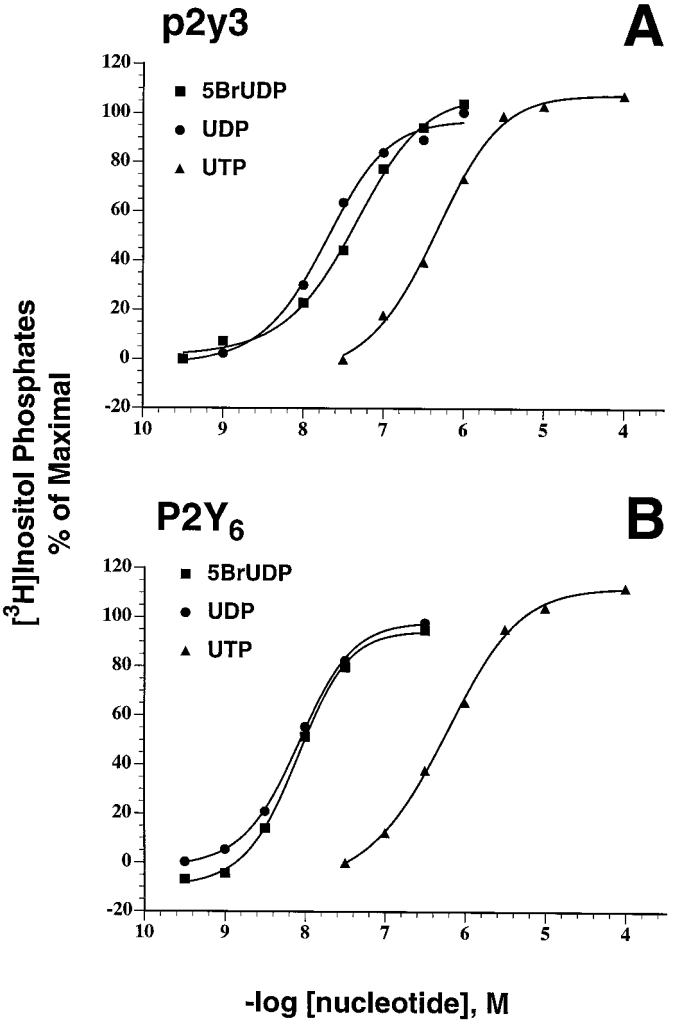

Fig. 1. Pharmacological selectivities of the turkey p2y3 and rat $P2Y_6$ receptors for uridine nucleotides. Inositol phosphate accumulation was measured in 1321N1 cells expressing either the turkey p2y3 receptor (A) or the rat $P2Y_6$ receptor (B), after a 5-min incubation with the indicated nucleotides in the presence of LiCl. Nucleoside diphosphates were pretreated with hexokinase and glucose as described previously (Nicholas *et al.,* 1996). The data were normalized to the maximal effect of UDP in each experiment and represent results from at least three different experiments. The standard error of each value was always <10% of the mean. The values for basal and agonist-induced [^{3}H]inositol phosphate accumulation were ~600 and ~2700 cpm, respectively, for p2y3 receptor-expressing 1321N1 cells and ~900 and ~3800 cpm, respectively, for $P2Y_6$ receptor-expressing 1321N1 cells. *5BrUDP*, 5-bromo-UDP.

and the issue of clone representation in cDNA libraries could be avoided. We reasoned that, if a mammalian homologue of the avian p2y3 receptor exists, it would be more homologous to a turkey p2y3 probe than to a rat $P2Y_6$ receptor probe. Therefore, hybridization and washing conditions were optimized for filters containing $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor genomic bacteriophage λ clones (previously isolated from the leukocyte genomic library), using a turkey p2y3 receptor probe. This optimization of screening parameters identified conditions that ensured clear identification of human $P2Y_6$ clones during library screening.

Fifteen clones that hybridized to the turkey p2y3 receptor probe were isolated from two different human genomic libraries, and all 15 of these clones encoded P2Y receptors (Table 2). The hybridization intensities of these clones fell into three classes: high intensity, medium intensity, and weak intensity. After plaque purification, the identity of each clone was determined both by high-stringency screening with human P2Y receptor probes and by PCR using P2Y receptor-specific primers. Ten of these clones encoded the $P2Y_6$ receptor gene, and these clones corresponded to the clones that hybridized with the highest intensity to the p2y3 receptor probe (Table 2). Four clones that hybridized to the probe with

TABLE 1

EC_{50} values of nucleotides for stimulation of inositol phosphate accumulation by turkey p2y3 and rat $P2Y_6$ receptors expressed in 1321N1 cells

Nucleotide	EC_{50}	
	Turkey p2y3 receptor	Rat $P2Y_6$ receptor
	nM	
UDP	11 ± 9	6 ± 4
UTP	615 ± 178	467 ± 143
ADP	468 ± 186	6,305 ± 1,558
ATP	9,193 ± 2,427	NE[a]
5-Bromo-UDP	44 ± 6	5 ± 4
ADP-β-thiol	1,763 ± 357	25,704 ± 27,948
2-Methylthio-ADP	279 ± 99	1,784 ± 1,224

[a] NE, no effect.

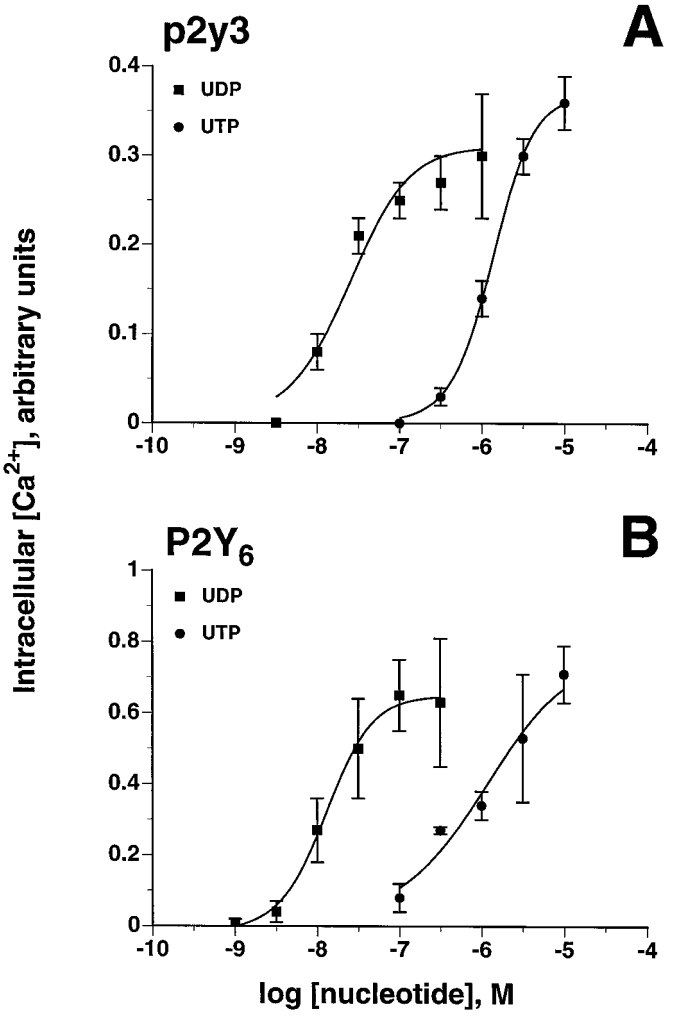

Fig. 2. Promotion of intracellular Ca^{2+} mobilization by HPLC-purified UTP and UDP in 1321N1 cells expressing the turkey p2y3 receptor (A) or the rat $P2Y_6$ receptor (B). Cells were plated on glass coverslips at low density and were loaded with fura-2/acetoxymethyl ester on the day of the experiment. The cells were placed in a sealed chamber under constant superfusion, and intracellular Ca^{2+} levels were measured in response to a 30-sec application of HPLC-purified nucleotides. The 340/380-nm ratios for 10–15 cells in a single optical field for each concentration of agonist were averaged and plotted versus time, to yield the area under the curve.

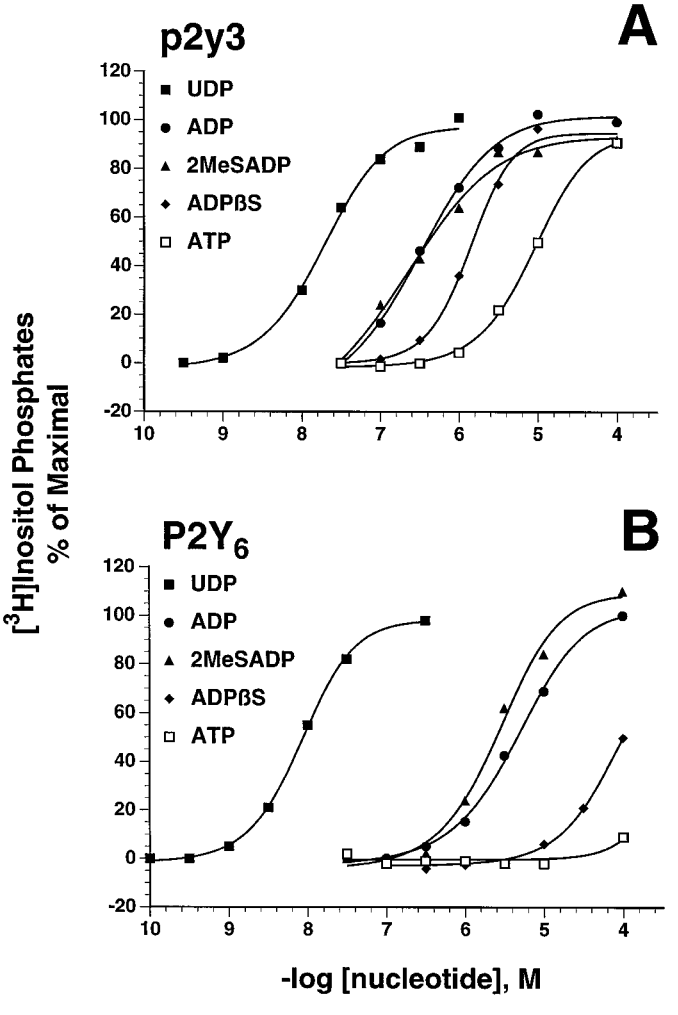

Fig. 3. Pharmacological selectivities of the turkey p2y3 and rat $P2Y_6$ receptors for adenine nucleotides and nucleotide derivatives. Inositol phosphate accumulation was measured in 1321N1 cells expressing either the turkey p2y3 receptor (A) or the rat $P2Y_6$ receptor (B), after a 5-min incubation with the nucleotides in the presence of LiCl. Nucleoside diphosphates were pretreated with hexokinase and glucose as described previously (Nicholas *et al.,* 1996). The data were normalized to the maximal effect of UDP in each experiment and represent results from at least three different experiments. The standard error of each value was always <10% of the mean. *2MeSADP*, 2-methylthio-ADP; *ADPβS*, ADP-β-thiol.

medium intensity were shown to encode the $P2Y_2$ receptor gene, and a single, weakly hybridizing clone encoded the $P2Y_4$ receptor gene.

Although this possibility is unlikely, it could be argued that clones encoding a potential human homologue of the avian p2y3 receptor were not adequately represented in either of the genomic libraries. To address this concern, we carried out Southern blot analysis of human DNA. Identical aliquots of DNA that had been digested with four different restriction enzymes were separated on agarose gels, blotted onto nylon filters, and screened with either a turkey p2y3 receptor probe at low stringency or a rat $P2Y_6$ receptor probe at high stringency (Fig. 4). The rat $P2Y_6$ receptor probe hybridized to a single fragment in each of the four lanes (Fig. 4, *left*). Notably, the p2y3 receptor probe hybridized with highest intensity to the same fragments as did the rat $P2Y_6$ receptor probe, indicating that no other gene exists in the human genome that is more homologous to the turkey p2y3 receptor than the human $P2Y_6$ receptor. An additional band, of lower intensity, was present in three of the four lanes of the blot probed at low stringency with the turkey p2y3 receptor probe (Fig. 4, *right*). Although the identities of these bands remain unknown, these same bands were observed in Southern blots probed with the rat $P2Y_6$ receptor probe at low stringency.

TABLE 2

Identities of clones isolated with the turkey p2y3 receptor probe

One million plaques each from two genomic libraries were screened, in duplicate, with a ^{32}P-labeled turkey p2y3 receptor probe. Positively hybridizing clones were plaque-purified, and their identities were confirmed by both high-stringency screening and PCR, as described in Experimental Procedures.

Human genomic library	No. of clones			
	Total clones isolated	$P2Y_6$ receptor clones	$P2Y_2$ receptor clones	$P2Y_4$ receptor clones
Library 1	7	6	1	0
Library 2	8	4	3	1
Total	15	10	4	1

Discussion

Webb *et al.* (1996) reported the cloning and characterization of a chick P2Y receptor that is 65% identical to the rat $P2Y_6$ receptor. We have cloned the turkey homologue (~98% identical to the chick sequence) of the chick p2y3 receptor and expressed it in 1321N1 cells, to directly compare its pharmacological selectivity with that of the rat $P2Y_6$ receptor. These studies show that the two receptors have very similar, but not identical, pharmacological selectivities. Both the turkey p2y3 and rat $P2Y_6$ receptors were activated most potently by UDP and much less potently by UTP. Similarly, 5-bromo-UDP was equipotent with UDP at both receptors. In contrast to the similar effects of uridine nucleotides, adenine nucleotides were more potent at the turkey p2y3 receptor than at the rat $P2Y_6$ receptor. The pharmacological selectivity of the turkey p2y3 receptor for stimulation of inositol lipid hydrolysis in 1321N1 cells was very similar to that described for the chick p2y3 receptor for mobilization of intracellular Ca^{2+} in Jurkat cells but very different from the selectivity reported for the chick receptor expressed in *X. laevis* oocytes. The source of this difference in apparent agonist selectivity in these expression systems is not clear.

The status of UTP as an agonist at the p2y3 and $P2Y_6$ receptors also was assessed. The stimulatory activity observed during incubation with UTP potentially could have resulted entirely from breakdown of UTP to UDP by ecto-

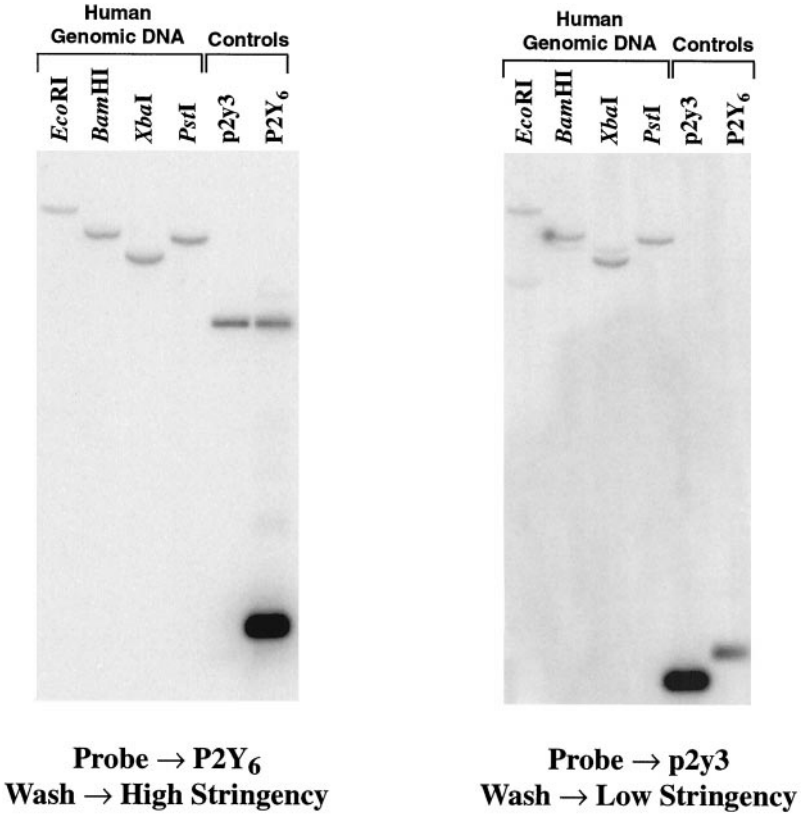

Fig. 4. Southern blots of human genomic DNA screened with either a turkey p2y3 receptor (*right*) or rat $P2Y_6$ receptor (*left*) probe. Southern blots of human genomic DNA were prepared and hybridized with full-length probes derived from either the turkey p2y3 or rat $P2Y_6$ receptor. The blot hybridized with the rat $P2Y_6$ receptor probe was washed at high stringency and the blot hybridized with the turkey p2y3 receptor probe was washed at low stringency, as described in Experimental Procedures. The extra bands observed in the control lanes of the blot hybridized with the rat $P2Y_6$ receptor probe were likely the result of hybridization of the probe with vector sequences (i.e., the probe contained a small amount of vector DNA).

nucleotidases or from contamination of the UTP stocks with UDP. Therefore, we took advantage of a constant flow system for measurement of intracellular Ca^{2+} levels in cells plated at low density, to eliminate the contribution of ecto-nucleotidase activity during determination of nucleotide selectivities. HPLC-purified UTP was a full agonist, with ~50-fold lower potency than UDP, in 1321N1 cells expressing either the turkey p2y3 or rat $P2Y_6$ receptor.

We reported previously that ADP was a partial agonist at the rat $P2Y_6$ receptor (Nicholas *et al.,* 1996), whereas in this study it was apparently a full agonist. The agonist effects of ADP reported here may be the result of higher receptor expression levels in the clonal cell line used in this study, compared with the population of cells assayed previously. Higher receptor densities could result in full-agonist effects of partial agonists and in apparent increases in the potencies of full agonists (Harden *et al.,* 1997). This possibility is supported by the nearly 10-fold lower EC_{50} of UDP for stimulation of inositol lipid hydrolysis in the clonal line of $P2Y_6$ receptor-expressing cells, compared with the value previously observed with the cell population.

Webb *et al.* (1996) proposed that the avian p2y3 receptor is a novel P2Y receptor subtype. This conclusion was based on the assumption that the 65% sequence identity of the chick p2y3 receptor to the rat $P2Y_6$ receptor was too low for these receptors to be species homologues. However, this percent identity is much greater that the percent identity (~40%) of the p2y3 receptor to other mammalian P2Y receptors. Furthermore, the percent identity between the two receptors increases to 74% when only the putative membrane-spanning regions are considered. The avian p2y3 receptor and mammalian $P2Y_6$ receptor have similar pharmacological selectivities, and we observed no evidence for the existence of a gene in the human genome that is more homologous to the turkey p2y3 receptor than the human $P2Y_6$ receptor. The most parsimonious interpretation of these results is that the avian p2y3 and human $P2Y_6$ receptors are species homologues.

There are parallels between the P2Y and β-AR subtypes in both birds and mammals. Turkeys have three known β-AR subtypes. The turkey β_2-AR is 85% identical to its mammalian orthologue and exhibits nearly identical pharmacological selectivity (Del Toro F and Nicholas RA, manuscript in preparation), the turkey β_1-AR is 68% identical to the mammalian β_1-AR and has similar but not identical pharmacological selectivity (Minneman *et al.,* 1980; Yarden *et al.,* 1986), and the turkey β_{4C}-AR is approximately 50% identical to all known β-AR subtypes and has unique pharmacological selectivity (Chen *et al.,* 1994). No mammalian homologue of the β_{4C}-AR has yet been identified. A nearly identical comparison can be made for avian and mammalian P2Y receptors. Thus, the avian and mammalian $P2Y_1$ receptors are 85% identical and exhibit nearly identical pharmacological selectivities (Schachter *et al.,* 1996), the avian p2y3 and mammalian $P2Y_6$ receptors are 65% identical and exhibit similar but not identical pharmacological selectivities, and a newly identified avian P2Y receptor (Boyer *et al.,* 1997) has 40–50% identity to the four mammalian P2Y receptor subtypes and pharmacological selectivity similar to that of the $P2Y_2$ receptor. As with the β_{4C}-AR, no mammalian homologue for this avian P2Y receptor has been identified.

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