P2Y ATP receptors are widely expressed in mammalian tissues and regulate a broad range of activities. Multiple subtypes of P2Y receptors have been identified and are distinguished both on a molecular basis and by pharmacologic substrate preference. Functional evidence suggests that hepatocytes from the little skate Raja erinacea express a primitive P2Y ATP receptor lacking pharmacologic selectivity, so we cloned and characterized this receptor. Skate hepatocyte cDNA was amplified with degenerate oligonucleotide probes designed to identify known P2Y subtypes. A single polymerase chain reaction product was found and used to screen a skate liver cDNA library. A 2314-base pair PCR product was found and used to screen a skate liver cDNA library. A 2314-base pair open reading frame encoding a 357-amino acid product with 61–64% similarity to P2Y1 receptors and 21–37% similarity to other P2Y receptor subtypes. Pharmacology of the putative P2Y receptor was examined using the Xenopus oocyte expression system and revealed activation by a range of nucleotides. The receptor was expressed widely in skate tissue and was expressed to inositol 1,4,5-trisphosphate-mediated increases in $[Ca^{2+}]_i$ (9). Multiple P2Y receptor subtypes have been cloned (10–19) and share approximately 30% identity between classes, with few motifs conserved among these. P2Y receptor subtypes are differentiated not only on a molecular level but also by nucleotide specificity (20). Indeed, pharmacologic selectivity provides an alternative means to identify P2Y receptor subtypes (21–23).

Hepatocytes from the little skate Raja erinacea are primitive polarized secretory epithelia (24). Like mammalian hepatocytes (25), skate hepatocytes express P2Y receptors that link to $Ca^{2+}$ signaling and secretion (26, 27). However, skate hepatocytes respond to a variety of nucleotide stimuli (26), so we identified the P2Y1 receptor expressed by these cells.

EXPERIMENTAL PROCEDURES

Animals and Materials—Male skates (R. erinacea, 0.7–1.2 kg), female sea urchins (Strongylocentrotus droebachiensis), female blue crabs (Callinectes sapidus), male dogfish sharks (Squalus acanthias), and male flounders (Pseudopleuronectes americanus) were caught by net in Frenchmen's Bay, Maine, during the summers of 1998 and 1999. Marine organisms were maintained in large tanks with flowing 15 °C seawater at Mount Desert Island Biological Laboratory (Salisbury Cove, ME) for up to 4 days before use. ATP, ADP, ATP-$\gamma$S, UTP, and UDP were purchased from Sigma. 2-Methylthioadenosine triphosphate (2MeSATP) was purchased from Research Biochemical (Natick, MA). Flu-3 was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality commercially available.

Degenerate Oligonucleotide RT-PCR—Skate liver was freshly excised under RNase-free conditions, minced, and homogenized. Total RNA was prepared using an organic-chloroform reagent (RNAzol; Ambion, Austin, TX) and then treated with RNase-free DNase (Promega, Madison, WI) at 37 °C for 15 min, extracted in phenol/chloroform/isooamyl alcohol (25:24:1), serially precipitated in isopropanol and 70% ethanol (v/v), and resuspended in diethylpyrocarbonate-containing water. cDNA was prepared from total RNA using Moloney murine leukemia virus reverse transcriptase and dT$_\text{m}$ primers (Advantage cDNA Polymerase; CLONTECH, Palo Alto, CA). Degenerate oligonucleotides designed to amplify conserved sequences within transmembrane domains 3 and 7 in the P2Y subtypes P2Y$_1$, and P2Y$_2$ (5'-CTACGTGACGATGAATCCTGATCAGAGGCTGC-3') and 5'-CCGTTGATGAATCCTGATCAGAGGCTGC-3' and 5'-GGATGATGAATCCTGATCAGAGGCTGC-3' (10) were used for cDNA PCR under the following conditions: 94 °C for 1 min; 30 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; and 72 °C for 5 min (Advantage cDNA Polymerase; CLONTECH). Products were analyzed by gel electrophoresis and purified (Qiagen, Valencia, CA).

Molecular Cloning and Sequence Analysis—A custom skate liver A fibrosis cDNA library was prepared (Stratagene, La Jolla, CA). Plaques were titered, plated, and lifted onto charged nylon membranes. A 3$\text{P}$-labeled probe was prepared from the sequenced PCR product using random labeling at room temperature for 4 h (Roche Molecular Biochemicals). Membranes were screened with the radiolabeled probe and
and obtained in RNA controls not exposed to reverse transcriptase (lanes 1 and 2). No such product was obtained in RNA controls not exposed to reverse transcriptase (lanes 3 and 4) or in water controls (not shown).

washed according to manufacturer instructions (Quik-Hyb; Stratagene). One positive clone was identified from 1,200,000 plaques. The clone was excised, dissolved in chloroform, and replated for secondary screening. Positive clones were identified in secondary screening plates as described above and isolated using an in vivo excision protocol. A positive clone was analyzed using PCR and fully sequenced in both directions using an automated sequencer by primer walking. Nucleotide and predicted amino acid sequences were analyzed using Lasergene software (DNASTar, Madison, WI), BLAST search (NCBI Protein Database), and Prosite. Phylogenetic analysis was performed using the Clustal method (29).

Pharmacologic Characterization of the Cloned Receptor—Pharmacologic behavior of the putative receptor was determined by expressing the cloned gene product in Xenopus laevis oocytes (15, 30, 31). Capped skate liver P2Y cRNA was prepared using T3 RNA polymerase according to the manufacturer’s instructions (MAXIscript; Ambion). Xenopus oocytes were isolated and defolliculated in collagenase for approximately 90 min, because only defolliculated oocytes lack endogenous P2Y receptors (32). The oocytes were injected with putative skate liver P2Y cRNA or water 2–3 days before use and then injected with the Ca2+-sensitive dye fluo-3 (1 μM; 40 nl/cell) 30–60 min before use. Oocytes were transferred to a specially designed chamber then examined by time lapse confocal microscopy (30). Cells were excited at 488 nm, and wavelengths of 515 nm were collected. A Bio-Rad MRC 1024 or Olympus Fluoview confocal imaging system was used to collect serial images as oocytes were perfused with ATP, 2MeSATP, ADP, UTP, UDP, or ATPγS. Responses to each nucleotide always were compared with responses to ATP from the same group of oocytes, to account for variability among different oocyte preparations.

Semi-quantitative Multiple Tissue RT-PCR—CDNA PCR of skate liver was performed under the following conditions: 94 °C for 5 min; 16–30 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min (Advantage cDNA Polymerase; CLONTECH) with skate P2Y-specific primers (5'-GAAGTCGCTGGGCAGGCTGAAGAA-3' and 5'-ACGTGGCGTAAACCCTTCGG TTCC-3'). PCR amplification was found to be exponential for 16–22 cycles under these conditions. Negative control reactions were performed on RNA unexposed to reverse transcriptase (lanes 3 and 4) or in water controls (not shown).

FIG. 1. Skate liver expresses a single P2Y receptor. RT-PCR was used to probe skate liver RNA with two sets of degenerate oligonucleotides designed to amplify regions conserved among P2Y1, P2Y2, P2Y4, and P2Y6. A single PCR product was amplified from skate liver cDNA using either set of primers (lanes 1 and 2). No such product was obtained in RNA controls not exposed to reverse transcriptase (lanes 3 and 4) or in water controls (not shown).

P2Y-specific primers (5'-GAAGTCGCTGGGCAGGCTGAAGAA-3' or 5'-ACGTGGCGTAAACCCTTCGGTTCC-3'). PCR amplification was found to be exponential for 16–22 cycles under these conditions. Negative control reactions were performed on RNA unexposed to reverse transcriptase (lanes 3 and 4) or in water controls (not shown).

FIG. 2. Nucleotide and predicted amino acid sequences of the skate liver P2Y receptor. The skate liver P2Y gene contains 2314 base pairs (A) with a 1074-base pair open reading frame (bold type) and encodes a predicted 357 base pair amino acid sequence (B) with seven predicted transmembrane helical domains and a G protein-coupled receptor signature motif. The skate liver P2Y amino acid sequence is aligned with human P2Y1 and P2Y2. Residues indicated by white letters on a black background are conserved among all three sequences, and boxed letters indicate residues conserved between any two sequences.

Sunnyvale, CA) was used to quantitate hybridization products.

Genomic DNA Dot Blots—Genomic DNA was prepared from sea urchin, blue crab, skate, dogfish shark, flounder, and frog according to manufacturer's instructions (Qiagen). Genomic DNA from chicken, rat, mouse, and human was purchased (CLONTECH). Lysophosphatidyl aliquots of DNA (25 μg) were reconstituted in 3 μl of water and then loaded onto a charged nylon membrane. The membrane was prehybridized at 68 °C for 1 h, hybridized at 66 °C for 2 h with a radiolabeled probe prepared...
as described under “Molecular Cloning and Sequence Analysis,” and then washed according to manufacturer instructions (QuikHyb; Stratagene). Identically prepared membranes were hybridized to a radiolabeled human glucose 3-phosphate dehydrogenase probe (CLONTECH) under the same conditions. X-ray films were placed atop the membrane at 280 °C for 24 h and developed. A laser densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnydale, CA) was used to quantitate hybridization products.

**RESULTS**

Molecular Cloning of a Skate Liver P2Y Receptor—To examine whether homologues of cloned mammalian P2Y receptors could be identified in skate liver, skate hepatocyte RNA was screened by RT-PCR using two separate but overlapping degenerate oligonucleotide primers designed to amplify sequences conserved among all known P2Y receptors (10, 28). Only a single 500–600-base pair PCR product was identified with either primer set, whereas no product was identified in water or RNA controls (Fig. 1). Sequences of both products contained an overlapping and identical segment and shared 50–60% identity to P2Y1 receptors of avians and mammals and 30–40% identity to other cloned P2Y receptors. The shorter PCR product was used to screen a skate liver bacteriophage cDNA library and produced a single hybridizing plaque. The plaque was excised, analyzed by gel electrophoresis, and sequenced fully in both directions. This clone (GenBank™ accession number AF242850) was 2314 base pairs in length and contained a 1074-base pair open reading frame, encoding a predicted protein sequence of 357 amino acids (Fig. 2). Sequence analysis of this clone revealed 61–64% identity to avian and mammalian P2Y1 receptors, 22–34% identity to avian and mammalian P2Y2, P2Y4, P2Y6, and P2Y11 receptors, and 37% identity to the frog P2Y1 receptor at the amino acid level (Table I). The deduced amino acid sequence contained seven predicted transmembrane helical domains, with an extracellular N terminus and intracellular C-terminal tail. Other motifs present included a G protein-coupled receptor signature motif, four protein kinase C phosphorylation sites, four cAMP-dependent protein kinase phosphorylation sites, four casein kinase II phosphorylation sites, five N-glycosylation sites, and four N-myristoylation sites. A computer-generated phylogenetic analysis (DNAStar) suggested that the skate P2Y receptor is most closely related to P2Y receptors of the P2Y1 subtype (Fig. 3A) and emerged early in evolution from a common ancestor to P2Y1 receptors of higher organisms (Fig. 3B).

Nucleotide Specificity of the Cloned Receptor—To assess whether this newly cloned gene product functioned as a P2Y receptor, the product was expressed in *Xenopus* oocytes. The oocytes then were injected with the Ca$^{2+}$-sensitive dye fluo-3 and then serially monitored using time lapse confocal microscopy to detect nucleotide-induced increases [Ca$^{2+}$]$_i$. Extracellular ATP (1 μM -100 μM) increased [Ca$^{2+}$]$_i$ in cRNA-injected but not water-injected oocytes (Fig. 4). Increases in [Ca$^{2+}$]$_i$ also were seen in cRNA-injected oocytes perifused with ATP in Ca$^{2+}$-free medium. These findings demonstrate that the putative skate liver P2Y receptor functions as a P2Y ATP receptor.

To determine the nucleotide specificity of this receptor, oocytes also were examined during perifusion with ADP, 2-MeSATP, UTP, UDP, and ATPγS. Perifusion with 100 μM concentrations of ATP, ADP, 2-MeSATP, UTP, UDP, and ATPγS each induced a single intense [Ca$^{2+}$]$_i$ wave that crossed the oocyte (Fig. 4, A and B, and Table II). Stimulation with lower concen-
receptor, semi-quantitative multiple tissue RT-PCR was performed. This technique was chosen because P2Y receptors frequently are found at concentrations in which they can be detected by RT-PCR but not by Northern blot (19). Skate P2Y receptor message was detected in all tissues examined (liver, common bile duct, duodenum, rectal gland, gill, brain, heart, spleen, and testis) but not in RNA controls, water controls, HepG2 human hepatoblastoma cells, or human brain (Fig. 5A). Strongest expression was seen in duodenum, spleen, rectal gland, brain, and liver (Fig. 5B), whereas expression in other organs was considerably lower. This demonstrates that this P2Y receptor is not a liver-specific gene but rather is expressed throughout in the skate to varying degrees.

Phylogenetic Distribution of the Skate Liver P2Y Receptor—To assess whether other members of the animal kingdom express homologues of the skate liver P2Y receptor, genomic DNAs of representative species were examined by dot blot (Fig. 6A). Strong hybridization was seen to crab, skate, shark, and flounder, whereas weak hybridization was seen to other species (Fig. 6B). This suggests that similar genes are expressed throughout the animal kingdom with more closely related genes found in marine species.

DISCUSSION

P2Y receptors are expressed widely in higher organisms. Humans and rodents each express at least four P2Y receptor subtypes (8). Although some tissues express only one or two P2Y subtypes at either the apical or basolateral plasma membrane (1–3), others express multiple P2Y subtypes, each of which may be expressed at either the apical, basolateral, or both membrane domains (3, 21, 23, 34). This highlights the complex organization of purinergic signaling in higher organisms.

In contrast to this complex expression of P2Y receptors in higher organisms, the current findings suggest that the primitive chordate R. erinacea expresses just a single P2Y receptor. The skate liver P2Y receptor has likely evolved within the subfamily of P2Y1 receptors yet is molecularly distinct from either avian or mammalian P2Y1 subtypes. Because this receptor is activated by a variety of diphosphate and triphosphate nucleotides, its pharmacologic specificity may be less developed than P2Y receptors of higher organisms. Furthermore, other receptors that link to inositol 1,4,5-trisphosphate formation and [Ca2+]i, signaling are not detectable in skate liver (26), suggesting that nucleotide receptors are among the most primitive extracellular receptors expressed in the animal kingdom. Moreover, the skate liver P2Y receptor is distributed throughout the skate, so purinergic signaling may be controlled similarly in all skate tissues. Despite its widespread tissue distribution and primitive pharmacology, this P2Y receptor regulates bile secretion in liver (26). Thus, this simple extracellular receptor is linked to specific physiologic responses.

The primitive nature of the skate liver P2Y receptor is highlighted by the finding that it is related to similar molecules in even more primitive organisms. Strong hybridization was seen with blue crab, indicating that a P2Y receptor is likely expressed in this invertebrate. Weak hybridization was seen with sea urchin, suggesting that a P2Y receptor may be expressed in this primitive invertebrate as well. Because the skate liver P2Y receptor is most closely related to the P2Y, subfamily, it is likely that the common ancestor of P2Y receptors arose in a simpler invertebrate such as the sea urchin rather than in an early chordate such as the skate. It has been suggested that nucleotides may have arisen as signaling molecules early in evolution because they were already plentiful as intracellular energy sources (9). A related hypothesis is that the initial efflux of nucleotides occurred as cells were injured or dying, and
evolution of early P2Y receptors allowed neighboring cells to become aware of cell injury or death. Thus, in addition to being among the most primitive signaling mechanisms, purinergic signaling may be among the oldest signaling mechanisms.

What advantage could be conferred by the evolution of single P2Y receptors with less selective pharmacology and widespread tissue distribution to the complex expression of multiple P2Y receptor subtypes in higher organisms to allow tissue-specific responses to release of particular nucleotides. The recent finding that some P2Y receptors may have distinct signal transduction mechanisms (18) suggests there may be even further fine tuning of responses through cross-talk between second messenger pathways. Further identification and characterization of P2Y receptors from organisms that evolved after the skate but before mammals and birds may help to address this important question.

TABLE II

| Nucleotide concentration | ATP   | 2-MeSATP | ADP   | UTP   | UDP   | ATP-s | ATP (Ca\(^{2+}\)-free) |
|--------------------------|-------|----------|-------|-------|-------|-------|------------------------|
| cRNA-injected oocytes    |       |          |       |       |       |       |                        |
| 100                      | 24/32 | 4/4      | 8/9   | 4/4   | 4/4   | 3/5   |                        |
| 10                       | 2/4   | 5/5      |       |       |       |       |                        |
| 1                        | 2/6   | 5/7      |       |       |       |       |                        |
| H\(_2\)O-injected oocytes| 100   | 0/3      | 0/3   | 0/3   | 0/3   |       |                        |

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