Molecular Cloning of a Novel P2 Purinoceptor from Human Erythroleukemia Cells*  


From the Departments of Physiology and **Pharmacology, the †Sol Sherry Thrombosis Research Center, and the °School of Medicine, Temple University Medical School, Philadelphia, Pennsylvania 19140 and the ¶Molecular Neurobiology Unit, Royal Free Hospital School of Medicine, London NW3 2PF, United Kingdom.

Screening of a human erythroleukemia cell cDNA library with radiolabeled chicken P2Y, cDNA at low stringency revealed a cDNA clone encoding a novel G protein-coupled receptor with homology to P2 purinoceptors. This receptor, designated P2Y7, has 352 amino acids and shares 23–30% amino acid identity with the P2Y1–P2Y6 purinoceptors. The P2Y7 cDNA was transiently expressed in COS-7 cells; binding studies thereon showed a very high affinity for ATP (37 mM), much less for UTP and ADP (~1,300 mM), and a novel rank order of affinities in the binding series studied of 8 nucleotides and suramin. The P2Y7 receptor sequence appears to denote a different subfamily from that of all the other known P2Y purinoceptors, with only a few of their characteristic sequence motifs shared. The P2Y7 receptor mRNA is abundantly present in the human heart and the skeletal muscle, moderately in the brain and liver, and not in the other tissues tested. The P2Y7 receptor mRNA was also abundantly present in the rat heart and cultured neonatal rat cardiomyocytes. The P2Y7 receptor is functionally coupled to phospholipase C in COS-7 cells transiently expressing this receptor. The P2Y7 gene was shown to be localized to human chromosome 14. We have thus cloned a unique member of the P2Y purinoceptor family which probably plays a role in the regulation of cardiac muscle contraction.

The widespread occurrence of metabotropic receptors for extracellular ATP has long been inferred from physiological and pharmacological evidence (1). A number of such G protein-coupled ATP receptors have been characterized and a consensus on their nomenclature has termed all of these P2Y purinoceptors (to be individually named P2Y1 to P2Y6), regardless of previous terminology such as P2U or P2T for subclasses thereof (2). The first such receptors to be characterized by DNA cloning and expression were the P2Y1 receptor (where UT is inactive) (3) and the P2Y2 receptor (ATP and UTP are equally active) (4). True species homologues (or orthologues) of P2Y7 have since been obtained, e.g. bovine (5) and human (5, 6), and of P2Y2, e.g. from human airway epithelium (7) or human erythrocytes (8). Further types identified by cloning have been the P2Y3 receptor (UDP > ADP > ATP) (9) and P2Y4 (UTP > ATP, and more strongly related to P2Y2) (10, 11). Further novel P2Y receptors have recently been identified from their cdnas from chicken activated T lymphocytes (12) and rat vascular smooth muscle cells (13) and designated P2Y5 and P2Y6 receptors. Previously we have demonstrated at least three P2 purinoceptors on the hematopoietic cell line, HEL cells, by intracellular calcium mobilization and by photoaffinity labeling (8). Here we report the molecular cloning and characterization of one of these, a novel P2 purinergic receptor designated P2Y7.

EXPERIMENTAL PROCEDURES

Materials—The HEL cell cDNA library was a gift from Dr. Jerry Ware, Salk Institute, La Jolla, CA. α-[32P]dCTP, α-[32P]dCTP, and α-[55S]dATP were from DuPont NEN; other materials (and procedures where not specified) were as described previously (6, 14, 15). A panel of mouse/human monochromosomal hybrid cell lines, each containing a single different human chromosome, was used as previously (6).

Cloning and Sequencing—The HEL cell cDNA library was screened as described earlier (6, 14) except that here chicken P2Y2 cDNA (labeled by random priming with α-[32P]dCTP) was the probe and the membranes were washed finally with 2× SSC containing 0.1% SDS at 50 °C (4 × 30 min). The DNA insert from the positive clone was subcloned into Bluescript KS vector by procedures described earlier (14). The complete nucleotide sequence of both strands was determined by dideoxy chain termination using T3 and T7 primers and synthetic oligonucleotides corresponding to the previously determined sequence. The nucleotide sequence analysis and dendrogram construction were performed using the GCG package.

Cell Culture and Transfection—The P2Y, cDNA was subcloned into the EcoRI site of the pcDNA3 expression vector (Invitrogen, San Diego, CA). The expression construct (pcDNA3-P2Y,) was transferred into COS-7 cells, and the transfected cells were harvested. Those procedures and also the binding assays with [35S]GTPγS (at ~22 °C) on membranes of transfected cells were as described elsewhere (15), except that transfection was with Lipofectamine (Life Technologies, Inc.), used as specified by the manufacturer, and not with DEAE-dextran. Nonspecific binding rose from 5% of the total binding up to 20% at saturation and was subtracted. Vector-transfected cells gave no specific binding (15).

Measurements of Inositol Phosphates and Cyclic AMP Contents—The abbreviations used are HEL, human erythrocytes; kb, kilobase(s); bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction, 2-MeSATP, 2-methylthio-ATP; βγ-meATP, βγ-methyl-ene ATP; ATPγS, adenosine 5′-O-(thiotriphosphate).
Cloning of a Novel P2Y Purinoceptor

Molecular Cloning of a Novel P2Y Purinoceptor cDNA—We have previously cloned the human P2Y1 (6) and P2Y2 (8) receptor cDNAs from a HEL cell cDNA library. In the present study, we screened the same library in Xgt11 with the radiolabeled chicken P2Y3 cDNA under low stringency hybridization conditions. Upon screening ~500,000 plaques, a single cDNA clone, designated HP212, was isolated and sequenced. The full-length cDNA clone is ~1.6 kb in length and contains an open reading frame of 1056 bp which is flanked by 304 bp of 5’-untranslated and 250-bp of 3’-untranslated sequence. The 5’-untranslated sequence is relatively GC rich and the 3’-untranslated sequence ends with a poly(A) tail.

RESULTS AND DISCUSSION

Molecular Cloning of a Novel P2Y Purinoceptor cDNA—We have previously cloned the human P2Y1 (6) and P2Y2 (8) receptor cDNAs from a HEL cell cDNA library. In the present study, we screened the same library in Xgt11 with the radiolabeled chicken P2Y3 cDNA under low stringency hybridization conditions. Upon screening ~500,000 plaques, a single cDNA clone, designated HP212, was isolated and sequenced. The full-length cDNA clone is ~1.6 kb in length and contains an open reading frame of 1056 bp which is flanked by 304 bp of 5’-untranslated and 250-bp of 3’-untranslated sequence. The 5’-untranslated sequence is relatively GC rich and the 3’-untranslated sequence ends with a poly(A) tail.

Analysis of the P2Y7 Receptor cDNA—The initiator methionine, encoded by the first in-frame ATG in the sequence, has a purine at the +3 position, which is the most conserved position in the Kozak (19) consensus sequence. The amino acid sequence derived from the nucleotide sequence of HP212 revealed that this cDNA codes for 352 amino acids (Fig. 1) with an estimated molecular weight of 37,682 daltons, without accounting for glycosylation. The hydrophobicity analysis of this cDNA indicates the seven transmembrane domains (Fig. 1, I–VII) of the G protein-coupled receptor superfamily. The receptor encoded by HP212 has two potential N-linked glycosylation sites at identical positions to those of the P2Y3 receptor sequence (9): one (residue 4) in the extracellular amino-terminal segment and the other (residue 166) in the second extracellular domain. The sequence also contains several serine/threonine phosphorylation residues in the carboxyl-terminal segment (14 out of 62 residues). The receptor has 27% amino acid identity to P2Y1 (6) and P2Y2 (7), 24% identity to P2Y3 (9), 28% identity to P2Y4 (10, 11), 23% identity to P2Y5 (12), and 30% identity to P2Y6 (13) purinoceptors, whereas among other cloned nucleotide receptors the minimum for the shared amino acid identity is ~34%. As the next such clone reported, we have designated the receptor encoded by HP212 as P2Y7. The P2Y7 receptor sequence appears to denote a different subfamily from that of the other known P2Y purinoceptors, with only a few of their characteristic sequence motifs shared: these include the conserved histidine in deduced transmembrane domain VI, but not the nearby arginine and a basic residue near the start of domain VII but not at a second therein (Fig. 1). However, adjacent basic residues occur just before domain VII. Construction of a dendrogram of all the known P2Y purinoceptors confirmed that the P2Y7 receptor forms a distinct branch (Fig. 2). Thus the P2Y7 receptor is a unique member of the P2Y purinoceptor family.

Pharmacological and Functional Characterization—The P2Y7 receptor was expressed transiently in COS-7 cells and the equilibrium binding was measured on membranes from the transfected cells of [35S]dATPαS, a radioligand with high affinity for some P2Y purinoceptor subtypes (12, 15) but very low affinity for the endogenous nucleotide binding sites on COS-7 cells. Saturable, specific binding sites for this radioligand were expressed, as illustrated in Fig. 3: analysis indicated a single class of high affinity binding sites, the Hill coefficient being 0.98 ± 0.08. The dissociation constant was 4.03 ± 1 nm. The expression was consistently efficient, with the maximal binding (Bmax) 12.3 ± 1.9 pmol of dATPαS bound/mg of membrane protein. (All values quoted are means ± S.E. for at least three transfections, with triplicate measurements made on each). This binding could be displaced (Table I) by a range of P2 purinoceptor-active nucleotides and also by suramin, an antagonist at micromolar concentrations at most P2Y receptors (2).
The affinity series found (ATP, 2-Cl-ATP, 5′-b-g-MeATP, 5′-MeSATP, b-g-MeATP, ADP, UTP) is quite distinct from any known for the P2Y1–P2Y6 receptors. The significant affinity for b-g-MeATP is unique for a P2Y receptor.

We have investigated whether the P2Y7 receptor is coupled to phospholipase C activation, as is common in this series. As seen in Fig. 4, in COS-7 cells transiently expressing the P2Y7 receptor 20 μM ATP stimulated the production of total inositol phosphates about 2-fold, while the stimulation in the cells transiently expressing the mouse P2Y2 receptor is 5.3-fold. In contrast, 20 μM ATP has no effect on the cells transfected with vector alone, as also found previously (15). These results indicate that the P2Y7 receptor is coupled to phospholipase C. For comparison, COS-7 cells transiently expressing the P2Y1 receptor have been shown to stimulate inositol 1,4,5-triphosphate formation about 2.7-fold with 1 μM ATP (15). The effect for P2Y7 is lower, which may indicate that the natural G protein partner is different and is low or absent, and inefficiently substituted by another, in COS-7 cells.

To examine potential coupling of the P2Y7 receptor to adenylyl cyclase through a stimulatory G protein, Gs, the effects of nucleotides on cellular cyclic AMP levels were examined in COS-7 cells transiently expressing the vector alone or the P2Y7 expression vector (Fig. 5). As shown in Fig. 5, neither P2Y7 receptor-transfected nor vector-transfected COS-7 cells caused stimulation of cAMP levels in response to 20 μM ATP. Under identical conditions, COS-7 cells transfected, as a positive control, with the prostaglandin IP receptor caused a 13.5-fold increase in intracellular cAMP levels in response to 100 nM iloprost.

Chromosomal Localization of P2Y7 Receptor Gene—PCR amplification of human genomic DNA with human P2Y7 receptor specific primers resulted in a PCR product of 821 bp as expected, while no amplification occurred with mouse or hamster DNA. When PCR was carried out with DNA from the panel of somatic cell hybrids as template, amplification of the expected size product was observed only in the DNA from the hybrid that contained human chromosome 14. Hybridization of a P2Y7 specific probe to the Southern blots of positive PCR products, including the control human DNA, resulted in a signal (Fig. 6). No signal was observed in the lanes with mouse or hamster DNA (Fig. 6) or with any of the hybrids that did not yield PCR products of the expected size (not shown). These results confirm that the PCR product represents the human gene and is not due to nonspecific amplification. The genes so far localized for human P2 purinoceptors are on different chromosomes: P2Y1, P2Y4, P2Y6, P2Y12, P2Y13, P2Y14, P2Y15, P2Y16, P2Y18, P2Y20, P2Y24, and P2Y26.
on chromosome 3 (6), P2Y2 on chromosome 11 (20), and P2Y4 on the X chromosome (10).

Northern Blot Analysis—The tissue distribution of P2Y7 was analyzed by Northern hybridization on a human multiple tissue Northern blot containing 20 µg of poly(A)1 RNA from eight human tissues. A radiolabeled cRNA of P2Y7 hybridized to an mRNA of 1.6 kb, with the highest levels in the human heart and skeletal muscle (Fig. 7A). There are much lower levels in the brain and liver, and low to negligible levels in the other tissues tested (Fig. 7A). The tissue distribution of P2Y7 is in contrast to other P2 purinoceptors. The 7.0-kb P2Y1 mRNA is present in the human placenta, skeletal muscle, and pancreas (6), while the 2.1-kb human P2Y2 mRNA is expressed abundantly in skeletal muscle and nasal epithelium (7). On the other hand, the P2Y4 mRNA is exclusively expressed in the human placenta (11). The unique tissue distribution of the P2Y7 receptor (Fig. 7A) among the P2 purinoceptor family could imply a physiological function in regulating cardiac muscle contraction, while P2Y1, P2Y2, and P2Y7 play a role in skeletal muscle contraction (as well as elsewhere).

Northern hybridization was also performed on total RNA from the rat heart and cultured neonatal rat cardiomyocytes and showed that the P2Y7 receptor mRNA is abundantly present in both these samples (Fig. 7B). The rat orthologue of the P2Y2 receptor mRNA is larger (~2.0 kb) than the human P2Y2 receptor mRNA. The P2Y7 receptor is at a lower abundance in HEL cells than in rat cardiomyocytes or heart, since it was below the level of detection in a Northern blot under the same conditions for all these samples. However, RT-PCR analysis of the HEL cell mRNA resulted in a PCR product of 821 bp, indicating that the P2Y7 receptor mRNA is expressed in HEL cells (Fig. 8).

We have found the P2Y7 receptor mRNA to be the most abundant in cardiac and skeletal muscle. For adult skeletal muscle, ATP has been found to facilitate acetylcholine stimulation via an unidentified second messenger pathway (21) and in rat diaphragm ATP increases inositol phosphate formation (22). For cardiac myocytes, while some ATP-induced cationic currents seen there may be due to P2X purinoceptors, the positive inotropic effects of ATP and its potent ability to increase calcium transients in stimulated rat heart and to increase the L-type calcium current in cardiac myocytes have been attributed to metabotropic (P2Y) purinoceptors (23–26). ATP was found to be active on ventricular myocytes with EC50 ~300 nM and 2-MeSATP and ATP were similarly highly potent (24). The cardiac P2 purinoceptors have been shown to couple to stimulation of inositol phosphate stimulation through phospholipase C (27) and have been suggested also to couple to a Gs type without stimulation of cAMP levels (28). Based upon these and similar reports, and the binding properties, functional characteristics, and cardiac mRNA abundance of the P2Y7 receptor, we suggest that this subtype, in addition to being one of the few P2Y receptors expressed in a hematopoietic cell lineage, is involved in cardiac muscle regulation.

In conclusion, we have cloned a novel P2 purinergic receptor with unique pharmacological profile and tissue distribution, and localized the P2Y7 gene to human chromosome 14. This
receptor is most probably the cardiac P2Y purinoceptor involved in the regulation of cardiac muscle contraction through modulation of L-type calcium currents.

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