Cloning of a Human Purinergic P2Y Receptor Coupled to Phospholipase C and Adenylyl Cyclase*

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Clones encoding a new human P2Y receptor, provisionally called P2Y₁₁, have been isolated from human placenta complementary DNA and genomic DNA libraries. The 1113-base pair open reading frame is inter- rupted by one intron. The P2Y₁₁ receptor is charac- terized by considerably larger second and third extracellular loops than the subtypes described so far. The deduced amino acid sequence exhibits 33% amino acid identity with the P2Y₁ receptor, its closest homolog. Northern blot analysis detected human P2Y₁₁ receptor messenger RNA in spleen and HL-60 cells. The level of P2Y₁₁ transcripts was strongly increased in these cells after granulocyte differentiation induced by retinoic acid or dimethyl sulfoxide. The new receptor was stably expressed in 1321N1 astrocytoma and CHO-K₁ cells, where it couples to the stimulation of both the phosphoinositide and adenyl cyclase pathways, a unique feature among the P2Y family. The rank order of agonist potency was: ATP > 2-methylthio-ATP >> ADP, whereas UTP and UDP were inactive, indicating that it behaves as a selective purinoceptor.

An impressive number of P2 receptor subtypes has been cloned since 1993. A molecular nomenclature has been established in which G protein-coupled P₂ receptors are named P₂Y, whereas P₂ receptors having an intrinsic ion channel activity are named P₂X (1, 2). The P₂Y family encompasses selective purinoceptors (the P₂Y₁ receptor (3, 4) activated preferentially by ADP and ATP), nucleotides receptors responsive to both adenine and uracil nucleotides (the P₂Y₂ receptor (5, 6) activated equipotently by ATP and UTP, and the P₂Y₆ receptor (7) activated equally by all triphosphate nucleotides), and pyrim- idinoceptors (the P₂Y₅ (8) and P₂Y₇ (9–11) receptors activated preferentially by UDP, and the P₂Y₁ receptor (11–13) activated preferentially by UTP). All these P₂Y subtypes are coupled exclusively to the phosphoinositide pathway. The inclusion of other receptors (P₂Y₅ (14) and P₂Y₁₅ (15)) within the P₂Y family remains controversial (16–18). Here we report the cloning of a new human gene encoding a G protein-coupled receptor that behaves as a selective purinoceptor and is coupled to the stimulation of both adenylyl cyclase and phospholipase C.

**EXPERIMENTAL PROCEDURES**

Materials—Trypsin was from Flow Laboratories (Bioggio, Switzerland). Culture media, G418, fetal calf serum (FCS), restriction enzymes, and Taq polymerase were purchased from Life Technologies, Inc. The radioactive products myo-[²-H]-inositol (17.7 Ci/mmol) and [α³²P]ATP (800 Ci/mmol) were from Amersham (Ghent, Belgium). Dextran, G15x8H (formate form) was from Bio-Rad, ADP, AMP, adenosine, UTP, UDP, AP₆, AP₄, all-trans retinoic acid, and 12-O- tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma. 2-Methylthio-ATP (2MeSATP), 2-methylthio-ADP (2MeSADP) and 8-(p-sulfophenyl) theophylline were from Research Biochemicals Interna- tional (Natick, MA). Forskolin was purchased from Calbiochem. (Bierges, Belgium). Indomethacin and Me₂SO were from Merck. Rolip- ram was a gift from the Laboratoires Jacques Logeais (Trappes, France). The HL-60 human cell line was obtained from the American Type Culture Collection (Rockville, MD). The human genomic DNA library was from Stratagene (La Jolla, CA). The human placenta cDNA library was kindly given by Prof. P. Chambon (Strasbourg, France). pEF5n3 is an expression vector developed by Euroscreen (Brussels, Belgium). Multiple human tissue Northern blot (MTN) were from Clontech (Palo Alto, CA).

Cloning and Sequencing—A human placenta cDNA library was screened at moderate stringency with an [α-³²P]ATP-labeled P₂Y receptor probe corresponding to a partial sequence covering the third to the seventh transmembrane segments. Three overlapping clones encoding a new G protein-coupled receptor were isolated but did not contain the 3' end of the coding region. A human genomic DNA library was then screened with this partial sequence to obtain the complete sequence of this new receptor. The hybridization conditions for screening the two libraries were 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 40% formamide at 42 °C for 14 h, and the final washing conditions were 0.5 x SSC, 0.1% SDS at 60 °C. Four genomic clones were purified and shown to contain the 3' end of the open reading frame missing in the cDNA clones. The sequence was obtained on both strands after subcloning of overlapping restriction fragments in M13mp18 and M13mp19 using the Sanger dyeoxy nucleotide chain termination method.

Northern Blot Analysis—Two blots of human organs (MTN I and II; 2 μg of poly(A)) RNA/lane) and a blot containing total RNA from differentiated and undifferentiated HL-60 cells (10 μg of total RNA/lane) were hybridized with a probe corresponding to the new receptor to characterize its tissue distribution. The HL-60 cells were maintained in RPMI 1640 supplemented with 10% FCS, 5 mM L-gluta- mine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C with 5% CO₂. The HL-60 cells were incubated for 6 days with or without 1 μM retinoic acid or 1.25% Me₂SO or for 8 h with 25 nM TPA. The RNA from the differentiated or undifferentiated HL-60 cells was prepared with the RNeasy kit (Qiagen). The blots were prehybridized 8 h at 42 °C in 1 The abbreviations used are: FCS, fetal calf serum; TPA, 12-O- tetradecanoylphorbol-13-acetate; 2MeSATP, 2-methylthio-ATP; 2Me- SADP, 2-methylthio-ADP; MTN, multiple human tissue Northern blot; DMEM, Dulbecco's modified Eagle's medium; IP₃, inositol triphos- phate; kb, kilobase(s).
a 50% formamide, 2% SDS solution and hybridized for 18 h in the same solution supplemented with the α-32P-labeled probe. The final washing conditions were 0.1 × SSC and 0.1% SDS at 55 °C. The blots were exposed for 12 days and visualized as an autoradiograph or using the PhosphorImager SI (Molecular Dynamics).

Cell Culture and Transfection—The complete sequence of the new receptor was subcloned between the HindIII and NheI sites of the bicistronic pEFIN3 expression vector. 1321N1 and CHO-K1 cells were transfected with the recombinant pEFIN3 plasmid or with the plasmid alone using the calcium phosphate precipitation method as described (19). The transfected cells were selected with 400 μg/ml G418 in complete medium (10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B in Dulbecco’s modified Eagle’s medium (DMEM)) 2 days after transfection and maintained in the same medium (10).

Inositol Phosphate Measurement—1321N1 cells were labeled for 24 h with 10 μCi/ml [3H]inositol in inositol-free DMEM containing 5% FCS, antibiotics, amphotericin, sodium pyruvate, and 400 μg/ml G418. Cells were washed twice with Krebs-Ringer Heps (KRH) buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.45 mM CaCl2, 1.25 mM KH2PO4, 25 mM Heps, pH 7.4, and 8 mM glucose) and incubated in the same medium for 30 min. The cells were then challenged by various nucleotides for 30 s. The incubation was stopped by the addition of an ice-cold 5% perchloric acid solution. Inositol phosphates were extracted and separated on Dowex columns as described previously (20).

Cyclic AMP Measurements—Stably transfected CHO-K1 or 1321N1 cell lines were spread on Petri dishes (150,000 cells/dish) and cultured in Ham’s F-12 or DMEM medium containing 10% FCS, antibiotics, amphotericin, sodium pyruvate, and 400 μg/ml G418. Cells were preincubated for 30 min in KRH buffer with Rolipram (25 μM) and incubated for different times in the presence of the agonists (15 min in most experiments). The incubation was stopped by the addition of 1 ml of 0.1 M HCl. The incubation medium was dried up, resuspended in water, and diluted as required. Cyclic AMP was quantified by radioimmunoassay after acetylation as described previously (21).

RESULTS

Cloning and Sequencing—A human cDNA placenta library was screened at moderate stringency with a human P2Y4 probe. Nine clones that hybridized weakly with the P2Y4 probe were obtained, purified, and analyzed. Six of them corresponded to the sequence of the P2Y4 receptor (10), whereas the three overlapping clones corresponded to a partial sequence encoding a new G protein-coupled receptor, displaying about 33 and 28% amino acid identity with the human P2Y1 and P2Y2 receptors, respectively (Fig. 2). The pool of G418-resistant clones was tested for its functional response to several nucleotides (13, 14). The putative membrane-spanning domains are underlined and numbered I–VII. The putative phosphorylation sites by protein kinase C and by calmodulin-dependent protein kinases are indicated by a black circle (●) and a black square (■), respectively. The potential N-glycosylation site is indicated by a black square (■). The weakest signal was observed in small intestine (MTN II). All the lanes in MTN I (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) were negative (data not shown). We also detected specific 2-kb transcripts in HL-60 cells. The signal was very weak in the undifferentiated HL-60 cells but increased when the cells had been treated with retinoic acid or Me3SO. No increase was observed when the HL-60 cells were stimulated with TPA. A weak nonspecific hybridization with 18 S rRNA was observed. These data were confirmed with a non-overlapping probe corresponding to the first 300 base pairs of the coding region, presenting limited homologies with the other P2Y subtypes.

Functional Expression of the New Receptor in 1321N1 Astrocytoma Cells—The complete sequence of the new receptor was introduced in the pEFIN3 expression vector to transfect the 1321N1 astrocytoma cell line used previously to characterize several P2Y subtypes (6, 10, 12). The pool of G418-resistant clones was tested for its functional response to several nucleotides. ATP (100 μM) induced a strong inositol trisphosphate (IP3) accumulation in cells transfected with the recombinant plasmid, whereas ADP, AMP, adenosine, UTP, UDP, AP3A, and AP6A were inactive at the same concentration (data not shown). All nucleotides were totally inactive on the cells trans-
fected with the vector alone. We then tested ATP, 2MeSATP, ADP, and 2MeSADP in a large range of concentrations. As shown in Fig. 4A, ATP was the most potent agonist (EC\textsubscript{50} \text{ATP} = 38 \pm 7 \text{ M} \mu \text{M}; EC\textsubscript{50} 2MeSATP = 118 \pm 15 \text{ M} \mu \text{M}; means \pm range of two independent experiments). The effects of ADP and 2MeSADP were minimal. Pertussis toxin (50 ng/ml; 24 h pre-treatment) had no effect on the ATP response (data not shown), whereas a lower concentration of pertussis toxin was previously shown to abolish the response to UTP in P2Y4 transfected 1321N1 astrocytoma cells (22). A response to ATP (10 \text{ M} \mu \text{M}) was also obtained following [Ca\textsuperscript{2+}]\textsubscript{i} measurements performed on the 1321N1 transfected cells, whereas ATP was inactive at this concentration (data not shown).

**Functional Expression of the New Receptor in CHO-K1 Cells**—The 1321N1 cells transfected with the new receptor displayed a strong cAMP increase in response to ATP. A much lower but significant endogeneous response due to the degradation of adenine nucleotides into adenosine was also obtained in the 1321N1 cells transfected with the vector alone (data not shown). The CHO-K1 cells express an endogeneous P2Y\textsubscript{2} receptor coupled to the phosphoinositide pathway (23) but do not possess adenosine receptors coupled to adenylyl cyclase. We therefore used CHO-K1 cells to characterize the coupling of the new receptor to the cAMP pathway. A pool of G418-resistant CHO-K1 clones was first tested for its response to several nucleotides at a concentration of 100 \text{ M} \mu \text{M}. ATP was able to induce a strong increase in the cAMP content, whereas it was inactive on cells transfected with the vector alone (data not shown). ADP, AMP, adenosine, UTP, and UDP were completely inactive. Concentration-action curves were established for ATP, 2MeSATP, ADP, and 2MeSADP (Fig. 4B). The rank order of potency was the same as in the inositol phosphate study on 1321N1 cells. The curves were obtained after 15 min of stimulation by the agonists; however, a significant cAMP response to ATP was already obtained after 2 min of stimulation (data not shown). The response to ATP (30 \text{ M} \mu \text{M}) was inhibited neither by indomethacin (10 \text{ M} \mu \text{M}, present from 30 min before the stimulation and readded in the stimulation medium) nor by 8-(p-sulfophenyl) theophylline (100 \text{ M} \mu \text{M}) (data not shown).

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**Fig. 2.** Dendrogram representing the structural relatedness of the P2Y\textsubscript{11} receptor with the other P2Y subtypes. The plot was constructed using the multiple sequence alignment program Pileup of the GCG package. The P2Y\textsubscript{2}-like published sequence (18) is identical to the P2Y\textsubscript{5} sequence submitted to the GenBank/EMBL Data Bank.

**Fig. 3.** Northern blot analysis of P2Y\textsubscript{11} messenger expression. Each lane of the MTN blot contains 2 \mu g of poly(A)\textsuperscript{+} RNA from several human tissues. Each lane of the HL-60 blot contains 10 \mu g of total RNA from differentiated or undifferentiated HL-60 cells. Hybridization with the probe was performed as described under "Experimental Procedures." The pictures of the MTNII blot and the HL-60 blot were obtained from an autoradiography and from a PhosphorImager SI (Molecular Dynamics), respectively. The 2-kb P2Y\textsubscript{11} transcripts are indicated by black arrowheads.

**Fig. 4.** Concentration-action curves of several nucleotides on IP\textsubscript{3} and cAMP accumulation in cells transfected with the P2Y\textsubscript{11} receptor. 1321N1 and CHO-K1 transfected cells were assayed for the accumulation of IP\textsubscript{3} (A) or cAMP (B), respectively, in response to various concentrations of the following nucleotides: ATP, 2MeSATP, ADP, and 2MeSADP. Incubation times were 30 s for IP\textsubscript{3} measurements and 15 min for cAMP assays. The data represent the means \pm S.D. of triplicate experimental points and are representative of two independent experiments.
We describe here the cloning of a new human receptor of the P2Y family. This receptor presents some structural peculiarities that differentiate it from some other P2Y subtypes. Concerning its gene structure, the coding sequence is interrupted by an intron. Comparison between the cDNA and the genomic DNA sequences has clearly demonstrated the absence of intron in the coding regions of the human P2Y11 receptor (24, 25), the rat P2Y2 receptor (26), and the rat P2Y7 receptor (11). In terms of protein structure, the second and third extracellular loops are significantly longer than those of the other P2Y receptors. The homology with the other subtypes is relatively weak (about 30%). The closest G-coupled receptor is the human P2Y7 receptor (33%), which is also a receptor responsive to adenine nucleotides (3, 4). Mutagenesis experiments with the P2Y2 receptor have identified three positively charged amino acids in the sixth and seventh transmembrane domains (His262, Arg265, and Arg295), which play a crucial role in nucleotide binding (presumably by neutralizing the negative charge of the phosphate groups) (27). These three residues are conserved in this new receptor.

So far, eight P2Y receptor subtypes are described in the literature (P2Y1–P2Y8). In addition, two sequences related to the P2Y7 receptor and named P2Y9 and P2Y10 have been recently submitted to the GenBank/EMBL™ Data Bank. The P2Y9 sequence is identical to that recently published under the name “P2Y7-like” (18). Therefore, the new receptor described in this paper might be called P2Y11. However, it is already clear that the nomenclature needs a revision. It was recently demonstrated that the P2Y7 receptor is actually a receptor for leukotriene B4 (16), and there is no functional evidence that the P2Y7 and related receptors (P2Y7-like or P2Y9 and P2Y10) are nucleotide receptors (17, 18).

Among the 16 human organs tested by Northern blotting, P2Y11 transcripts of 2 kb were only detectable in spleen and with lower intensity in small intestine. This distribution is reminiscent of that of the human P2Y4 1.7-kb messenger. We have also observed expression of the P2Y11 receptor in the HL-60 cell line; this expression was strongly increased following treatment by MeSO4 or retinoic acid, two agents known to induce the differentiation of these cells into granulocytes (28). On the contrary, TPA, which is known to induce the monocytic differentiation of the HL-60 cells (29), did not stimulate the expression of the P2Y7 receptor. The confirmation of these data with a second probe of the P2Y11 cDNA, which shares little similarity with other P2Y sequences, excludes possible cross-hybridization with another P2Y receptor transcript. In view of the Northern blot results, it is tempting to speculate that the P2Y11 receptor is involved in the recently described accumulation of cAMP in ATP-stimulated HL-60 cells (30). A possible role of this new receptor in the immune system has to be investigated.

Among the P2Y receptors, the P2Y1 subtype has the unique property of activating both the phosphoinositide and the cAMP pathways. Other cloned P2Y receptors are coupled to phospholipase C exclusively (except for the P2Y2 receptor for which the transduction mechanisms remain unknown). The rank order of potency of agonists was the same for the two pathways. ATP was clearly much more potent than ADP. This difference may be even underestimated as a result of low level ATP contamination in ADP preparation or conversion of ADP into ATP during assays (4, 11). On the other hand, 2MeSATP had the same maximal effect as ATP but presented a lower potency, whereas 2MeSADP, a potent activator of the P2Y1 and P2X7 subtypes (4), was almost inactive. The EC50 values were comparable with those obtained in the study concerning the effects of extracellular nucleotides on the cAMP accumulation in the HL-60 cells (30).

Stimulatory effects of adenine nucleotides on the cAMP pathway have been described in different cell types (31, 32). In most cases, these stimulatory effect of nucleotides was inhibited by xanthines. These data suffer from the fact that it is difficult to exclude that the effect of adenine nucleotides is mediated by their degradation into adenosine due to the ubiquitous presence of ectonucleotidases expressed at the cell surface. The cAMP study has been performed with CHO-K1 cells to avoid the endogeneous cAMP response to adenosine in the astrocytoma cell line. Neither in untransfected CHO-K1 cells nor in P2Y7-transfected CHO-K1 cells did adenosine increase cAMP accumulation. Furthermore the cAMP response to ATP was insensitive to xanthine inhibition. It was also insensitive to indomethacin, indicating that is not mediated by the release of prostaglandins. It is unlikely that the cAMP response would be an indirect consequence of the calcium response because the use of ATP, which activates the phosphoinositide pathway by the activation of P2Y2 endogeneous receptors, or the use of calcium ionophores in the CHO-K1 cells failed to stimulate cAMP accumulation (33). Therefore our data constitute the first strong evidence that a P2 receptor can be coupled to the stimulation of adenylyl cyclase. The physiological relevance of this effect as well as the physiological role of the P2Y11 receptor remain to be determined.

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