The cloning of several receptors for ATP has been reported since 1993. In keeping with the latest nomenclature proposal, these P2 purinergic receptors can be subdivided into two classes: G protein-coupled receptors, or P2Y receptors, and receptors with intrinsic ion channel activity or P2X receptors (2). Two distinct rat P2X receptors have been cloned, respectively, from the vas deferens (3) and pheochromocytoma PC12 cells (4); they have a characteristic topology, with two hydrophobic putatively membrane-spanning segments and an ion pore motif reminiscent of potassium channels. In the P2Y family, the sequences of two subtypes, both coupled to phospholipase C, have been published: chick (5), turkey (6), bovine (7), and mouse and rat (8) P2Y2 receptors (formerly called P2U); murine (9, 10), rat (11), and human (12) P2Y2 receptors (previously named P2U) on the other hand. In addition, a P2Y3 receptor, with a preference for ADP over ATP, has been cloned from chick brain, but its sequence is not yet published (13). Furthermore, the 6H1 orphan receptor, cloned from activated chicken T lymphocytes, exhibits a significant degree of homology to the P2Y1 and P2Y2 receptors, suggesting that it also belongs to the P2Y family, although its responsiveness to nucleotides has not yet been demonstrated (14). In this paper, we describe the cloning of a new member of the P2Y family, tentatively called P2Y16, which exhibits a preference for uridine over adenine nucleotides and therefore constitutes the first example of a pyrimidinergic receptor.

EXPERIMENTAL PROCEDURES

Materials—Trypsin was from Flow Laboratories (Bioggio, Switzerland), and the culture media, reagents, G418, fetal calf serum (FCS), restriction enzymes and Taq polymerase were purchased from Life Technologies, Inc. The radioactive products myo-[a-32P]inositol (17.7 Ci/mmol) and [a-32P]ATP (800 Ci/mmol) were from Amersham (Ghent, Belgium). Dowex AG 1-X8 (formate form) was from Bio-Rad Laboratories. UTP, UDP, ATP, ADP, carbachol, LiCl, and apyrase grade VII were obtained from Sigma. 2-Methylthio-ATP was from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen.

Cloning and Sequencing—Degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments between the murine P2Y2 and the chick P2Y1 receptor sequences. These primers were used to amplify novel receptor gene fragments by low stringency PCR starting from human genomic DNA. The amplification conditions were as follows: 93 °C, 1 min, 50 °C, 2 min, 72 °C, 3 min, 35 cycles. The PCR products with sizes compatible with P2 receptor gene fragments were subcloned in M13mp18 and M13mp19 and sequenced by the Sanger dideoxy nucleotide chain termination method. One of the resulting clones sharing similarities with P2 receptors was labeled by random priming and used to screen a human genomic DNA library constructed in the λ Charon 4a vector. The hybridization was in 6 × SSC (1 × SSC: 0.15 × NaCl, 0.015 × sodium citrate) and 40% formamide at 42 °C for 14 h, and the final wash conditions were 0.1 × SSC, 0.1% SDS at 65 °C. A preparation of λ phages (15) was made for several purified clones which hybridized strongly with the probe. A restriction map and a Southern blotting analysis allowed to isolate a 1.4-kyb Nhel-EcoRV fragment that was subcloned into the pluBluescript SK+ vector (Stratagene). The complete sequence of the new receptor coding sequence was obtained on both strands after subcloning of overlapping fragments in M13mp18 and M13mp19.

Cell Culture and Transfection—The P2Y16 receptor coding sequence was subcloned between the HindII and the EcoRV sites of the pcDNA3 expression vector for transfection into 1321N1 human astrocytoma...
cells, a cell line which does not respond to nucleotides and which has already been used for the expression of purinergic receptors (6, 12). Cells were transfected with the recombinant pcDNA3 plasmid using the calcium phosphate precipitation method as described (16). 1321N1 cells were incubated for 6 h at 37 °C in the presence of pcDNA3 vector alone or vector containing the P2Y4 receptor coding sequence, then washed, and incubated in culture 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). The selection with G418 (400 μg/ml) was started 2 days after transfection. From the pool of transfected 1321N1 cells, individual clones were isolated by limiting dilution with the aim of selecting clones with high IP stimulation factors in response to nucleotides. The different clones were maintained in a medium containing 400 μg/ml G418.

Inositol Phosphates (IP) Measurement—1321N1 cells were labeled for 24 h with 10 μCi/ml [3H]inositol in isositol-free Dulbecco’s modified Eagle’s medium containing 5% FCS, antibiotics, amphotericin, and 2 units/ml aprotase as described (6). Cells were washed twice with Krebs-Ringer Modified Hepes (KRMH) 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 Hepes (pH 7.4), and 8 mM glucose) and incubated in the same medium supplemented with 10 mM LiCl for 5 min before the addition of the agonists. The incubation was stopped after 20 min by the addition of an ice-cold 3% perchloric acid solution. IP were extracted and separated on Dowex columns as described previously (17).

RESULTS

Cloning and Sequencing—In order to isolate new subtypes of P2 receptors, sets of degenerate oligonucleotides primers were synthetized on the basis of the best conserved segments in the published sequences of the chick brain P2Y1 (5) and murine neuroblastoma P2Y2 (9) receptors. These primers were used in low-stringency PCR on human genomic DNA as described (18).

Some combinations generated discrete bands with a size compatible with that expected for P2 receptors. For example, the primer [5′-CAGATCTAGATATGTTGTC(G)/(A)GGTGCTCATCTTGC-3′] corresponding to the second transmembrane region and the primer [5′-CTTAACTCGTG(A/G)TC(TCA)GCTT-3′] corresponding to the seventh transmembrane region amplified a 712-bp fragment. The partial sequences obtained after sequencing were translated into peptic sequences and compared to a local databank which contains G protein-coupled receptor sequences. Most of the clones resulting from these PCR products encoded a part of a new receptor which displayed 58% identity with the murine P2Y2 receptor and 42% identity with the chick P2Y1 receptor partial sequences. In addition, some clones encoded a peptic sequence presenting 87% identity with the chick P2Y1 receptor and are therefore believed to represent fragments of the human P2Y1 gene (data not shown).

The partial sequence of the new receptor was used as a probe to screen a human genomic DNA library. Several clones that strongly hybridized with the probe at high stringency conditions were obtained and purified. The inserts of the clones varied from 12 to 17 kb, and restriction analysis revealed that all clones belonged to a single locus. The full sequence of a 1.4-kb Nhel-EcoRV fragment was obtained, and an intronless open reading frame of 1095 bp was identified (Fig. 1). The predicted molecular mass of the encoded protein is 36.5 kDa. This molecular mass is unlikely to be modified in vivo, since no N-glycosylation consensus sequences are found in the putative exofacial regions. In contrast with the human P2Y2 receptor, there is no RGD motif, an integrin binding consensus sequence, in the putative first extracellular loop. Some potential sites of phosphorylation by protein kinase C or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyl-terminal part of the receptor (Fig. 1). The four positively charged amino acids which have been reported to play a role in the P2Y1 receptor activation by ATP and UTP (1) are conserved in the P2Y4 sequence: His262, Arg265, Lys289, and Arg292 (Fig. 1). The P2Y4 amino acid sequence was compared to the chick P2Y1 and the murine P2Y2 amino acid sequences and to their closest neighbors in the G protein-coupled receptor family (Fig. 2). It is clear that, from a structural point of view, the newly cloned receptor is more closely related to the human P2Y2 receptor (51% of identity between the complete sequences) than to the chick P2Y1 receptor (35%).

Tissue Distribution of the P2Y4 Receptor—The tissue distribution of P2Y4 transcripts was investigated by Northern blotting. A number of rat tissues (heart, brain, liver, testis, and kidney) were tested using a probe at low stringency, but no hybridization signal could be obtained. No P2Y4 transcript could be detected in the following human cell lines: K562 leukemia cells (Fig. 3) and HL-60 leukemia cells and SH-SY5Y human neuroblastoma cells (data not shown). On the contrary, a strong signal, corresponding to a 1.8-kb mRNA, was found in human placenta (Fig. 3).

Functional Expression of the New P2Y4 Receptor—After transfection of the pcDNA3-P2Y4 construction in 1321N1 cells, the pool of G418-resistant clones was tested for

FIG. 1. Nucleotide and deduced amino acid sequence of the human P2Y4 receptor. The putative membrane-spanning domains are underlined and numbered I to VII. The consensus sequence contained in both the P2Y4 and the three amino acids (AHN) corresponding to the RGD sequence in the first extracellular loop of the P2Y2 receptor are represented in bold. The putative phosphorylation sites by protein kinase C or by calmodulin-dependent protein kinases and protein kinase C are indicated respectively by black squares (■) and by open circles (○).
their functional response (IP₃ accumulation) to ATP and UTP. Both nucleotides were found to be agonists of the P2Y₄ receptor, but the response to UTP was more robust; as previously reported (6, 12), none of the tested nucleotides (UTP, UDP, ATP, 2-methylthio-ATP, and ADP) produced a detectable effect in cells transfected with the pcDNA3 vector, whereas carbachol elicited strong responses (data not shown). About 20 transfected donors were then isolated and tested for their response to UTP. The clone present in the highest IP₃ stimulation factor in response to UTP was selected and used in all subsequent experiments (13 experiments in all). UTP and UDP produced the same maximal effect, with a similar potency (EC₅₀ around 2 μM) (Fig. 4). ATP had a lower potency (EC₅₀ around 20 μM), and its maximal effect was only 35% of that of UTP and UDP (mean of four independent experiments). The effect of ADP was barely detectable (Fig. 4), and 2-methylthio-ATP was completely inactive (data not shown).

DISCUSSION

We describe here the cloning of a human gene encoding a novel member of the P₂ purinergic receptor family. This receptor was provisionally named P2Y₄; this assignment is tentative since we are aware of other recently cloned subtypes that may qualify for number 4. No N-glycosylation consensus sequence can be detected in the putative extracellular regions. This situation is rare in G protein-coupled receptors but not unique: it has also been reported for the rat α₁₃-adrenergic receptor (19), a human receptor for the LD78 chemokine (20) and the rat RBS11 orphan receptor (21). Despite its structural relatedness to the human P2Y₂ receptor, the newly cloned receptor exhibits one conspicuous difference in the putative first extracellular loop: the RGD motif, an integrin binding consensus sequence, present in the P2Y₂ receptor is not conserved, although clusters of four amino acids are perfectly conserved on both sides. Site-directed mutagenesis studies have demonstrated the crucial influence of charged amino acids in transmembrane helices 6 and 7 of the P2Y₂ receptor on agonist potency and specificity (1). His²⁶₂, Arg²⁶⁵, and Arg²⁹² seem to be directly involved in the binding of the negatively charged phosphate groups, whereas a mutation of Lys²⁸⁹ into Arg decreases the affinity for ATP/UTP and increases that for ADP/UDP. These four residues are conserved in the P2Y₄ subtype, whereas Arg²⁶₅ and Lys²⁸⁹ are replaced respectively by Lys and Gin in the P2Y₁ receptor (5). The P2Y₄ receptor sequence presents also a series of 10 amino acids (LFLTCISVHR) which is a consensus sequence between all the P2Y receptors cloned until now. Potential sites of phosphorylation by protein kinase C or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyl-terminal part of the receptor. These regions are also particularly rich in serine and threonine residues that could be targets for the family of G protein-coupled receptor kinases. A strong signal corresponding to a 1.8-kb mRNA was detected in human placenta. No other human organ has been tested so far. Various rat tissues tested at low stringency and several human cell lines were negative. This suggests a rather restricted expression of the P2Y₄ receptor.

In the late eighties, it became apparent that extracellular uridine nucleotides exert effects on many tissues and cells. It was then proposed that these actions are mediated by pyrimidinoceptors distinct from the purinoceptors involved in the response to adenosine nucleotides (22). The existence of nucleotide receptors common to ATP and UTP constituted an alternative possibility, in favor of which experimental evidence started to accumulate, for instance the lack of additivity and cross-desensitization of the responses to the two nucleotides (23, 24). The final demonstration of this concept was provided by the cloning and functional expression of the P₂₅/P₂Y₂ receptor at which ATP and UTP are equally active and equipotent (9, 10, 12). However, the pyrimidinoceptor hypothesis re-emerged with the observation that UTP and UDP stimulate inositol phosphates formation in C6–2B rat glioma cells, whereas ATP and ADP are totally inactive (25). However, the C6 glioma cells, from which the C6–2B subclone was isolated, express a typical P₂₅ response: a mutation of that receptor, with selective loss of the adenine nucleotides responsiveness,
might thus explain the peculiar behavior of C6–2B cells. Our results conclusively demonstrate the existence of a human gene encoding a receptor with a preference for pyrimidine over purine nucleotides. Indeed, at the P2Y₄ receptor, UTP and UDP were full agonists, whereas ATP behaved as a partial agonist with a lower affinity than UTP and ADP was barely active. However, on the basis of its structure, this receptor clearly belongs to the P2Y family, which thus encompasses selective purinoceptors (P2Y₁), nucleotide receptors responsive to both adenine and uridine nucleotides (P2Y₂), and pyrimidinoceptors (P2Y₄).

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