



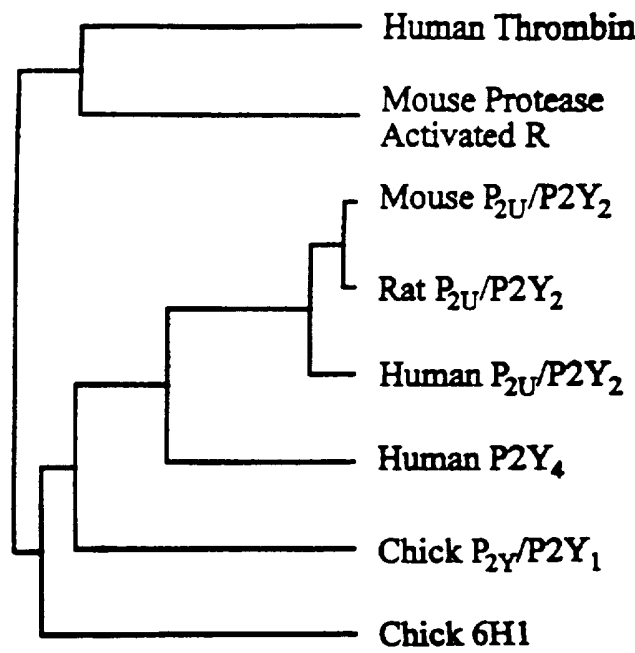
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : C12N 15/12, C07K 14/705, C12N 15/85, 15/86, 5/10, C12Q 1/68, C07K 16/28, A01K 67/027, G01N 33/53</p>	<p>A1</p>	<p>(11) International Publication Number: <b>WO 97/19170</b> (43) International Publication Date: 29 May 1997 (29.05.97)</p>
<p>(21) International Application Number: PCT/BE96/00123 (22) International Filing Date: 21 November 1996 (21.11.96)  (30) Priority Data: 95870124.5 21 November 1995 (21.11.95) EP (34) Countries for which the regional or international application was filed: AT et al.  (71) Applicant (for all designated States except US): EURO- SCREEN S.A. [BE/BE]; Avenue des Bécassines 7, B-1160 Bruxelles (BE).  (72) Inventors; and (75) Inventors/Applicants (for US only): COMMUNI, Didier [BE/BE]; Groendallaan 19, B-1800 Vilvoorde (BE). PIROTON, Sabine [BE/BE]; Avenue Marius-Renard 27a, B-1070 Bruxelles (BE). PARMENTIER, Marc [BE/BE]; Chaussée d'Uccle 304, B-1604 Linkebeek (BE). BOEY- NAEMS, Jean-Marie [BE/BE]; Avenue Peter-Benoit 5, B-1780 Wemmel (BE).  (74) Agents: VAN MALDEREN, Michel et al.; Office Van Malderen, Place Reine-Fabiola 6/1, B-1083 Bruxelles (BE).</p>	<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR

(57) Abstract

The present invention concerns a new receptor having a preference for pyrimidine nucleotides, preferably UTP, over purine nucleotides, and which has an amino acid sequence having more than 60 % homology with the amino acid sequence shown in Figure 1.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<b>AM</b>	Armenia	<b>GB</b>	United Kingdom	<b>MW</b>	Malawi
<b>AT</b>	Austria	<b>GE</b>	Georgia	<b>MX</b>	Mexico
<b>AU</b>	Australia	<b>GN</b>	Guinea	<b>NE</b>	Niger
<b>BB</b>	Barbados	<b>GR</b>	Greece	<b>NL</b>	Netherlands
<b>BE</b>	Belgium	<b>HU</b>	Hungary	<b>NO</b>	Norway
<b>BF</b>	Burkina Faso	<b>IE</b>	Ireland	<b>NZ</b>	New Zealand
<b>BG</b>	Bulgaria	<b>IT</b>	Italy	<b>PL</b>	Poland
<b>BJ</b>	Benin	<b>JP</b>	Japan	<b>PT</b>	Portugal
<b>BR</b>	Brazil	<b>KE</b>	Kenya	<b>RO</b>	Romania
<b>BY</b>	Belarus	<b>KG</b>	Kyrgystan	<b>RU</b>	Russian Federation
<b>CA</b>	Canada	<b>KP</b>	Democratic People's Republic of Korea	<b>SD</b>	Sudan
<b>CF</b>	Central African Republic	<b>KR</b>	Republic of Korea	<b>SE</b>	Sweden
<b>CG</b>	Congo	<b>KZ</b>	Kazakhstan	<b>SG</b>	Singapore
<b>CH</b>	Switzerland	<b>LI</b>	Liechtenstein	<b>SI</b>	Slovenia
<b>CI</b>	Côte d'Ivoire	<b>LK</b>	Sri Lanka	<b>SK</b>	Slovakia
<b>CM</b>	Cameroon	<b>LR</b>	Liberia	<b>SN</b>	Senegal
<b>CN</b>	China	<b>LT</b>	Lithuania	<b>SZ</b>	Swaziland
<b>CS</b>	Czechoslovakia	<b>LU</b>	Luxembourg	<b>TD</b>	Chad
<b>CZ</b>	Czech Republic	<b>LV</b>	Latvia	<b>TG</b>	Togo
<b>DE</b>	Germany	<b>MC</b>	Monaco	<b>TJ</b>	Tajikistan
<b>DK</b>	Denmark	<b>MD</b>	Republic of Moldova	<b>TT</b>	Trinidad and Tobago
<b>EE</b>	Estonia	<b>MG</b>	Madagascar	<b>UA</b>	Ukraine
<b>ES</b>	Spain	<b>ML</b>	Mali	<b>UG</b>	Uganda
<b>FI</b>	Finland	<b>MN</b>	Mongolia	<b>US</b>	United States of America
<b>FR</b>	France	<b>MR</b>	Mauritania	<b>UZ</b>	Uzbekistan
<b>GA</b>	Gabon			<b>VN</b>	Viet Nam

5

10 RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR.Object of the present invention.

The present invention concerns a new receptor having a preference for pyrimidine nucleotides preferably uridine triphosphate over purine nucleotides and the nucleic acid molecule encoding said receptor, vectors comprising said nucleic acid molecule, cells transformed by said vector, antibodies directed against said receptor, nucleic acid probes directed against said nucleic acid molecule, pharmaceutical compositions comprising said products and non human transgenic animals expressing the receptor according to the invention or the nucleic acid molecule according to said receptor.

The invention further provides methods for determining ligand binding, detecting expression, screening for drugs, molecular binding specifically to said receptor and treatment involving the receptor according to the invention.

Background of the invention.

30 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 phaeochromocytoma 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), mouse and rat (8) P2Y1 receptors (formerly called P2Y); 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).

**Summary of the invention.**

This invention provides a receptor having a preference for pyrimidine nucleotides preferably uridine triphosphate over purine nucleotides. A receptor having a preference for pyrimidine nucleotides over purine nucleotides means a receptor for which pyrimidine nucleotides and purine nucleotides are not equally active and equipotent. This means that the receptor according to the invention in presence of these agonists presents a functional response (preferably the accumulation of Inositol triphosphate (IP3), diacylglycerol (DAG), or calcium ions) to lower concentration of pyrimidine nucleotides, preferably uridine triphosphate, than to purine

nucleotides or a more important functional response to similar concentration of pyrimidine nucleotide than to purine nucleotide.

The inositol phosphate (IP3) accumulation after addition of said agonists is described in the specification thereafter.

Advantageously, the receptor according to the invention has at least a twofold, preferably a tenfold to one hundredfold preference for pyrimidine nucleotides over purine nucleotides.

A preferred embodiment of the receptor according to the invention is characterized by a preference for uridine triphosphate over adenine nucleotides.

The receptor according to the invention is a receptor, preferably a G protein-coupled receptor, which belongs structurally to the purinergic receptor family (P2Y family) but functionally is a pyrimidinergic receptor, preferably a UTP-specific receptor.

According to a preferred embodiment of the present invention, the receptor is a human receptor.

Said receptor has an amino acid sequence having more than 60% homology with the amino acid sequence shown in figure 1. Preferably, the amino acid sequence of the receptor according to the invention has at least the amino acid sequence shown in figure 1 or a portion thereof.

A portion of the amino acid sequence means a peptide or a protein having the same binding properties as the receptor according to the invention (i.e. peptide or a protein which is characterized by a preference for pyrimidine nucleotides, preferably UTP, over purine nucleotides).

The present invention is also related to a nucleic acid molecule, such as a DNA molecule or an RNA molecule,

encoding the receptor according to the invention.

Preferably, said DNA molecule is a cDNA molecule or a genomic DNA molecule.

Preferably, said nucleic acid molecule has more than 60% homology to the DNA sequence shown in figure 1.

Preferably, the nucleic acid molecule according to the invention is at least the DNA sequence shown in figure 1 or portion thereof. "A portion of a nucleic acid sequence" means a nucleic acid sequence encoding at least a portion of amino acid sequence as described above.

The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements necessary for expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells. The vector according to the invention is a plasmid or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

The plasmid may be the pcDNA3-P2Y4.

The present invention concerns also the cell (preferably a mammalian cell, such as a 1321N1 cell) transformed by the vector according to the invention. Advantageously, said cell is preferably non neuronal in origin and is chosen among the group consisting of a COS-7 cell, an LM(tk-) cell, an NIH-3T3 cell or a 1321N1 cell.

The present invention is also related to a nucleic acid probe comprising the nucleic acid molecule according to the invention, of at least 15 nucleotides capable of

specifically hybridizing with a unique sequence included in the sequence of the nucleic acid molecule encoding the receptor according to the invention. Said nucleic acid probe may be a DNA or an RNA molecule.

5           The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA molecule encoding the receptor according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a  
10           sequence capable of specifically hybridizing to the cDNA molecule encoding the receptor according to the invention.

          Said antisense oligonucleotide may comprise chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyme  
15           activity.

          Another aspect of the present invention concerns a ligand other than purine and pyrimidine nucleotides (preferably an antibody) capable of binding to a receptor according to the invention and an anti-ligand (preferably  
20           also an antibody) capable of competitively inhibiting the binding of said ligand to the receptor according to the invention.

          Preferably, said antibody is a monoclonal antibody.

          The present invention concerns also the monoclonal  
25           antibody directed to an epitope of the receptor according to the invention and present on the surface of a cell expressing said receptor.

          The invention concerns also the pharmaceutical composition comprising an effective amount of oligonucleotide  
30           according to the invention, effective to decrease the activity of said receptor by passing through a cell membrane and binding specifically with mRNA encoding the receptor

according to the invention in the cell so as to prevent its translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier capable of passing through said cell membrane.

5                    Preferably, in said pharmaceutical composition, the oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA.

                  Preferably, the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell  
10                    capable of being taken up by cell after binding to the structure. The structure of the pharmaceutically acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

15                    Preferably, said pharmaceutical composition comprises an amount of the antibody according to the invention effective to block the binding of a ligand to the receptor according to the invention and a pharmaceutically acceptable carrier.

20                    The present invention concerns also a transgenic non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the receptor according to the invention.

                  The present invention also concerns a transgenic  
25                    non human mammal comprising a homologous recombination knockout of the native receptor according to the invention.

                  According to a preferred embodiment of the invention, the transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic  
30                    acid according to the invention is so placed as to be transcribed into antisense mRNA which is complementary to the mRNA encoding the receptor according to the invention and



which hybridizes to mRNA encoding said receptor, thereby reducing its translation. Preferably, the transgenic non human mammal according to the invention comprises a nucleic acid molecule encoding the receptor according to the invention and comprises additionally an inducible promoter or a tissue specific regulatory element.

Preferably, the transgenic non human mammal is a mouse.

The invention relates to a method for determining whether a ligand can be specifically bound to the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

The invention relates to a method for determining whether a ligand can specifically bind to a receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor. Preferably, said method is used when the ligand is not previously known.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises contacting a cell transfected

with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in second messenger concentration or a modification in the cellular metabolism, (preferably determined by the acidification rate of the culture medium) a decrease in the

receptor activity, thereby determining whether the ligand is a receptor antagonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises preparing a cell  
5 extract from cells transfected with an expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor  
10 agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

15 Preferably, the second messenger assay comprises measurement of intracellular cAMP, intracellular inositol phosphate (IP3), intracellular diacylglycerol (DAG) concentration or intracellular calcium mobilization.

Preferably, the cell used in said method is a  
20 mammalian cell non neuronal in origin, such as a COS-7 cell, a CHO cell, a LM(tk-) cell an NIH-3T3 cell or 1321N1.

In said method, the ligand is not previously known.

The invention is also related to the ligand isolated and detected by any of the preceding methods.

25 The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the receptor according to the invention, effective to reduce the activity of said receptor and a pharmaceutically acceptable carrier.

30 For instance, said agonist or antagonist may be used in a pharmaceutical composition in the treatment of cystic fibrosis, and the method according to the invention

may be advantageously used in the detection of improved drugs which are used in the treatment of cystic fibrosis.

Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the receptor according to the invention.

The invention is also related to the drugs isolated and detected by any of these methods.

The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

The invention is also related to a method of detecting expression of a receptor according to the invention by detecting the presence of mRNA coding for a receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention under hybridizing conditions and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

Said hybridization conditions are stringent conditions.

The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of cystic fibrosis.

The present invention concerns also a method for diagnosing a predisposition to a disorder associated with the activity of the receptor according to the invention. Said method comprises:

- a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;

- c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;
- 5 e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;
- 10 f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.
- 15

A last aspect of the present invention concerns a method of preparing the receptor according to the invention, which comprises:

20

- a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- 25 b) inserting the vector of step a in a suitable host cell;
- 30 c) incubating the cell of step b under conditions allowing the expression of the receptor according to the invention;

- d) recovering the receptor so obtained; and
- e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

**Short description of the drawings.**

5 **Figure 1** represents nucleotide and deduced amino acid sequence of a human P2Y<sub>4</sub> receptor according to the invention. The putative membrane-spanning domains are underlined and numbered I to VII. The consensus sequence conserved between all the P2Y  
10 receptors and the three amino acids (AHN) corresponding to the RGD sequence in the first extracellular loop of the P2Y<sub>2</sub> receptor are represented in bold. The putative phosphorylation sites by PKC or by calmodulin-dependent protein  
15 kinases and PKC are indicated respectively by black squares (■) and by open circles (O).

**Figure 2** is a dendrogram representing structural relatedness among the cloned P2Y receptor and the closest neighbour in the G protein-coupled  
20 receptor family. The plot was constructed using the multiple sequence alignment program Pileup of the GCG package (26). For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains.

25 **Figure 3** represents a northern blot analysis of P2Y<sub>4</sub> receptor expression. The Northern blot was performed with 15 µg of total RNA from human placenta and 4 µg of poly(A)+ RNA from K562 cells and from two different human placentas. The probe  
30 was a human P2Y<sub>4</sub> gene fragment amplified by PCR (TM2 to TM7).

- Figure 4** represents the time course of  $\text{InsP}_3$  accumulation in 1321N1 cells expressing the human  $\text{P2Y}_4$  receptor.  $^3\text{H}$  inositol labelled cells were incubated for the indicated time with UTP ( $100 \mu\text{M}$ ), UDP ( $100\mu\text{M}$ ) and ATP ( $100 \mu\text{M}$ ) in the absence of  $10 \text{ mM LiCl}$  (panel A) or in its presence (panel B). The data represent the mean of triplicate experimental points and are representative of two independent experiments.
- Figure 5** Represents the effect of ATP on the accumulation of  $\text{InsP}_3$  induced by UTP in 1321N1 transfected cells. Concentration-action curves of ATP in the presence of UTP  $10$  or  $100 \mu\text{M}$  at  $30 \text{ s}$  (panel A) and  $20 \text{ min}$  (panel B). Concentration-action curve of ATP with or without UTP ( $10 \mu\text{M}$ ) at  $20 \text{ min}$  (panel C). The data represent the mean  $\pm$  S.D. of triplicate experimental points and are representative of two (panel A), five (panel B) or three (panel C) independent experiments.
- Figure 6** represents the concentration-action curves of UTP and UDP on the  $\text{InsP}_3$  accumulation in three different clones of 1321N1 transfected cells. The cells were incubated in the presence of various UTP (●) and UDP (■) concentrations ( $0, 0.1, 1, 3, 10$  and  $100 \mu\text{M}$ ) for  $30 \text{ s}$  or  $20 \text{ min}$ . The data represent the mean  $\pm$  S.D. of triplicate experimental points obtained in one representative experiment. The  $\text{EC}_{50}$  values were determined by curve fitting (Sigma Plot: version 2.0).
- Figure 7** Represents the effect of various nucleotides on the  $\text{InsP}_3$  production in 1321N1 transfected cells.

The cells were incubated with UTP, UDP, 5BrUTP, dUTP, ITP, AP<sub>3</sub>A, AP<sub>4</sub>A, AP<sub>5</sub>A and AP<sub>6</sub>A at the same concentration of 100  $\mu$ M or without agonist (Cont) for 30 s or 20 min. The data represent the mean  $\pm$  S.D. of triplicate experimental points and are representative of three independent experiments. The EC<sub>50</sub> values were determined by curve fitting (Sigma Plot: version 2.0).

**Figure 8** Represents concentration-action curves of various nucleotides on the InsP<sub>3</sub> accumulation in 1321N1 cells expressing a human P2Y<sub>4</sub> receptor. 1321N1 cells were incubated in the presence of various concentrations of UTP, UDP, dUTP, 5BrUTP, ITP and ATP for a period of time of 20 min. The data are the mean  $\pm$  range of duplicate experimental points obtained in an experiment representative of two.

**Figure 9** Represents the action of various P<sub>2</sub> antagonists on the InsP<sub>3</sub> production induced by UTP in 1321N1 transfected cells. Cells were incubated in the presence of suramin, reactive blue 2 and PPADS at a concentration of 100  $\mu$ M and different UTP concentrations (0, 2 and 10  $\mu$ M) for 20 min. The data represent the mean  $\pm$  S.D. of triplicate experimental points and are representative of two independent experiments.

**Figure 10** Represents the effect of PPADS on the UTP stimulation of InsP<sub>3</sub> in 1321N1 transfected cells. The cells were exposed to various concentrations of UTP in the presence or in the absence of PPADS (100  $\mu$ M) for 20 min. The data are the mean  $\pm$  S.D. of triplicate experimental points obtained in an



experiment representative of two.

5 **Figure 11** Represents the effect of pertussis toxin on the UTP-induced accumulation of InsP<sub>3</sub> in 1321N1 cells expressing a human P2Y<sub>4</sub> receptor. The cells were preincubated for 18 hours in the presence or in the absence of 20 ng/ml pertussis toxin. The cells were then incubated with or without UTP 100 μM and with or without pertussis toxin (20 ng/ml) for various times: 30 s, 5 min or 20 min. The data represent the mean ± S.D. of triplicate experimental points and are representative of two independent experiments.

#### Detailed description of the invention.

##### EXPERIMENTAL PROCEDURES

##### 15 1. 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 GIBCO BRL (Grand Island, NY). The radioactive products myo-D-[2-<sup>3</sup>H]inositol (17.7 Ci/mmol) and [<sup>32</sup>P]ATP (800 Ci/mmol) were from Amersham (Gent, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Richmond, Calif.). UTP, UDP, ATP, ADP, carbachol, LiCl and apyrase grade VII were obtained from Sigma Chemical Co. (St. Louis, MO). 2MeSATP was from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen (San Diego, CA).

##### 2. Cloning and sequencing

30 Degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments between the murine P2Y<sub>2</sub> and the chick P2Y<sub>1</sub> 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 labelled by random priming and used to screen a human genomic DNA library constructed in the  $\lambda$  Charon 4a vector. The hybridization was in 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 wash conditions were 0.1 x SSC, 0.1% SDS at 65 °C. A preparation of  $\lambda$  phages (15) was made for several clones which hybridized strongly with the probe. A restriction map and a Southern blotting analysis allowed to isolate a 1.4 kb NheI-EcoRV fragment that was subcloned into the pBluescript SK<sup>+</sup> vector (Stratagene). The complete sequence of a new receptor coding sequence was obtained on both strands after subcloning of overlapping fragments in M13mp18 and M13mp19.

### 3. Cell culture and transfection

The P2Y<sub>4</sub> receptor coding sequence was subcloned between the HindIII 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 (pcDNA3-P2Y<sub>4</sub>) using the calcium phosphate precipitation method as described (16). 1321N1 cells were incubated for 6 hours at 37 °C in the presence of pcDNA3 vector alone or vector containing the P2Y<sub>4</sub> receptor coding sequence, then washed and incubated in

culture medium (10% FCS, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin and 2.5  $\mu\text{g/ml}$  amphotericin B in Dulbecco's modified Eagle's medium (DMEM)). The selection with G418 (400  $\mu\text{g/ml}$ ) was started two 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  $\mu\text{g/ml}$  G418.

#### 10 4. Inositol phosphates (IP) measurement

1321N1 cells were labelled for 24 hours with 10  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ] inositol in inositol-free DMEM (Dulbecco's modified Eagle's medium) medium containing 5% fetal calf serum, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, 2.5  $\mu\text{g/ml}$  amphotericin B and 400  $\mu\text{g/ml}$  G418. Cells were washed twice with KRH (Krebs-Ringer Hepes) buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM  $\text{MgSO}_4$ , 1.45 mM  $\text{CaCl}_2$ , 25 mM Hepes (pH 7.4) and 8 mM glucose) and incubated in this medium for 30 min. The agonists were added in the presence of LiCl (10 mM) and the incubation was stopped after 30 s, 5 min or 20 min by the addition of an ice-cold 3% perchloric acid solution. For the time course study, LiCl (10 mM) was added 5 min before the agonists and the incubation was stopped at different times. When tested, pertussis toxin (20 ng/ml) was added for 18 h during the labelling period time and during the stimulation by the agonist. Inositol phosphates were extracted and  $\text{InsP}_3$  was isolated by chromatography on Dowex column as described previously (17).

#### 25 5. Radioligand binding assay.

30 Binding assays of [ $\alpha^{32}\text{P}$ ] UTP to cell membranes were carried out in Tris-HCl (50 mM, pH 7.5), EDTA 1 mM in a final volume of 0.5 ml, containing 25-50  $\mu\text{g}$  of protein and 0.5 nM

of radioligand (27). The assays were conducted at 30°C for 5 min. Incubations were stopped by the addition of 4 ml of ice-cold Tris-HCl (50 mM, pH 7.5) and rapid filtration through Whatman GF/B filters under reduced pressure. The filters were then washed three times with 2 ml of the same ice-cold Tris-HCl buffer. Radioactivity was quantified by liquid scintillation counting, after an overnight incubation of the filters in liquid scintillation mixture.

#### 6. Northern blot and Southern blot analysis

Total and poly(A)<sup>+</sup> RNA were prepared from different tissues and human cell lines using the guanidinium thiocyanate-caesium chloride procedure (15), denatured by glyoxal and fractionated by electrophoresis on a 1% agarose gel in 10 mM phosphate buffer pH 7.0. DNA samples, prepared from the  $\lambda$  Charon 4a clones, were digested with restriction enzymes. Northern and Southern blots were prepared (15) and baked for 90 min at 80 °C. Membranes were prehybridized for at least 4 hours and hybridized overnight with the same probe as for the screening, at 42 °C in a solution containing 50% formamide for Northern blots and 40% formamide for Southern blots. Filters were washed twice for 15 min in 2 x SSC at room temperature and then twice for 30 min in 0.2 x SSC at 60 °C before being exposed at -70 °C in the presence of intensifying screens for 5 days (Northern blots) or 1 hour (Southern blots).

## RESULTS

### 1. Cloning and sequencing

In order to isolate new subtypes of P2 receptors, sets of degenerate oligonucleotide primers were synthesized 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'-CAGATCTAGATA(CT)ATGTT(CT)(AC)A(CT)(CT)T(ACGT)GC-3'] corresponding to the second transmembrane region and the primer [5'-TCTTAAGCTTGG(AG)TC(ACGT)A(CG)(AG)CA(AG)CT(AG)TT-3'] corresponding to the seventh transmembrane region amplified a 712 bp fragment. The partial sequences obtained after sequencing were translated into peptidic 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 peptidic sequence presenting 87% identity with the chick P2Y1 receptor and are therefore believed to represent fragments of the human P2Y1 gene.

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 NheI-EcoRV fragment was obtained and an intronless open reading frame of 1095 bp was identified. The sequence is depicted in figure 1 where the putative membrane-spanning domains are underlined and numbered I to VII. The predicted molecular weight of the encoded protein is 36.5 kDa. This molecular weight 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. The three amino acid (AHN) corresponding to the RGD sequence in the first  
5 extracellular loop of the P2Y2 receptor are represented in bold in figure 1. Some potential sites of phosphorylation by protein kinase C (PKC) or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyterminal part of the receptor. The putative  
10 phosphorylation sites by PKC or by calmodulin-dependent protein kinases and PKC are indicated respectively by black squares and by open circles in figure 1. The four positively charged amino acid which have been reported to play a role in the P2Y2 receptor activation by ATP and UTP (1) are conserved in the P2Y4 sequence: His<sup>262</sup>, Arg<sup>265</sup>, Lys<sup>289</sup> and Arg<sup>292</sup>  
15 (Figure 1). The P2Y4 amino acid sequence was compared to the chick P2Y1 and the murine P2Y2 amino acid sequences and to their closest neighbours in the G protein-coupled receptor family (Figure 2). The plot was constructed using the  
20 multiple sequence alignment program Pileup of the GCG package (26). For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains. It is clear that, from a structural point of view, the newly cloned receptor is more closely related to the  
25 human P2Y2 receptor (51% of identity between the complete sequences) than to the chick P2Y1 receptor (35%).

## 2. Tissue distribution of the P2Y4 receptor

The tissue distribution of P2Y4 transcripts was investigated by Northern blotting. A number of rat tissues  
30 (heart, brain, liver, testis and kidney) were tested using a human 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 (Figure 3), HL-60 leukemia cells and SH-SY5Y human neuroblastoma cells. The Northern blot was performed with 15  $\mu$ g of total RNA from human placenta and 4  $\mu$ g of poly(A)<sup>+</sup> RNA from K562 cells and from two different human placentas. The probe was the human P2Y<sub>4</sub> gene fragment amplified by PCR (TM2 to TM7). On the contrary, a strong signal, corresponding to a 1.8 kb mRNA, was found in human placenta (Figure 3).

### 3. Functional expression of the new P2 receptor in 1321N1 cells

After transfection of the pcDNA3-P2Y<sub>4</sub> construction in 1321N1 cells, the pool of G418-resistant clones was tested for their functional response (IP<sub>3</sub> accumulation) to ATP and UTP. Both nucleotides were found to be agonists of the P2Y<sub>4</sub> receptor, but the response to UTP was more robust. About 20 transfected clones were then isolated and tested for their response to UTP. The clone presenting the highest IP accumulation factor in response to UTP was selected and used in all subsequent experiments. Functional characterization of the P2Y<sub>4</sub> receptor was performed by determining the accumulation of InsP<sub>3</sub> after 20 min incubation with the agonists in the presence of 10 mM LiCl. We observed that the response to UTP was biphasic, with a peak reached at 30 s, followed by a more sustained stimulation of lower magnitude (Fig. 4A). With ATP, only that second phase was detectable: its effect became apparent after 1 min of stimulation only and was stable for at least 20 min (Fig. 4A and B). As for UTP, the stimulation by UDP was biphasic, but it was slightly delayed (Fig 4A and B). Inclusion of LiCl had little effect on the initial peak induced by UTP or UDP, but it strongly enhanced the following plateau phase (Fig. 4B).

The maximal effect of ATP observed after a 20 min

incubation represented about  $27 \pm 9\%$  of that of UTP (mean  $\pm$  S.D. of ten experiments). In order to demonstrate that ATP is able to antagonize the UTP response, incubations of 1321N1 cells were conducted with ATP alone or in combination with UTP. Figure 5 shows that at high concentration (500  $\mu$ M or more), ATP was able to inhibit the effect of UTP, both at 30 s and 20 min. At 30 s, the response to UTP 10  $\mu$ M was fully antagonized by ATP 2 mM, corresponding to the fact that ATP has no effect on the human P2Y<sub>4</sub> receptor at this early time (panel A). At 20 min, an inhibition of  $62 \pm 11\%$  of the UTP effect (10  $\mu$ M), corresponding to the difference between the UTP and the ATP effects, was observed in the presence of 2 mM ATP (mean  $\pm$  S.D. of five independent experiments) (panels B and C). The ATP concentration-inhibition curves were shifted to the right when the UTP concentration was increased, indicating the competitive nature of this inhibitory effect (panels A and B). On the other hand, at lower concentrations (30-300  $\mu$ M), ATP enhanced the response to UTP by 29% (range 12-47%, mean of four experiments) (panel B). ADP, which had almost no effect per se and did not inhibit the action of UTP, reproduced that enhancement: in the presence of ADP (100  $\mu$ M), the stimulation by UTP (10  $\mu$ M) represented  $158 \pm 15\%$  (mean of three independent experiments) of that by UTP alone (data not shown). However, this potentiating effect of ATP and ADP was not specific: indeed the action of carbachol mediated by muscarinic receptors endogenously expressed in the 1321N1 cells (6) was also increased in the presence of these nucleotides. This observation was reproduced with cells transfected with the recombinant P2Y<sub>4</sub>-pcDNA3 plasmid or with the vector alone and was also obtained with AMP and adenosine (data not shown).

We compared the concentration-action curves of UTP



and UDP on the  $\text{InsP}_3$  production for several clones of transfected cells. The study was made at two times (Fig. 6) : 30 s and 20 min. In the set of experiments performed on clone 11 (clone of 1321N1 transfected cells chosen for the pharmacological characterization), UTP appeared to be 10-fold more potent than UDP after a 20 min incubation and this difference was reproduced with two other clones (Fig. 6). The  $\text{EC}_{50}$  values were  $0.3 \pm 0.1 \mu\text{M}$  and  $3.3 \pm 0.6 \mu\text{M}$  in clone 2,  $2.4 \pm 0.1 \mu\text{M}$  and  $19.8 \pm 4.8 \mu\text{M}$  in clone 11 and  $0.3 \pm 0.1 \mu\text{M}$  and  $3.2 \pm 0.8 \mu\text{M}$  in clone 21, respectively, for UTP and UDP (mean  $\pm$  S.D. of two independent experiments). At 30 s of incubation, it was not possible to determine  $\text{EC}_{50}$  values because the curves were clearly shifted to the right, but we can observe that the difference between the two agonists potency was even more striking (Fig. 6). Several clones, including clones 2, 11 and 21 were tested in binding studies with  $[\alpha^{32}\text{P}]$  UTP but no increase in specific binding was observed as compared to the cells transfected with the vector alone (data not shown).

In view of the time differences observed in Figure 6, the testing of a range of nucleotides was performed at two times: 30 s and 20 min. As Figure 7 shows, several agonists were barely or not active at 30 s (UDP, 5BrUTP, dUTP, ITP) whereas they produced a significant effect at 20 min. Full concentration-action curves were obtained at 20 min. The rank order of potency was:  $\text{UTP} > \text{UDP} = \text{dUTP} > 5\text{BrUTP} > \text{ITP} > \text{ATP}$  (Fig. 8). The  $\text{EC}_{50}$  values obtained were the following:  $\text{EC}_{50}$  UTP =  $2.5 \pm 0.6 \mu\text{M}$ ,  $\text{EC}_{50}$  UDP =  $19.5 \pm 3.9 \mu\text{M}$  (mean  $\pm$  S.D. of eight independent experiments),  $\text{EC}_{50}$  dUTP =  $20.0 \pm 2.3 \mu\text{M}$ ,  $\text{EC}_{50}$  5BrUTP =  $27.1 \pm 1.9 \mu\text{M}$  and  $\text{EC}_{50}$  ITP =  $32.8 \pm 5.4 \mu\text{M}$  (mean  $\pm$  S.D. of two independent experiments). The approximative  $\text{EC}_{50}$  value obtained for ATP was:  $43 \pm 12 \mu\text{M}$  (mean  $\pm$  S.D. of five

independent experiments). The diadenosine polyphosphates also increased the  $\text{InsP}_3$  production in transfected cells with  $\text{EC}_{50}$  between 3 and 7  $\mu\text{M}$  (data not shown), but their maximal effect was only 20-25 % of that of UTP, a value close to that of ATP (range of four independent experiments) (Fig. 7). UMP, uridine, AMP, adenosine and  $\text{ATP}\gamma\text{S}$  were without any effect (data not shown).

No specific antagonist is available for any  $\text{P}_2\text{Y}$  subtype. Nonetheless, several non-selective antagonists such as suramin, RB2 or PPADS have been tested on  $\text{P}_2$  receptors and their relative actions on these subtypes may constitute a mean to discriminate them (27). So we tested the ability of these three antagonists to inhibit the UTP response in the model of the human  $\text{P}_2\text{Y}_4$  receptor. As we can see on figure 9, PPADS appeared to be the most active antagonist ( $73 \pm 14\%$  inhibition;  $\text{IC}_{50}$  around 15  $\mu\text{M}$  (data not shown)), suramin was inactive, and RB-2 produced an inhibition of  $33 \pm 5\%$  of the UTP response (mean  $\pm$  S.D. of two independent experiments). Figure 10 shows the mixed nature of the antagonism by PPADS of the UTP response: it affects both the  $\text{EC}_{50}$  value and the maximal effect of UTP. The  $\text{EC}_{50}$  value for UTP in the absence of PPADS was  $3.3 \pm 0.6 \mu\text{M}$  and  $12.2 \pm 4.5 \mu\text{M}$  in the presence of 100  $\mu\text{M}$  PPADS (mean  $\pm$  S.D. of two independent experiments).

The effect of pertussis toxin (20 ng/ml, 18 hours pretreatment) was studied at different times after UTP (100  $\mu\text{M}$ ) addition (Fig. 11). The UTP response was clearly inhibited at 30 s ( $62 \pm 5\%$  of inhibition: mean  $\pm$  S.D. of two independent experiments), whereas no significant effect was observed at 5 and 20 min.

## REFERENCES

1. Erb, L., Garrad, R., Wang, Y., Quinn, T., Turner, J.T.,  
and Weisman, G.A. (1995) *J. Biol. Chem.* **270**, 4185-4188.
- 5 2. Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly,  
J.W., Harden, T.K., Jacobson, K.A., Leff, P., and Williams,  
M. (1994) *Pharm. Rev.* **46**, 143-156.
- 10 3. Valera, S., Hussy, N., Evans, R.J., Adami, N., North,  
R.A., Surprenant, A., and Buell, G. (1994) *Nature* **371**, 516-  
519.
- 15 4. Brake, A.J., Wagenbach, M.J., and Julius, D. (1994) *Nature*  
**371**, 519-523.
5. Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N.,  
Smart, T.G., King, B.F., Burnstock, G., and Barnard, E.A.  
(1993) *FEBS* **324**, 219-225.
- 20 6. Filtz, T.M., Li, Q., Boyer, J.L., Nicholas, R.A., and  
Harden, T.K. (1994) *Mol. Pharm.* **46**, 8-14.
7. Henderson, D.J., Elliot, D.G., Smith, G.M., Webb, T.E.,  
25 and Dainty, I.A. (1995) *Biochem. Biophys. Res. Commun.* **212**,  
648-656.
8. Tokoyama, Y., Hara, M., Jones, E.M.C., Fan, Z., and Bell,  
G.I. (1995) *Biochem. Biophys. Res. Commun.* **211**, 211-218.
- 30 9. Lustig, K.D., Shiau, A.K., Brake, A.J., and Julius, D.

(1993) Proc. Natl. Acad. Sci. **90**, 5113-5117.

10. Erb, L., Lustig, K.D., Sullivan, D.M., Turner, J.T., and Weisman, G.A. (1993) Proc Natl Acad Sci **90**, 10449-10453.

5

11. Rice, W.R., Burton, F.M., and Fiedeldey, D.T. (1995) Am. J. Respir. Cell, Molec. Biol. **12**, 27-32.

12. Parr, C.E., Sullivan, D.M., Paradiso, A.M., Lazarowski, E.R., Burch, L.H., Olsen, J.C., Erb, L., Weisman, G.A., Boucher, R.C., and Turner, J.T. (1994) Proc. Natl. Acad. Sci. **91**, 3275-3279.

13. Barnard, E.A., Burnstock, G., and Webb, T.E. (1994) TIPS **15**, 67-70.

14. Kaplan, M.H., Smith, D.I., and Sundick, R.S. (1993) J. Immun. **151**, 628-636.

15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY).

16. Velu, T.J., Beguinot, L., Vass, W.C., Zhang, K., Pastan, I., and Lowry, D.R. (1989) J. Cell. Biochem. **39**, 153-166.

25

17. Communi, D., Raspe, E., Piroton, S., and Boeynaems, J.M. (1995) Circ. Res. **76**, 191-198.

18. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.J., Dumont, J.E., and

30

- Vassart, G. (1989) *Science* **244**, 569-572.
19. Zeng, D., Harrison, J.K., D'Angelo, D.D., Barber, C.M.,  
Tucker, A.L., Lu, Z., and Lynch, K.R. (1990) *Proc. Natl.*  
5 *Acad. Sci.* **87**, 3102-3106.
20. Nomura, H., Nielsen, B.W., and Matsushima, K. (1993) *Int.*  
*Immun.* **5**, 1239-1249.
- 10 21. Harrison, J.K., Barber, C.M., and Lynch, K.R. (1994)  
*Neuroscience Letters* **169**, 85-89.
22. Seifert, R. and Schultz, G. (1989) *TiPS* **10**, 365-369.
- 15 23. Brown, H.A., Lazarowski, E.R., Boucher, R.C., and Harden,  
T.K. (1991) *Mol. Pharm.* **40**, 648-655.
24. O'Connor, S.E., Dainty, I.A., and Leff, P. (1991) *TiPS*  
**12**, 137-141.
- 20 25. Lazarowski, E.R. and Harden, T.K. (1994) *J. Biol. Chem.*  
**269**, 11830-11836.
26. Devereux, J., Haerberli, P. and Smithies O.A. (1984)  
25 *Nucleic Acids Res.* **12**, 387-395.
27. Motte S., Swillens S. and Boeynaems J.M. (1996) *Eur.*  
*J. Pharmacol.* **307**, 201.
- 30 28. Boyer, J.L., Zohn, I.E., Jacobson, K.A. and Harden, T.K.  
(1994) *Br. J. Pharmacol.* **113**, 614.

CLAIMS.

1. Receptor which has an amino acid sequence having more than 60% homology with the amino acid sequence shown in Figure 1.

5           2. Receptor according to claim 1, which has at least the amino acid sequence shown in Figure 1 or a portion thereof.

10           3. Receptor according to claim 1 or 2 having a preference for pyrimidine nucleotides over purine nucleotides.

          4. Receptor according to claim 3, having at least a twofold preference, preferably tenfold to one hundredfold preference for pyrimidine nucleotides over purine nucleotides.

15           5. Receptor according to any of the claims 3 or 4, wherein the pyrimidine nucleotide is uridine triphosphate.

          6. Receptor according to any of the claims 3 to 5, having a preference for UTP over UDP.

20           7. Receptor according to claim 5 being a high affinity UTP-specific receptor.

          8. Receptor according to any of the preceding claims, belonging to the P2 receptor family.

          9. Receptor according to any of the preceding claims, being a G protein-coupled receptor.

25           10. Receptor according to any of the preceding claims, being a human receptor.

          11. Nucleic acid molecule encoding the receptor according to any of the preceding claims.

30           12. Nucleic acid molecule according to claim 11, wherein the nucleic acid molecule is DNA or RNA molecule.

          13. DNA molecule according to claim 12, which is a cDNA molecule or a genomic DNA molecule.

14. Nucleic acid molecule according to any of the claims 11 to 13, having more than 60% homology to the DNA sequence shown in Figure 1.

5 15. DNA molecule according to claim 14, which has at least the DNA sequence as shown in figure 1 or a portion thereof.

16. Vector comprising the nucleic acid molecule according to any of the claims 11 to 15.

10 17. Vector according to claim 16, adapted for expression in a cell, which comprises the regulatory elements necessary for expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule according to any of the claims 11 to 15 as to permit expression thereof.

15 18. Vector of claim 17, wherein the cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

19. Vector according to any of the claims 16 to 18, wherein the vector is a plasmid or a virus, preferably a  
20 baculovirus, an adenovirus or a Semliki Forest virus.

20. Vector according to claim 19, wherein the plasmid is pcDNA3-P2Y4.

21. Cell comprising the vector according to any of the claims 16 to 20.

25 22. Cell of claim 21, wherein the cell is a mammalian cell, preferably non neuronal in origin.

23. Cell of claim 21, wherein the cell is chosen among the group consisting of COS-7 cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

30 24. Nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the

nucleic acid molecule according to any of the claims 11 to 15.

25. Nucleic acid probe of claim 24, wherein the nucleic acid is DNA or RNA.

5 26. Antisense oligonucleotide having a sequence capable of specifically hybridizing to a mRNA molecule of claim 12, so as to prevent translation of the mRNA molecule.

10 27. Antisense oligonucleotide having a sequence capable of specifically hybridizing to the DNA molecule of claim 13.

28. Antisense oligonucleotide according to claim 26 or 27, comprising chemical analogs of nucleotides.

15 29. Ligand other than purine and pyridine nucleotides capable of binding to a receptor according to any of the claims 1 to 10.

30. Anti-ligand capable of competitively inhibiting the binding of the ligand according to claim 29 to the receptor according to any of the claims 1 to 10.

20 31. Ligand according to claim 29 which is an antibody.

32. Anti-ligand according to claim 30 which is an antibody.

33. Antibody according to claim 31 or 32, which is a monoclonal antibody.

25 34. Monoclonal antibody according to claim 33, directed to an epitope of the receptor according to any of the claims 1 to 10, present on the surface of a cell expressing said receptor.

30 35. Pharmaceutical composition comprising an amount of the oligonucleotide according to claim 26, effective to decrease activity of the receptor according to any of the claims 1 to 10 by passing through a cell membrane



and binding specifically with mRNA encoding said receptor in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

5           36.     Pharmaceutical composition of claim 35, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

          37.     Pharmaceutical composition of claim 36, wherein the substance which inactivates mRNA is a ribozyme.

10           38.     Pharmaceutical composition according to any of the claims 35 to 37, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure.

15           39.     Pharmaceutical composition of claim 38, wherein the structure of the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a selected cell type.

          40.     Pharmaceutical composition which comprises an effective amount of the anti-ligand of claim 30, effective to block binding of a ligand to the receptor according to any of the claims 1 to 10 and a pharmaceutically acceptable carrier.

25           41.     Transgenic non human mammal expressing the nucleic acid molecule according to any of the claims 11 to 15.

          42.     Transgenic non human mammal comprising a homologous recombination knockout of the native receptor according to any of the claims 1 to 10.

30           43.     Transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid molecule according to any of the claims 11 to 15 so

placed as to be transcribed into antisense mRNA which is complementary to the mRNA of claim 12 and which hybridizes to said mRNA thereby reducing its translation.

5 44. Transgenic non human mammal according to any of the claims 41 to 43, wherein the nucleic acid according to any of the claims 11 to 15 additionally comprises an inducible promoter.

10 45. Transgenic non human mammal according to any of the claims 41 to 44, wherein the nucleic acid according to claim 11 to 15 additionally comprises tissue specific regulatory elements.

46. Transgenic non human mammal according to any of the claims 41 to 45, which is a mouse.

15 47. Method for determining whether a ligand can specifically bind to a receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence  
20 of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

25 48. Method for determining whether a ligand can specifically bind to the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of  
30 the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said

receptor.

49. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

50. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

51. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism,

a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

52. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in a second messenger concentration, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

53. A method according to any of the claims 47 to 50, wherein the second messenger assay comprises measurement of intra-cellular cAMP, intra-cellular Inositol phosphate, intra-cellular diacylglycerol concentration or intra-cellular calcium mobilization.

54. Method according to any of the preceding claims 47 to 53, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of COS-7 cells, CHO cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

55. Method according to any of the preceding claims 47 to 54, wherein the ligand is not previously known.

56. Ligand detected by the method according to any of the preceding claims 47 to 55.

57. Pharmaceutical composition which comprises the ligand according to claim 56 and a pharmaceutically acceptable carrier.

58. Method of screening drugs to identify drugs

which specifically bind to the receptor according to any of the claims 1 to 10 on the surface of the cell, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs under conditions permitting binding of said drugs to the receptor, and determining those drugs which specifically bind to the transfected cell, thereby identifying drugs which specifically bind to the receptor.

59. Method of screening drugs to identify drugs which specifically bind to the receptor according to any of the claims 1 to 10 on the surface of the cell, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cells extract, contacting the membrane fraction with a plurality of drugs and determining those drugs which bind to the transfected cell, thereby identifying drugs which specifically bind to said receptor.

60. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs under conditions permitting the activation of a functional receptor response, and determining those drugs which activate such receptor using a bio-assay, such as a modification in a second messenger concentration or modification in the cellular metabolism, thereby identifying drugs which act as receptor agonists.

61. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from

cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs under conditions permitting the activation of a functional receptor response, and determining those drugs which activate such receptor using a bio-assay, such as a modification in a second messenger concentration, thereby identifying drugs which act as receptor agonists.

62. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 10, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a modification in a second messenger concentration or modification in the cellular metabolism, thereby identifying drugs which act as receptor antagonists.

63. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 10, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs in presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a modification in a second messenger

concentration, thereby identifying drugs which act as receptor antagonists.

64. Drug detected by any of the methods according to claims 58 to 63.

5 65. Pharmaceutical composition comprising a drug according to claim 64.

66. Method of detecting the expression of the receptor according to any of the claims 1 to 10, by detecting the presence of mRNA coding said receptor, which comprises  
10 obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to claim 24 under hybridizing conditions, and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

15 67. Method of detecting the presence of the receptor according to any of the claims 1 to 10 on the surface of a cell, which comprises contacting the cell with the antibody of claim 31 under conditions permitting binding of the antibody to the receptor, and detecting the presence  
20 of the antibody bound to the cell, thereby detecting the presence of the receptor on the surface of the cell.

68. Method of determining the physiological effects of expressing varying levels of the receptor according to any of the claims 1 to 10, which comprises  
25 producing a transgenic non human mammal according to any of the claims 41 to 46 whose levels of receptor expression are varied by use of an inducible promoter which regulates the receptor expression.

69. Method of determining the physiological  
30 effects of expressing varying levels of the receptor according to any of the claims 1 to 10, which comprises producing a panel of transgenic non human mammals according

to any of the claims 41 to 46, each expressing a different amount of said receptor.

5 70. Method for identifying an antagonist of the receptor according to any of the claims 1 to 10 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of the receptor, which comprises administering the antagonist to a transgenic non human mammal according to any of the claims 10 40 to 45 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal as a result of receptor activity, thereby identifying the antagonist.

71. Antagonist identified by the method of claim 70.

15 72. Pharmaceutical composition comprising an antagonist according to claim 71 and a pharmaceutically acceptable carrier.

20 73. Method for identifying an agonist of the receptor according to any of the claims 1 to 10 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said receptor, which comprises administering the agonist to a transgenic non human mammal according to any of the claims 41 to 46 and determining whether the antagonist alleviates the physical 25 and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the abnormalities indicating the identification of the agonist.

74. Agonist identified by the method of claim 73.

30 75. Pharmaceutical composition comprising an agonist according to claim 74 and a pharmaceutically acceptable carrier.

76. Method for diagnosing a predisposition to a



disorder associated with the activity of a specific allele of the receptor according to any of the claims 1 to 10, which comprises:

- 5 a) obtaining nucleic acid molecules of subjects suffering from said disorder;
- b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- 10 d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;
- e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable  
15 marker to create a unique band pattern specific to subjects suffering from said disorder;
- f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic  
20 acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the  
25 same.

77. Method of preparing the purified receptor according to any of the claims 1 to 10, which comprises:

- 30 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the

- cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;
- b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing  
5 the expression of the receptor according to the invention;
- d) recovering the receptor so obtained; and
- e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

AAGGGAGCTTGGGTAGGGGCCAGGCTAGCCTGAGTGCACCCAGATGCGCTTCTGTGACGCT 60  
CTCCCTAGTGCTTCAACCACTGCTCTCCCTGCTCTACTTTTTTTTGGCTCCAGCTCAGGGAT 120  
GGGGGTGGGCAGGGAAATCCTGCCACCCTCACTTCTCCCTTCCCATCTCCAGGGGGGCC 180

1 ATGGCCAGTACAGAGTCCTCCCTGTTGAGATCCCTAGGCCTCAGCCCAGGTCCTGGCAGC 240  
1 M A S T E S S L L R S L G L S P G P G S

51 AGTGAGGTGGAGCTGGACTGTTGGTTTTGATGAGGATTTCAAGTTCATCCTGCTGCCTGTG 300  
21 S E V E L D C W F D E D F K F I L L P V

121 AGCTATGCAGTTGTCTTTGTGCTGGGCTTGGGGCCTTAACGCCCCAACCCATGCGCTCTTC 360  
41 S Y A V V F V L G L G L N A P T L W L F

181 ATCTTCCGCTCCGACCCTGGGATGCAACGGCCACCTACATGTTCCACCTGGCATTGTCA 420  
61 L F R L R P W D A T A T Y M F H L A L S

241 GACACCTTGTATGTGCTGTGCTGCCACCCTCATCTACTATTATGCAGCCCACAACCAC 480  
81 D T L Y V L S L P T L I Y Y Y A A H N H

301 TGGCCCTTTGGCACTGAGATCTGCAAGTTCGTCGGCTTTCTTTTCTATTGGAACCTCTAC 540  
101 W P F G T E I C K F V R F L F Y W N L Y

361 TGCAGTGTCCCTTTTCTCACCTGCATCAGCGTGCACCGCTACCTGGGCATCTGCCACCCA 500  
121 L S V L F L T C I S V H R Y L G I C H P

421 CTTGGGGCACTACGCTGGGGCCGCCCTCGCCTCGCAGGCCCTTCTCTGCCCTGGCAGTTTGG 560  
141 L R A L R W G R P R L A G L L C L A Y W

481 TTGGTCTAGCCGGCTGCCTCGTGCCCAACCTGTTCTTTGTGCACAACCAGCAACAAGGG 720  
161 L V V A G C L V P N L F F V T T S N K G

541 ACCACCGTCCCTGTGCCATGACACCACCTCGGCCCTGAAGAGTTTGACCACCTATGTGCACTTC 780  
181 T T V L C H D T T R P E E F D H Y V H F

601 AGCTCGGGCGTACATGGGGCTGCTCTTTGGCGTCCCTGCCTGGTCACTCTTTGTTTCTAT 840  
201 S S A V M G L L F G V P C L V T L V C Y

661 GGACTCATGGCTCGTCCCTGTATCAGCCCTTGCCAGGCTCTGCACAGTCTGCTCTCTCGC 900  
221 G L M A R R L Y Q P L P G S A Q S S S R

721 CTCCGCTCTCTCCGACCATAGCTGTGGTGGCTGACTGTCTTTGCTGTCTGCTTCGTTCCCT 960  
241 L R S L R T I A V V L T V F A V C F V P

781 TTCCACATCACCCCGCACCATTTACTACCTGGCCAGGCTGTTGGAAGCTGACTGCCGAGTA 1020  
261 F H I T R T I V Y L A R L L E A D C R V

841 CTGAACATTGTCAACGTGGTCTATAAAGTGACTCGGGCCCTGGCCAGTGCCAACAGCTGC 1080  
281 L N I V N V V Y K V T R P L A S A N S C

901 CTGGATCCTGTGCTCTACTTTGCTCACTGGGGACAAATATCGACGTCAGCTCCGTCAGCTC 1140  
301 L D P V L Y L L T G D K Y R R Q L R Q L

961 TGTGGTGGTGGCAAGCCCCAGCCCCGACGGCTGCCTCTTCCCTGGCACTAGTGTCCCTG 1200  
321 C G G G K P Q P R T A A S S L A L V S L

1021 CCTGAGGATAGCAGCTGCAGGTGGGGCGCCACCCSCCAGGACAGTAGCTGCTCTACTCCT 1260  
341 P E D S S C R W A A T P Q D S S C S T P

1081 AGGGCAGATAGATTGTAACACGGGAAGCCGGGAAGTGAGAGAAAAGGGGATGAGTGCAGG 1320  
361 R A D R L \*

GCAGAGGTGAGGGAACCCAATAGTGATACCTGGTAAGGTGCTTCTTCCCTTTTCCAGGC 1380  
TCTGGAGAGAAGCCCTCACCTGAGGGTTGCCAGGGAGGCAGGGATATC 1429

FIG. 1

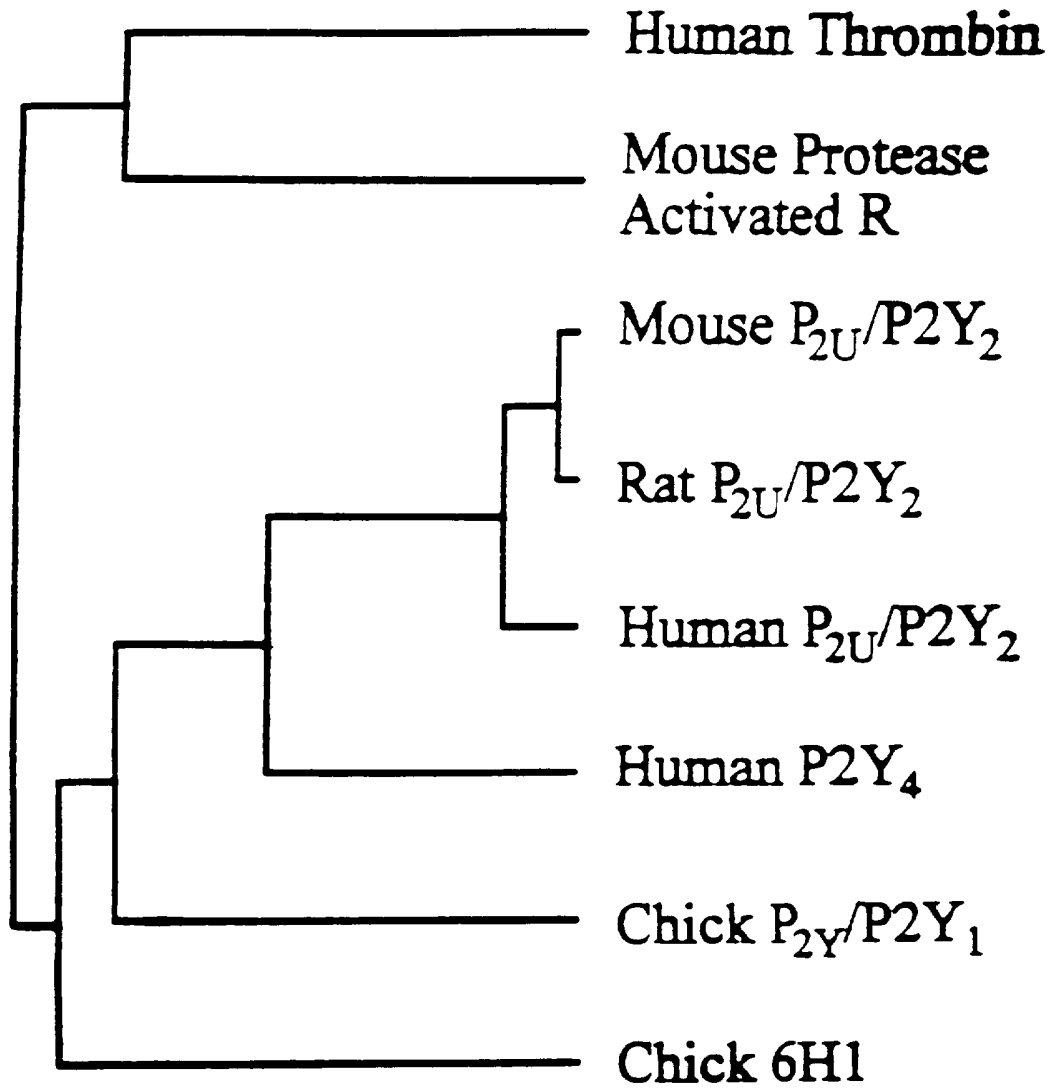


FIG. 2

Expression of P2Y<sub>4</sub> receptors

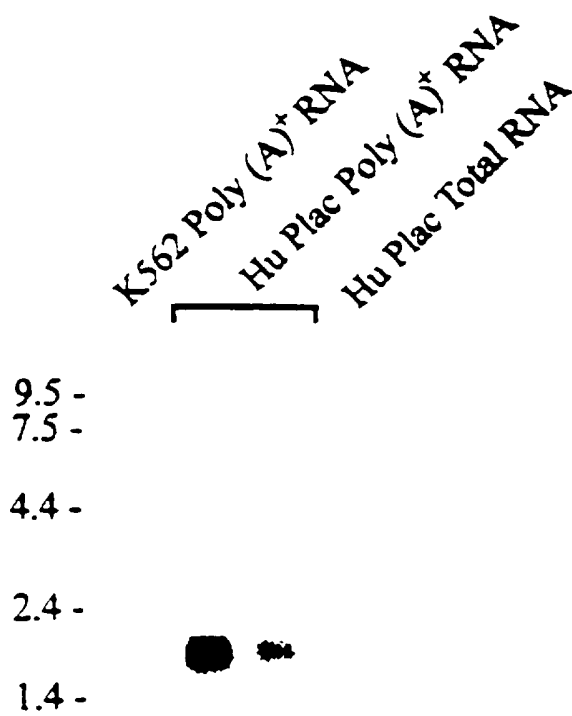


FIG. 3

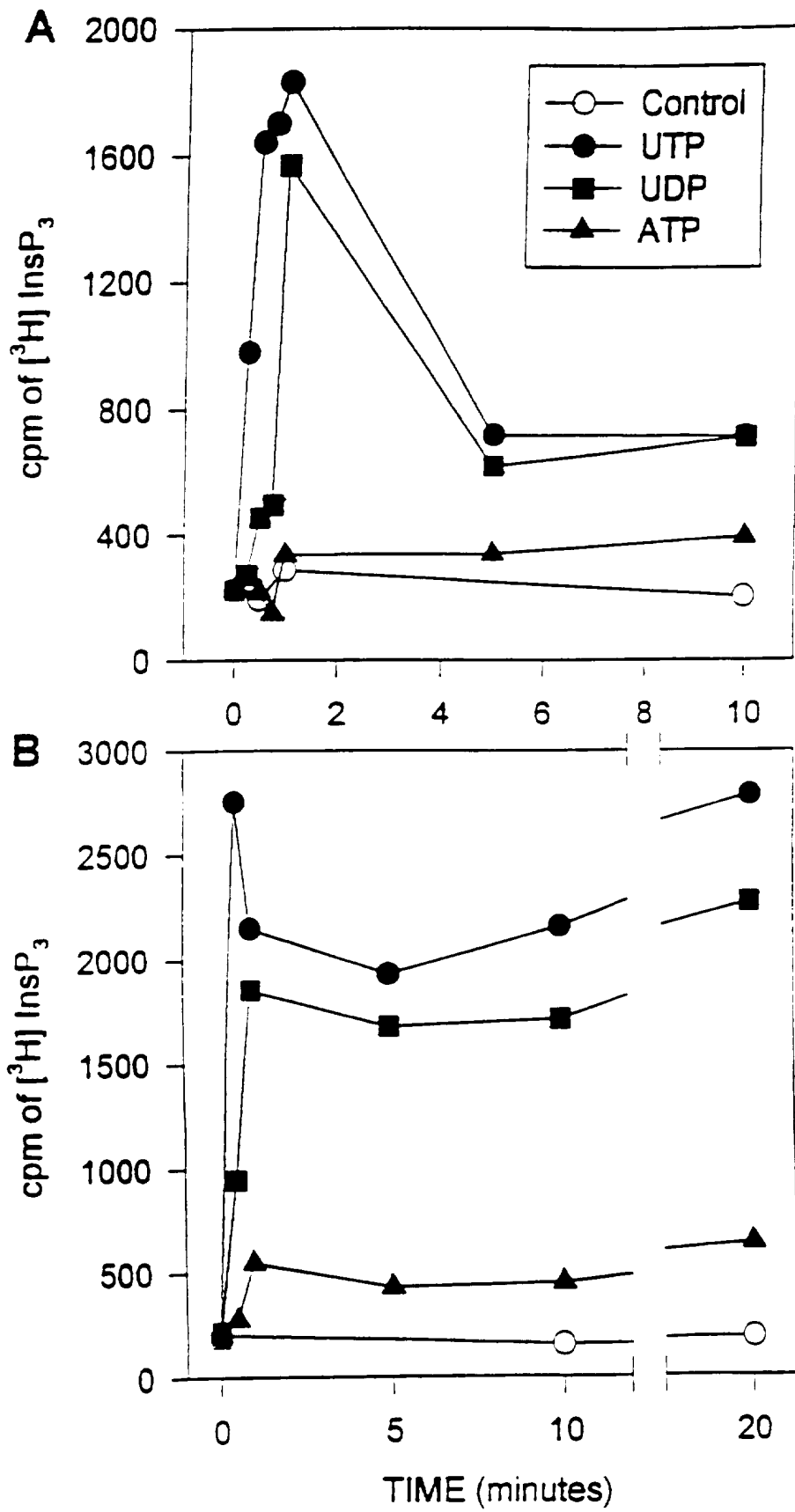


Fig. 4

5/12

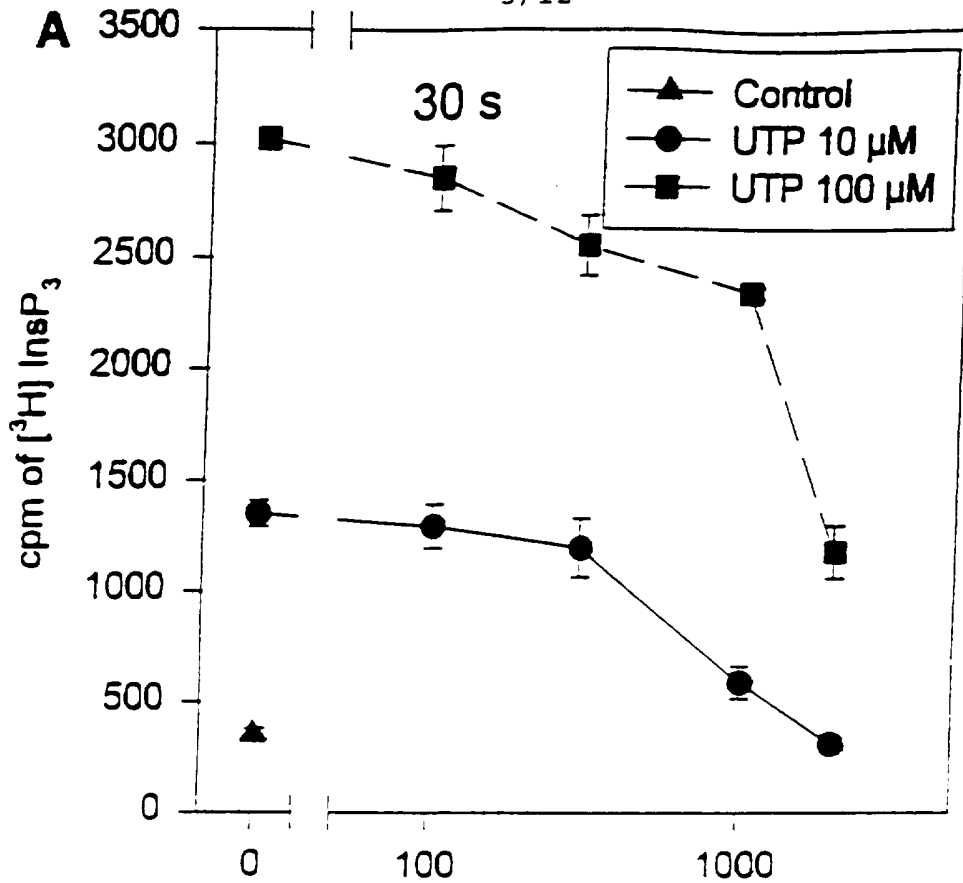


Fig. 5  
Panel A

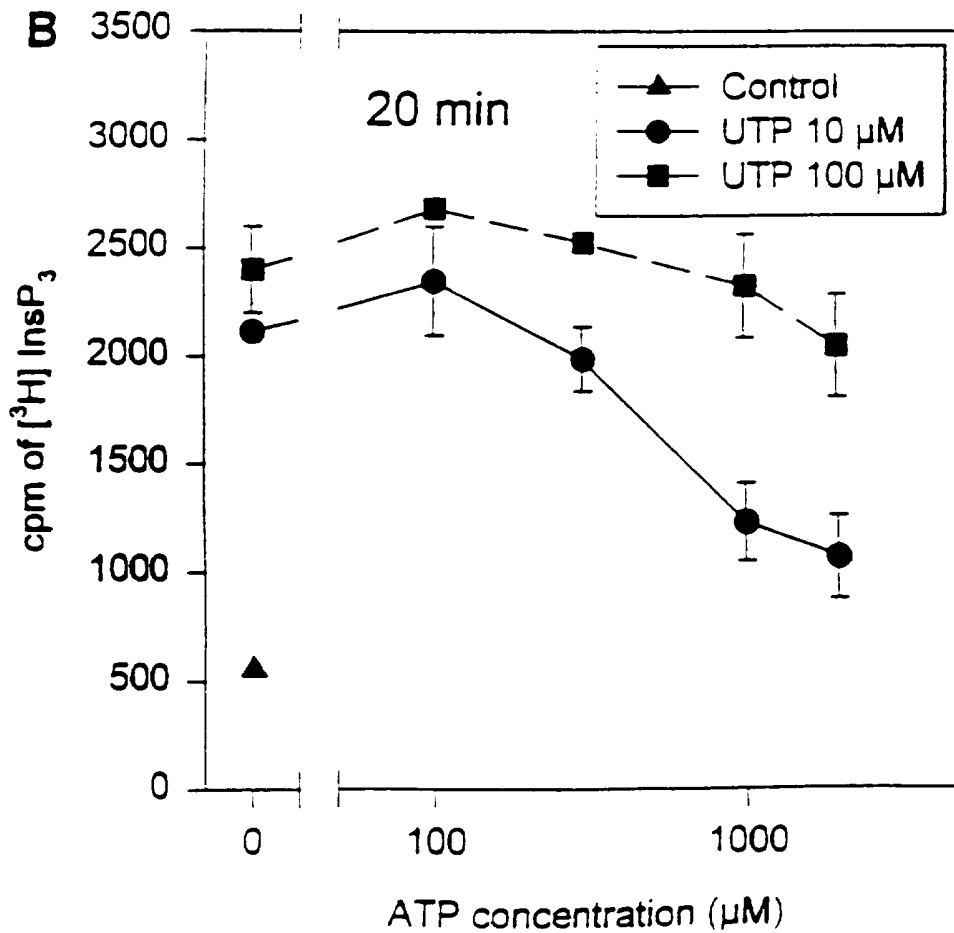


Fig. 5  
Panel B

6/12

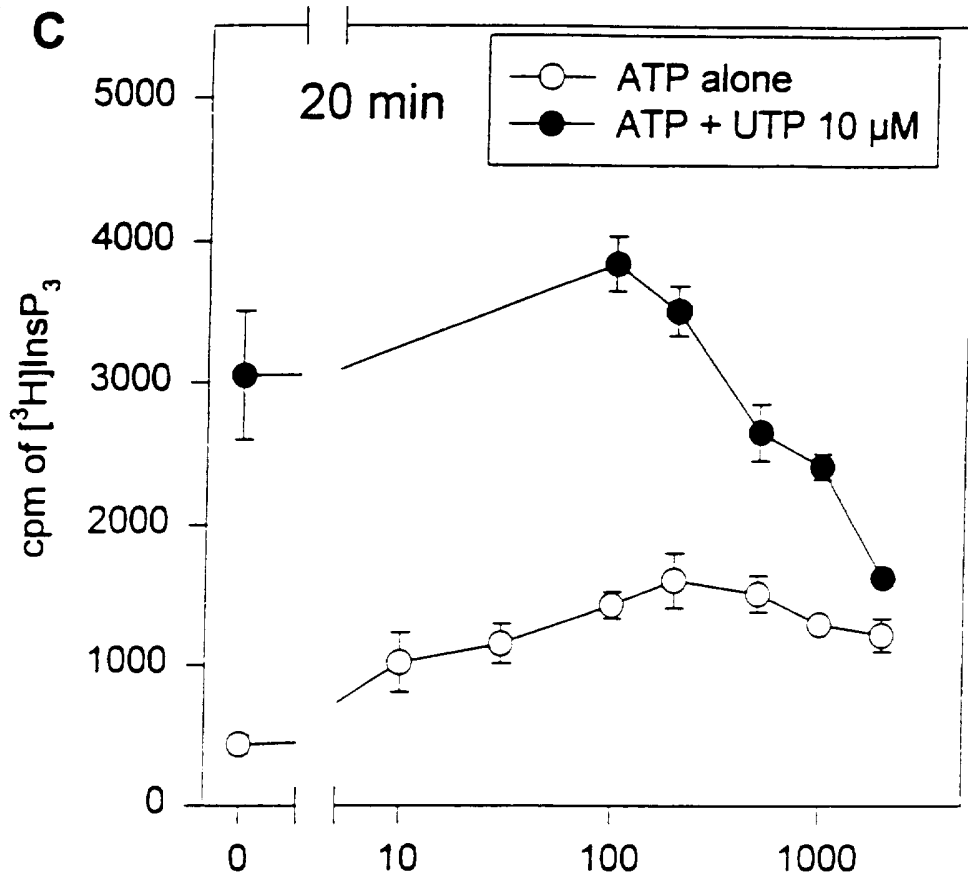


Fig. 5  
Panel C



7/12

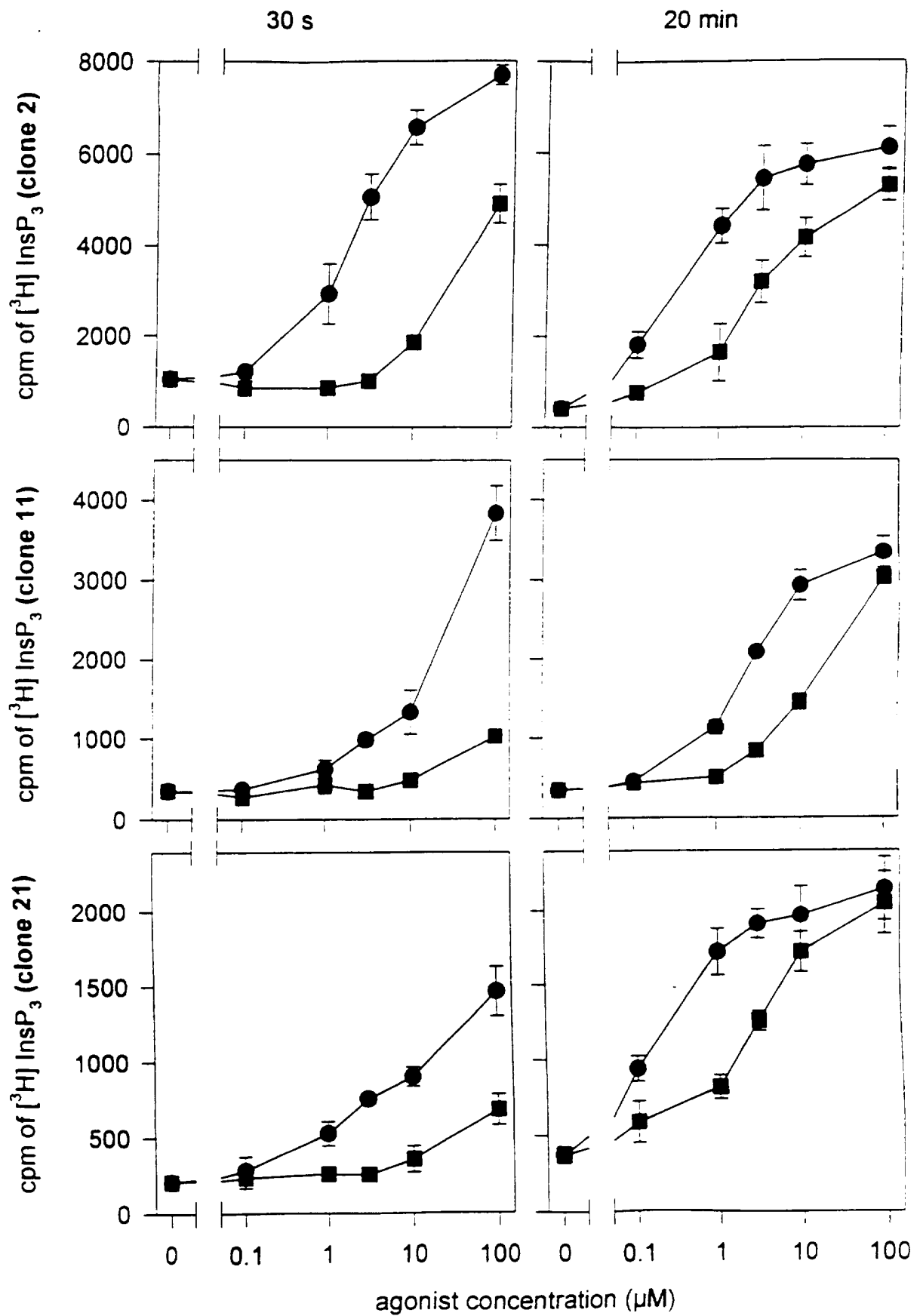


Fig. 6

8/12

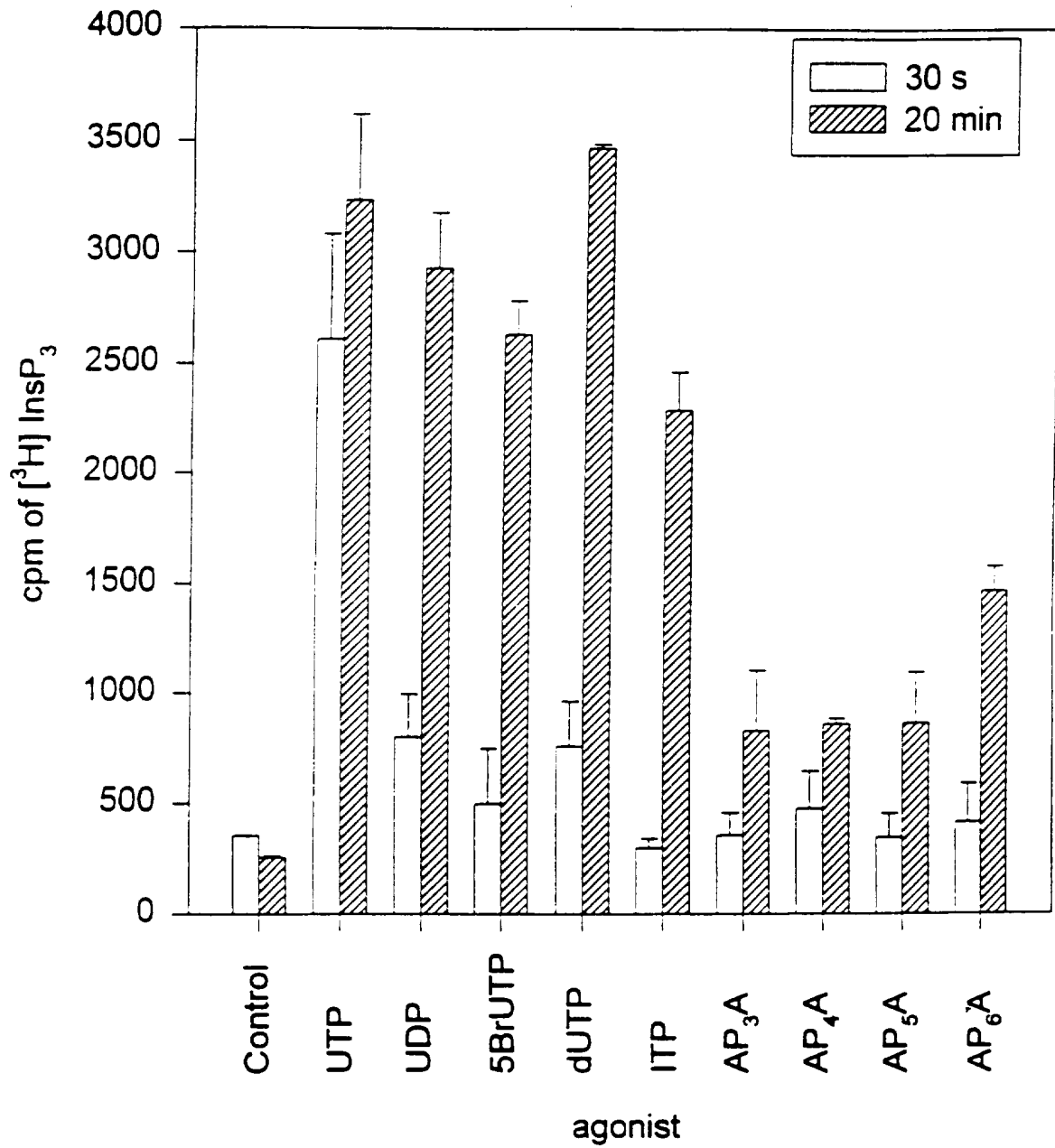


Fig. 7

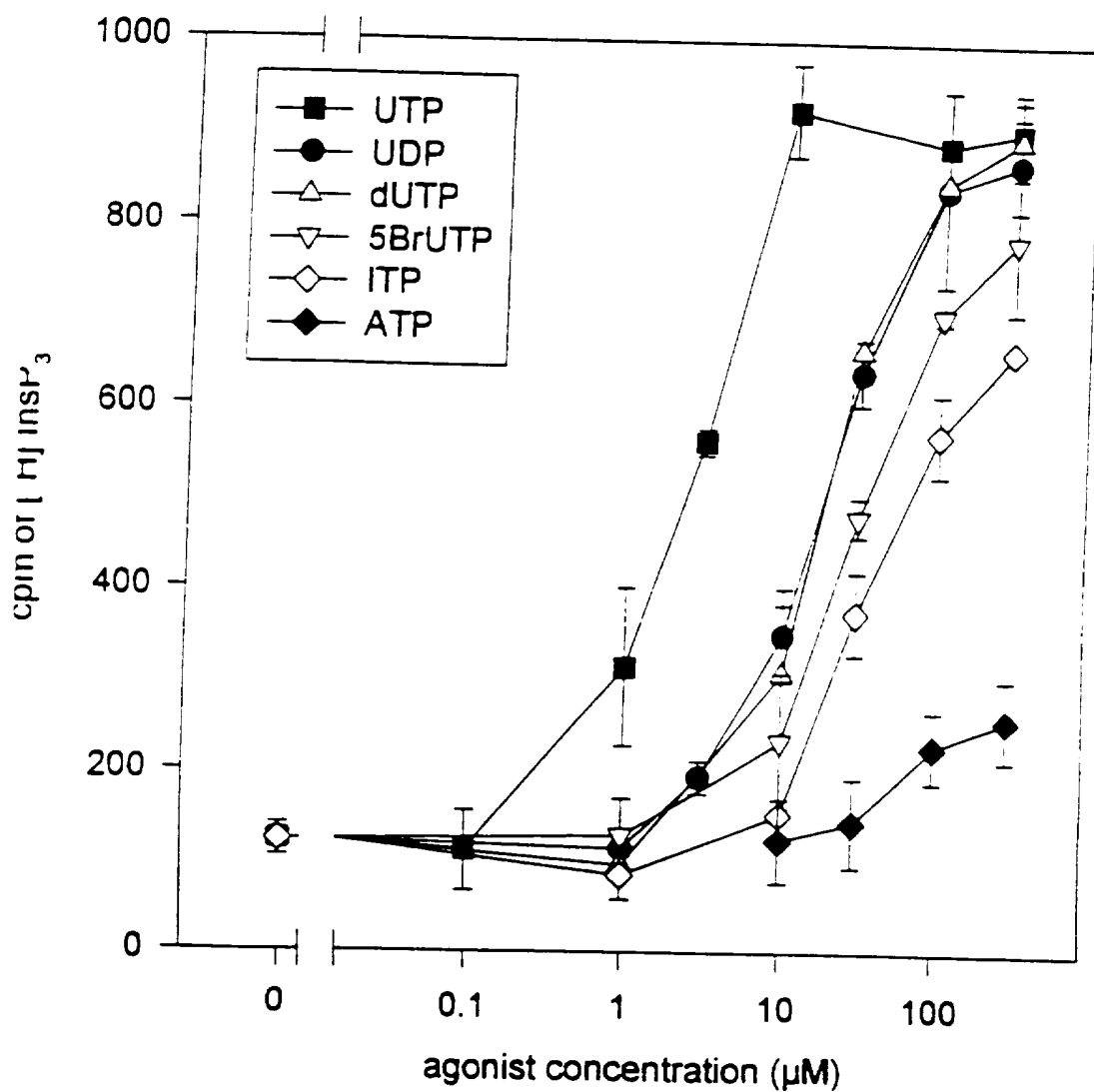


Fig. 8

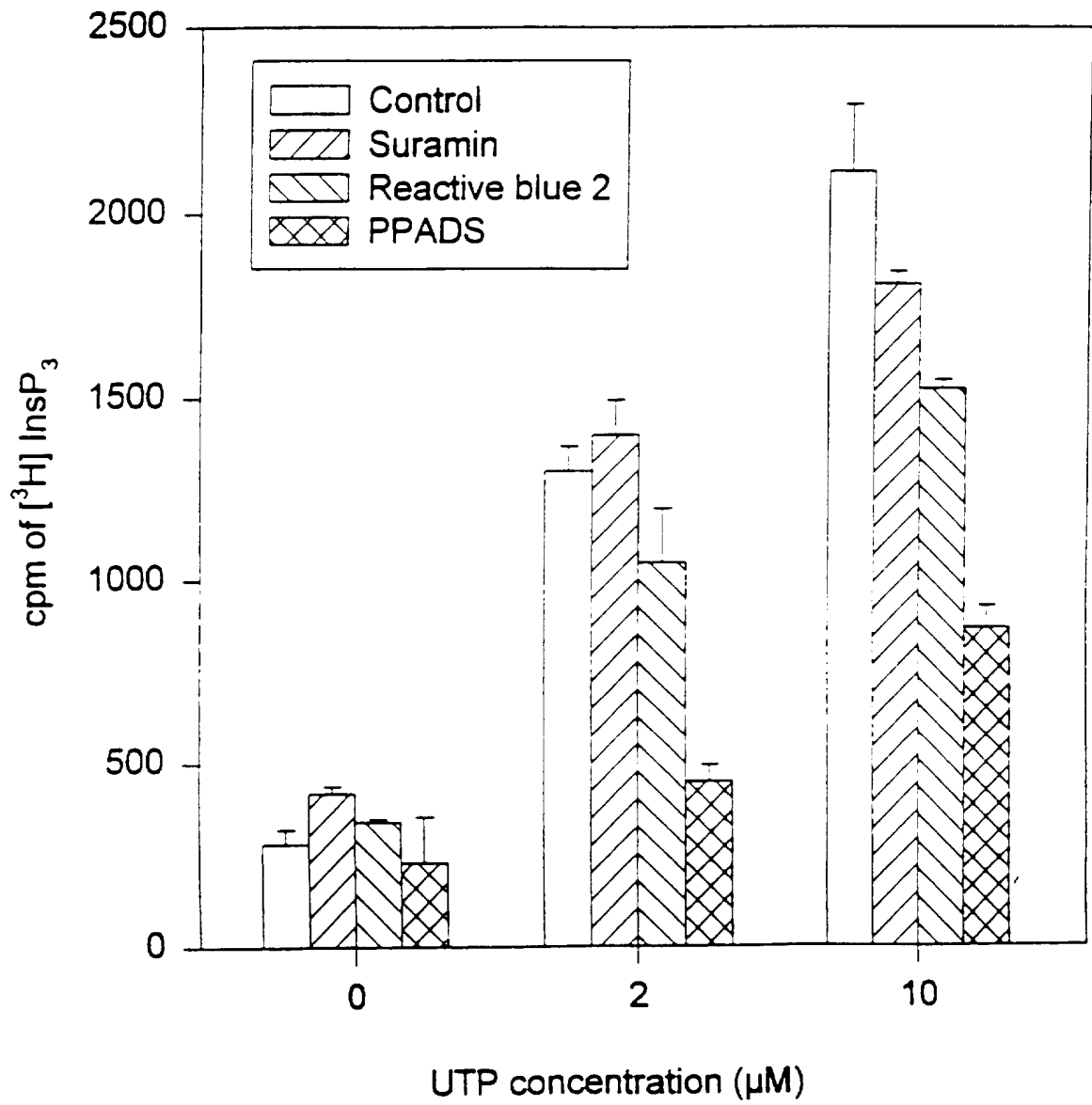


Fig. 9

11/12

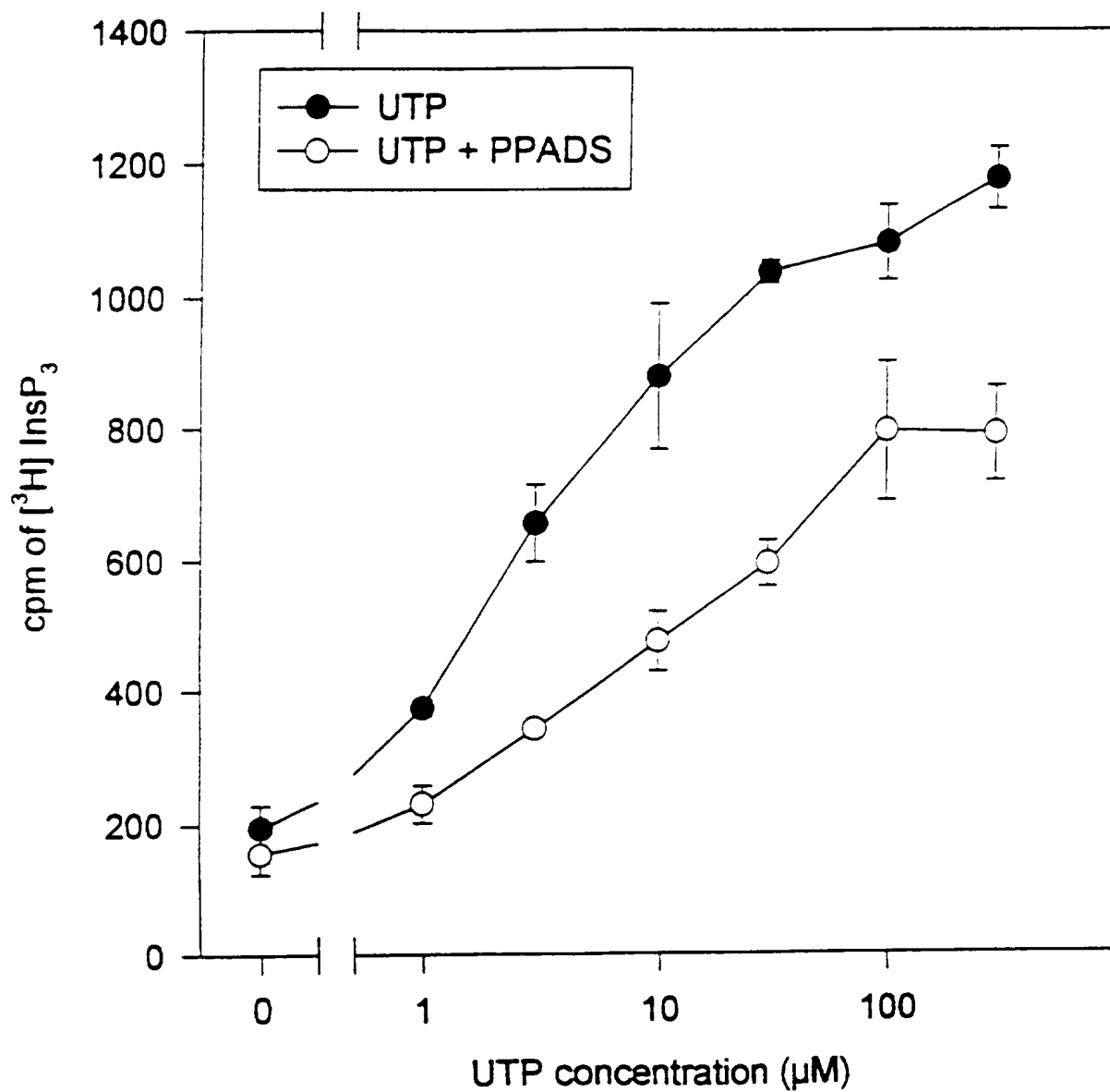


Fig. 10

12/12

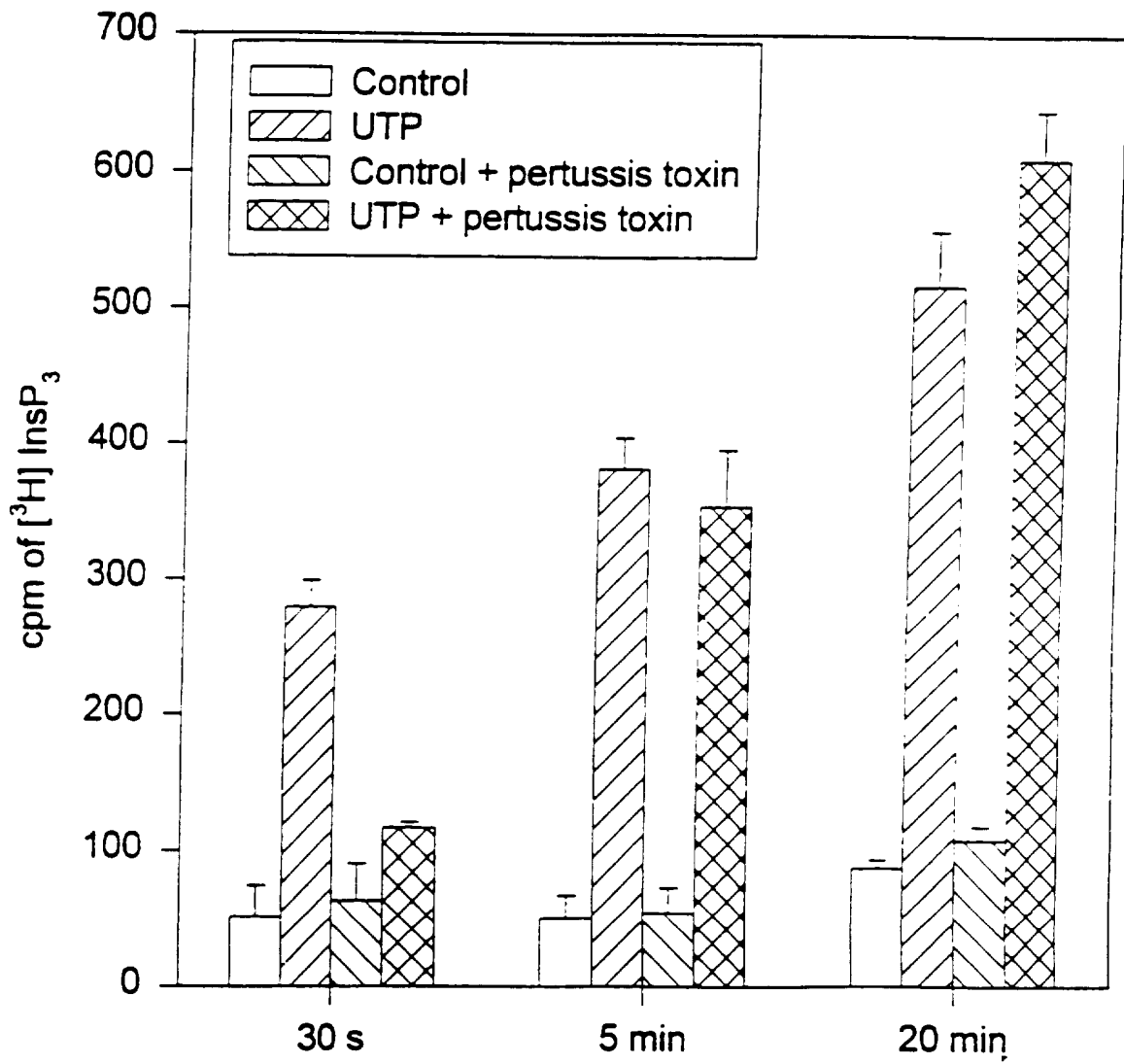


Fig. 11

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/BE 96/00123

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6    C12N15/12    C07K14/705    C12N15/85    C12N15/86    C12N5/10 C12Q1/68    C07K16/28    A01K67/027    G01N33/53					
According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6    C07K    C12N    C12Q    A01K    G01N					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.			
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, April 1994, pages 3275-3279, XP000611412 PARR C E ET AL: "CLONING AND EXPRESSION OF A HUMAN P2U NUCLEOTIDE RECEPTOR, A TARGET FOR CYSTIC FIBROSIS PHARMACOTHERAPY" cited in the application see the whole document <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	11-14, 16-19, 21-23			
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.			
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed		*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family			
Date of the actual completion of the international search  <p style="text-align: center;">24 April 1997</p>		Date of mailing of the international search report  <p style="text-align: center;">02. 05. 97</p>			
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer  <p style="text-align: center;">Kania, T</p>			

12

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/BE 96/00123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 10538 A (UNIV NORTH CAROLINA ;UNIV MISSOURI (US); BOUCHER RICHARD C (US); W) 20 April 1995	14-19, 21-25
A	see the whole document	1-13, 26-28, 35,36, 38-40, 47-67, 76,77
	---	
A	TRENDS IN PHARMACOLOGICAL SCIENCES, vol. 16, April 1995, pages 133-139, XP002030122 BOARDER M. ET AL.: "G protein-coupled P2 purinoceptors: from molecular biology to functional responses" * see the whole document, esp. p. 137 *	1-77
	---	
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 16, 22 April 1994, pages 11830-11836, XP002030123 LAZAROWSKI E. AND HARDEN T.: "Identification of a uridine nucleotide-selective G-protein-linked receptor that activates phospholipase C" cited in the application see the whole document	1-77
	---	
P,X	FEBS LETTERS, vol. 384, no. 3, 22 April 1996, pages 260-264, XP002030124 STAM N. ET AL.: "Molecular cloning and characterization of a novel orphan receptor (P2p) expressed in human pancreas that shows high structural homology to the P2u purinoceptor" see the whole document	1-16,24, 25,27
	---	
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 52, 29 December 1995, pages 30849-30852, XP002030125 COMMUNI D. ET AL.: "Cloning and functional expression of a human uridine nucleotide receptor" see the whole document	1-23
	---	
T	WO 96 38558 A (INCYTE PHARMA INC) 5 December 1996 see the whole document	1-77
	-----	



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/BE 96/00123

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 70,73  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No <b>PCT/BE 96/00123</b>
--

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9510538 A	20-04-95	AU 7965694 A	04-05-95
		US 5596088 A	21-01-97
		US 5607836 A	04-03-97
-----			
WO 9638558 A	05-12-96	AU 5972996 A	18-12-96
		AU 6032596 A	18-12-96
		WO 9638591 A	05-12-96
-----			