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Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled $P2Y_{13}$ receptor

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Abstract

The *human* $P2Y_{13}$ receptor is a new receptor characterized by coupling to Gi, responsiveness to adenine di-phospho-nucleotides and blockade by the P2Y antagonist AR-C69931MX. The *mouse* $P2Y_{13}$ ortholog has also been reported. Here we report, for the first time, the cloning of *rat* $P2Y_{13}$ receptor, its pharmacological analysis and tissue distribution. Rat $P2Y_{13}$ is 79% and 87% identical to human and mouse $P2Y_{13}$ receptors, respectively. Expression of $rP2Y_{13}$ receptor in 1321N1 cells induced the appearance of responses to the typical $P2Y_{13}$ receptor agonists ADP and 2MeSADP, as detected by stimulation of [^{35}S]GTPγS binding. Agonist activities were higher in cells transfected with $rP2Y_{13}$ receptor in the presence of the $G\alpha_{16}$ subunit; in all cases agonist effects were abolished by pertussis toxin pre-treatment. At variance from both *human* and *mouse* receptors, ADP was more potent than 2MeSADP. Other nucleotides and sugar-nucleotides were ineffective. Both in the absence and presence of $G\alpha_{16}$, activation of $rP2Y_{13}$ receptor by ADP and 2MeSADP was completely inhibited by nM concentrations of AR-C69931MX. In contrast, no inhibition of $rP2Y_{13}$ receptor was induced by the selective $P2Y_1$ receptor antagonist MRS2179. $rP2Y_{13}$ receptor showed highest expression levels in spleen, followed by liver and brain (with particularly high levels in cortex and striatum as reported in man), suggesting important roles in the nervous and immune systems. Expression levels comparable to those of the other cloned P2Y receptors were found in primary rat astrocytes, indicating a possible role in reactive astrogliosis. Hence, *rat* $P2Y_{13}$ receptor displays several similarities but also interesting differences with its *human* and *mouse* orthologs, that will have to be taken into account when characterizing the pathophysiological roles of this receptor in the rat animal models.

Keywords: $P2Y_{13}$ receptor; Cloning; Rat

1. Introduction

A wealth of compelling data supports a role for adenine (ATP, ADP) and uracyl (UTP, UDP) nucleotides as neurotransmitters/neuromodulators in diverse mammalian cells and tissues under both physiological and pathological conditions [1,2]. Many of these effects are mediated by specific membrane G-protein-coupled P2Y receptors [3]. For example, ATP and UTP regulate salt and fluid transport through epithelia via the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, which may hence represent potential targets for the therapy of cystic fibrosis and obstructive gut diseases [4–7]. ADP activates platelet $P2Y_1$ and $P2Y_{12}$ receptors that play a crucial role in thrombus formation and stabilisation [8,9]. ATP may also play a role in the immune system via the $P2Y_{11}$ receptor [10]. The recent recognition of the UDP-glucose receptor (previously known as GPR105/KIAA0001 [11]) as the $P2Y_{14}$ receptor [3] demonstrates that sugar-nucleotides may also serve as extracellular transmitters via the activation of specific members of this receptor family.

Eight distinct mammalian P2Y receptors (the $P2Y_{1,2,4,6,11,12,13,14}$ subtypes) are currently recognised [3]. Their human orthologs and several (but not all) rodent orthologs have been reported [2]. Following the cloning of the rat and human $P2Y_{12}$ receptor [12], it became clear that two phylogenetically- and structurally-distinct P2Y receptor subgroups can be identified [3,10]. Group 1 encompasses specific adenine nucleotide-responding receptors ($hP2Y_1$,

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hP2Y$_{11}$), uracyl-nucleotides preferring receptors (hP2Y$_4$, P2Y$_6$), and receptors of mixed selectivity (P2Y$_2$, rodent P2Y$_4$) (ibidem). P2Y$_{12}$, which is only distantly related to those subtypes, constitutes the first member of the second group. In 2001, Communi and coworkers identified human P2Y$_{13}$ receptor (previously known as GPR86) as a new ADP receptor phylogenetically and structurally related to P2Y$_{12}$ [13]. The pharmacology of this second member of group 2 was also confirmed by Zhang and coworkers (who also identified its murine ortholog [14]), and its similarities with that of the hP2Y$_{12}$ receptor have been further analysed recently [15]. P2Y$_{14}$ [3,11] constitutes the third member of group 2. Receptors in the second group share the highest degree of sequence identity: for example, human P2Y$_{12}$, P2Y$_{13}$ and P2Y$_{14}$ are 47–48% identical, with respect to a 29–46% identity of P2Y receptors in the first [3,10]. P2Y$_{12,13,14}$ also share several characteristic amino acid motifs in transmembrane (TM) 6 and 7 that are believed to play a role in binding to extracellular nucleotides. In particular, all cloned hP2Y receptors share a **H**-*X*-*X*-**R**/**K** motif in TM6 which is crucial for receptor activity [3]. In receptors of the first group (P2Y$_{1,2,4,6,11}$), a **Q/K**-*X*-*X*-**R** motif in TM7 also seems to participate in ligand binding (ibidem). In human P2Y$_{12,13,14}$ receptors, this motif is substituted with **E**-*X*-*X*-**L**, suggesting a different mode of agonist binding [3].

At present, very little is known on the pathophysiological roles of P2Y$_{13}$ receptor. The abundance of human and mouse P2Y$_{13}$ receptors is highest in brain and in spleen [13,14], suggesting important roles in the nervous and immune systems. Model organisms such as rat and mouse provide powerful systems in which to study the function of novel proteins and may be also used to elucidate the biological roles of P2Y$_{13}$ receptor. However, to make use of model systems, orthologous genes must be identified in these systems. While murine P2Y$_{13}$ receptor has been cloned and characterised [14], no data regarding the expression and functional properties of rat P2Y$_{13}$ receptor have been yet reported. The presence of a ''putative'' P2Y$_{13}$ receptor in rat chromosome 2 has been very recently (October 23, 2003) predicted by automated computational analysis, by applying the gene prediction method GNOMON on a previously annotated genomic sequence. In the present study, by utilising specific RT–PCR primers external to the coding sequence of the putative open reading frame (ORF) of this genomic sequence, we have cloned this sequence from rat tissues, heterologously expressed it in 1321N1 cells, and showed that this rat protein is specifically activated by adenine nucleotides, as are the human and mouse P2Y$_{13}$ receptors, although with some interesting pharmacological differences. In the present study, we also show that the tissue distribution of the newly-cloned rat protein receptor is similar (but not identical) to that previously reported for the human and mouse receptors [14,15], with highest levels in spleen, liver, brain and kidney. In particular, rat P2Y$_{13}$ receptor is expressed to significant levels in primary astrocytes from rat brain, where a yet-unidentified P2Y receptor mediating reactive astrogliosis had been previously reported [17,18]. This is the first report demonstrating that the P2Y$_{13}$ receptor is expressed and functional in rat tissues.

2. Materials and methods

2.1. Identification of the putative rat ortholog of $P2Y_{13}$ receptor in rat genome

The putative genomic sequence encoding for the rat P2Y$_{13}$ receptor was identified by database querying (NBLAST, see also below) with the human P2Y$_{13}$ sequence [13]. Database searching in HTGS revealed a similar rat sequence from clone CH230-189I10. Sequence determination of the rat clone confirmed an ORF consisting of 1011 nucleotides coding region (putative rat P2Y$_{13}$ receptor) that encoded a full-length open reading frame (ORF) of 336 amino acids. This ORF was amplified by PCR from rat genomic DNA as described below.

2.2. Cell culture

Primary astrocytic cultures were established from *C. striatum* and cerebral cortex of 7-day-old rats as previously described [19,20]. Cells were initially plated in serum-supplemented medium and after 48 h switched to a chemically defined serum-free medium. For RNA isolation, cells were grown in 10-cm diameter tissue culture dishes (2.4×10^6 cells per dish).

2.3. Total RNA/genomic DNA isolation and PCR analysis

Total RNA was extracted as previously described [20] using the TRIZOL® reagent (Invitrogen, Milan, Italy) according to manufacturer's instructions. In the case of primary astrocytic cultures, cells were scraped and lysed in TRIZOL® reagent directly in the culture dish. For the distribution study, the various rat tissues reported in Fig. 7 were disrupted in 1 ml of TRIZOL® reagent with a glass/teflon potter (20 strokes). RNA was quantified spectrophotometrically.

Genomic DNA was extracted from 0.5 cm of rat tail using Nucleospin Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions.

For PCR analysis, after treatment of total RNA with RQ1 RNase-free-DNase (Promega, Milan, Italy), one microgram of RNA was reverse-transcribed with Superscript II RNA H$^-$ Reverse Transcriptase (200 U per sample) (Invitrogen), in the presence of 100 pmoles of random hexamers (Applied Biosystems, Milan, Italy). Aliquots (5% of the reverse-transcribed c-DNA product) of either total cDNA or genomic DNA were amplified in each PCR

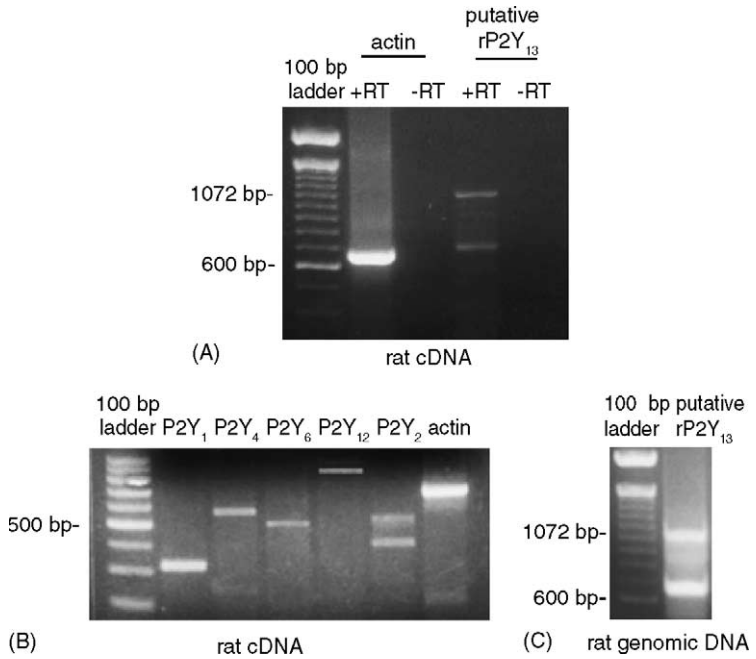

Fig. 1. (A) PCR amplification of putative $P2Y_{13}$ receptor in rat astrocytic cDNA. Reverse-transcribed total RNA isolated from striatal astrocytes was amplified with oligonucleotide primers specifically designed for the putative ORF of a rat genomic sequence highly homologous to human and mouse $P2Y_{13}$ receptor (see Section 3 for more details). PCR products were resolved on 1.5% of ethidium bromide-stained agarose gel and visualised under UV light. A specific PCR product of the expected mw (1072 bp) was found (indicated as +RT). No PCR products were detected in RNA samples from the same rat cells that were not subjected to RT (indicated as −RT). β-Actin was used as a positive control. (B) PCR amplification of the six already cloned rat P2Y receptors in rat astrocytic cDNA. A sample of the same RNA preparation utilised for panel A was retro-transcribed and expression of $P2Y_1$ $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_2$ assessed by PCR with oligonucleotide primers specifically designed for these receptor subtypes. PRC products were resolved and visualised as described above. Specific PCR products of 318 bp ($P2Y_1$), 550 bp ($P2Y_4$), 481 bp ($P2Y_6$), 895 bp ($P2Y_{12}$), and 499 bp ($P2Y_2$) were found. No PCR products were detected in RNA samples that were not subjected to RT (data not shown). β-Actin was used as a positive control. (C) PCR analysis of putative $rP2Y_{13}$ receptor in rat genomic DNA. Genomic DNA, isolated from the rat tail, was amplified by PCR with the same oligonucleotide primers utilised in A (see also Section 2). An amplified product of 1072 bp identical to that shown in panel A was obtained.

assay as previously described [20], with Platinum Taq DNA polymerase (1.25 U per sample) (Invitrogen) in a 25 μl reaction mixture containing 20 pmoles of 5′ and 3′ primers in a standard PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.4). For cDNA, control samples which did not undergo reverse transcription (indicated as −RT in Fig. 1) were processed in parallel with the same experimental protocol to check for contamination of RNA with genomic DNA. Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) for 40 cycles (typically 95 °C/45 s, 30 s at the annealing temperature ranging from 51 to 60 °C, depending on the specific receptor subtype; 72 °C/45 s) after an initially denaturation at 95 °C for 2 min for cDNA or for 5 min in the case of genomic DNA. The following forward (Fw) and reverse (Rw) oligonucleotide primers were utilised:

$P2Y_1$ Fw: 5′-CCTGCGAAGTTATTTCATCTA-3′
Rw: 5′-GTTGAGACTTGCTAGACCTCT-3′

$P2Y_2$ Fw: 5′-GCAGCATCCTCTTCCTCACCT-3′
Rw: 5′-CATGTTGATGGCGTTGAGGGT-3′

$P2Y_4$ Fw: 5′-GGCATTGTCAGACACCTTGTA-3′
Rw: 5′-AAGGCACGAAGCAGACAGCAA-3′

$P2Y_6$ Fw: 5′-CGCTTCCTCTTCTATGCCAA-3′
Rw: 5′-GTAGGCTGTCTTGGTGATGTG-3′

$P2Y_{12}$ Fw: 5′-TCCCATTGCTCTACACTGTC-3′
Rw: 5′-TGTCCTTTCTTCTTATTTGC-3′

For the putative $rP2Y_{13}$ receptor, two distinct sets of primers were utilised. For cloning, the following primers external to the putative ORF of the sequence identified in rat chromosome 2 WGS supercontig (GenBank accession no. NW_047625) were utilised:

Fw: 5′-CCTCTCTTTGTGTGCATGT-3′
Rw: 5′-TGAAATTAGCCTGTGGGAC-3′

For the distribution study, aliquots (2.5% of the reverse-transcribed c-DNA product) of the total cDNA were amplified fo 30 cycles with the following primers:

Fw: 5′-CAGGGACACTCGGATGACA-3′
Rw: 5′-TGTTCGGCAGGGAGATGA-3′

cDNA was also amplified with β-actin primers (Fw: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′; Rw: 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′) as positive control.

2.4. Cloning and heterologous expression of putative $rP2Y_{13}$ receptor

The PCR product of 1072 bp, obtained from amplification of rat genomic DNA, was excised and purified from agarose gel with a classical extraction with phenol/isoamylic alcohol/chloroform. An aliquot (~40 ng) of the purified PCR product was cloned into the pcDNA3.1 expression vector using the pcDNA3.1/V5-His©TOPO® TA Expression Kit (Invitrogen). The construct was verified by sequencing using the Applied Biosystems Terminator cycle sequencing kit.

Heterologous expression studies were carried out in 1321N1 astrocytoma cells seeded on 75 cm^2 flasks (1.3×10^6 cells) by utilising the calcium phosphate precipitation method [21]. 1321N1 astrocytoma cells were maintained in culture in Dulbecco's modified Eagle's medium high glucose (DMEM; Celbio, Milan, Italy) supplemented with 2.5 μg/ml amphotericin B, 1 mM sodium piruvate (Gibco-BRL Life Technologies, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS) (Celbio) [13].

For transfection, growth medium was replaced with DMEM (4.5 g/l glucose, 0.58 g/l glutamine, 0.11 g/l sodium piruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS) to which 1.5 ml of the calcium phosphate/DNA suspension were added. In the 1.5 ml suspension the DNA concentration was as follows: 3.75 μg of the reporter plasmid pEGFP (Enhanced Green Fluorescent Protein), 18.75 μg of $rP2Y_{13}$-pcDNA3.1 (or the empty plasmid) and filling DNA to a final concentration of 37.5 μg. In the case of cotrasfection with $hG\alpha_{16}$-pcDNA3.1, the DNA concentration was as follows: 3.75 μg of the reporter plasmid pEGFP, 15 μg of $rP2Y_{13}$-pcDNA3.1, 15 μg of $hG\alpha_{16}$-pcDNA3.1 and filling DNA to a final concentration of 37.5 μg. Fluorescence microscopy analysis of cells 48 h after transfection indicated that approximately 30% of cells expressed the transfected plasmids, as evaluated by quantifying the number of cells showing expression of EGFP over the total number of cells. In GTPγS-binding studies (see also below), inhibition of Pertussis-toxin (PTX)-sensitive Gi proteins was obtained by incubating transfected cells with 100 ng/ml PTX (Sigma, Milan, Italy) for 18 h before membrane preparation.

2.5. [^{35}S]GTPγS binding assay

1321N1 cells (control and transfected cells) were homogenised in 5 mM Tris–HCl, 2 mM EDTA, pH 7.4 and centrifuged at 48,000 × *g* for 15 min at 4 °C. The resulting pellets were washed in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4 and stored at −80 °C until use. Measurement of nucleotide-stimulated [^{35}S]GTPγS binding to membranes of cells expressing the putative $P2Y_{13}$ receptor in the absence or presence of co-transfected $G\alpha_{16}$ protein was performed as previously described [15]. Briefly, membrane fractions (10 μg) from control 1321N1 cells, cells transfected with $rP2Y_{13}$-pcDNA3.1, cells transfected with $hG\alpha_{16}$-pcDNA3.1, and cells transfected with both $rP2Y_{13}$-pcDNA3.1 and $hG\alpha_{16}$-pcDNA3.1 were incubated at room temperature in binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP) containing either ADP (50 nM–10 μM), or 2MeSADP (50 nM–10 μM), or ATP (10 μM), or αβMeATP (10–100 μM), or βγMeATP (10–100 μM), or UTP (50 μM), or UDP-glucose (50 μM) (Sigma). Moreover, concentration–response curve with two distinct P2Y receptor antagonists (MRS2179, Sigma and AR-C69931MX, Astra-Zeneca) were performed by pre-treating cell membranes with 1 μM ADP in the absence or presence of graded antagonist concentrations (1–100 μM for MRS2179, and 1 nM–1 μM for AR-C69931MX). After 15 min, [^{35}S]GTPγS (0.2 nM) was added, and samples were incubated for 30 min at 30 °C. Reactions were terminated by rapid filtration through GF/C glass fiber filters under vacuum. The filters were washed four times with binding buffer and then counted in a scintillation cocktail. Non-specific binding was determined in the presence of 10 μM GTPγS and was less than 10% of total binding. In selected experiments, transfected 1321N1 cells were treated overnight with 100 ng/ml pertussis toxin (PTX) added to culture medium.

2.6. Bioinformatic analysis

P2Y receptor sequences were obtained from GenBank™ (http://www.ncbi.nlm.nih.gov/Entrez/). Determination of putative open reading frame was performed with DNA Strider 1.2. Analysis of potential phosphorylation and *N*-glycosylation sites in the putative rat $P2Y_{13}$ protein was performed by utilising CBS Prediction Servers (NetPhos 2.0 and NetNGlyc 1.0 Server; http://www.cbs.dtu.dk/services). Structural analysis for putative transmembrane domain determination of the protein sequences were performed with SOSUI software (http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html). BLAST searches were performed through the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequences were aligned with ClustalX 1.8. Phylogenetic trees were generated with the program TreeView 1.5.

2.7. Densitometric analysis

Densitometric analysis of RT–PCR products were performed using NIH Image 1.52. Each image was imported into NIH Image by Adobe Photoshop, a gel-plotting macro was used to outline the bands, and the intensity was calculated on the uncalibrated OD setting. Results were normalised to the corresponding housekeeping gene β-actin and expressed as percentage of spleen signal set to 100%.

2.8. Statistical analysis

For the analysis and graphic presentation of [^{35}S]GTPγS binding data we used the non-linear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad). All data are presented as mean ± S.E.M. Statistical analysis was performed by one-way ANOVA. Significance refers to results where $P < 0.05$ was obtained.

3. Results

3.1. Cloning and sequence analysis

Interrogation of the rat genome database with the sequence of the human $P2Y_{13}$ receptor revealed the presence of an highly homologous sequence in chromosome 2 WGS supercontig (GenBank accession no. NW_047625). This sequence contains a 1011 base open reading frame (ORF) encoding 336 aminoacid residues and is 79 and 87% identical to the human and mouse $P2Y_{13}$ receptor, respectively. We designed specific oligonucleotide primers external to the putative ORF of this genomic sequence and utilised them to verify if it were expressed in rat primary astrocytes where a yet-unidentified P2Y-like receptor has been previously reported [17,18]. A specific PCR product of the expected mw (1072 base pairs, bp) was found in the cDNA obtained from astrocytes freshly isolated from rat *C. striatum* (Fig. 1A). No signal was detected in parallel RNA samples that did not undergo retro-transcription (indicated as −RT in Fig. 1A), demonstrating that the amplified product was not due to contamination of RNA with genomic DNA. An amplified product with identical mw was also detected in the cDNA obtained from rat cortical astrocytes (data not shown), in line with the expression data reported for the human receptor in cerebral cortex ([14]; see also below). In rat striatal astrocytes, the expression level of the putative $P2Y_{13}$ receptor was comparable with those of the other members of the rat P2Y receptor family, as shown in Fig. 1B reporting the RT–PCR analysis of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptors. The same oligonucleotide primers utilised in panel A to verify the expression of putative $P2Y_{13}$ receptor in rat astrocytes revealed a band of 1072 bp in genomic DNA isolated from rat tail (Fig. 1C), the tissue from which this sequence was then cloned. Another band with a lower molecular weight (approximately 700 bp) was also detected; the amplified product with the expected mw of 1072 bp was excised from the gel and cloned. The nucleotidic sequence and the deduced peptide sequence of this clone are reported in Fig. 2A. The clone is 99% identical to that reported in rat chromosome 2 WGS supercontig, with the only exception of a A-to-G nucleotide substitution in position 411, which has however no effect on the encoded aminoacid. This substitution was found in all the four clones analysed. The hydrophilicity profile of the putative protein revealed the presence of seven hydrophobic regions (highlighted in Fig. 2A), consistent with a seven transmembrane structure typical of a GPCR [22,23]. The putative peptidic sequence of the newly cloned rat receptor contains five potential sites for N-linked glycosylation and several putative phosphorylation sites (Fig. 2A). The alignment of the newly cloned rat sequence with the human and mouse $P2Y_{13}$ receptor is reported in Fig. 2B.

Comparison of the deduced rat protein (336 aminoacids) with the human (333 aminoacids) and mouse (337 aminoacids) $P2Y_{13}$ receptors shows full overlapping of the TM regions, including TM3, TM6 and TM7, which are believed to be particularly important for ligand interaction. The rat protein also shows full conservation of the typical amino acid motifs in TM6 and TM7 (in particular, an **H**-X-X-**R** and a **K**-**E**-X-X-**L** motifs, respectively, see also Section 1) that are also present in the human and mouse $P2Y_{13}$ receptors and have been proposed to be important for binding to extracellular nucleotides [3,24,25]. The similarity of the newly cloned rat protein to the other P2Y receptors cloned from rat (i.e. $P2Y_{1,2,4,6,12,14}$) is shown in Fig. 3A. The putative rat $P2Y_{13}$ receptor shows highest identity with $rP2Y_{12}$ (49%) and $rP2Y_{14}$ (43%), followed by $rP2Y_4$ (26%), $rP2Y_1$ and $rP2Y_2$ (25%) and $rP2Y_6$ (24%). The phylogenetic relationships among all P2Y receptors cloned from man, mouse and rat (including the newly cloned putative $P2Y_{13}$ receptor) are shown in Fig. 3B. The presence of two phylogenetically distinct P2Y receptor subgroups is evident (see also Section 1). The newly cloned rat protein clusters in the second subgroup that also includes the $P2Y_{12}$ and $P2Y_{14}$ receptors. Blast analysis showed that the newly cloned receptor is clustered on rat chromosome 2 together with $P2Y_1$, $P2Y_{12}$, $P2Y_{14}$, and several ‘‘orphan’’ GPCRs (i.e. receptors for yet-unidentified endogenous ligands) which are structurally and phylogenetically correlated to the P2Y receptor family (H963 and GPR87, Fig. 4). This clustering is remarkably similar to the clustering of these same receptors on both human chromosome 3 and mouse chromosome 2 (Fig. 4; [26,27]).

3.2. Pharmacology and G-protein coupling of the $rP2Y_{13}$ receptor

It is expected that orthologous receptors, in addition to having conserved sequences, will have similar responses to agonists. To verify if the newly cloned rat sequence responded to extracellular nucleotides, we heterologously expressed it in 1321N1 astrocytoma cells, a cell line that has been reported to be devoid of endogenous P2Y receptors and has been extensively utilised for the functional characterisation of cloned P2Y receptors [13,21,28,29]. Previous studies of our laboratory have confirmed these cells as an adequate experimental system to functionally express P2Y receptors, since the transient transfection of these cells with other already characterised P2Y receptor

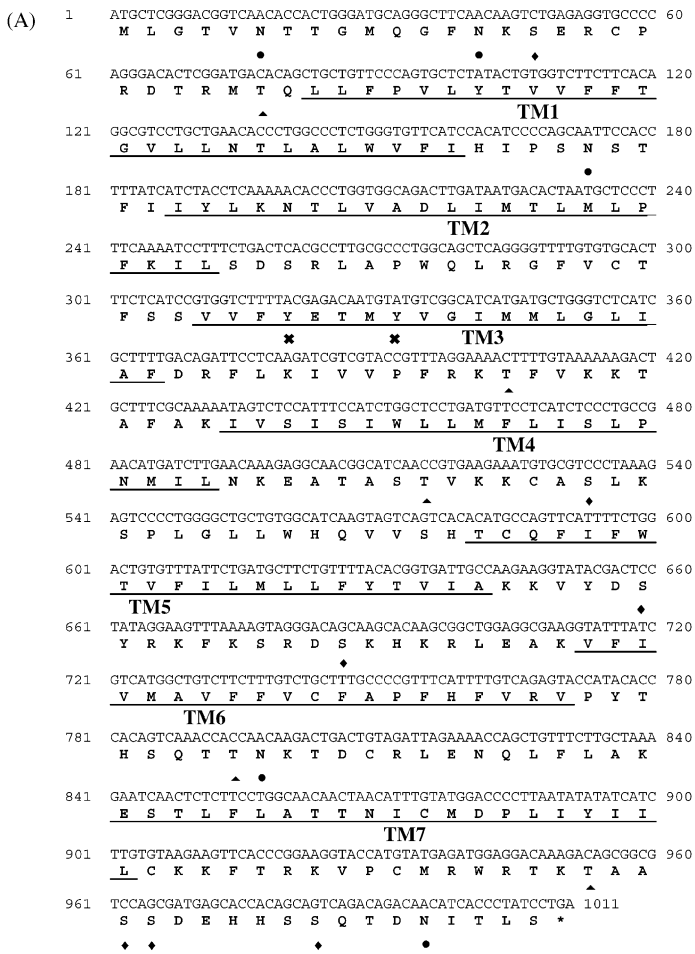

Fig. 2. (A) Nucleotide and deduced amino acid sequences of the ORF of the putative rat $P2Y_{13}$ receptor. Regions corresponding to the hydrophobic putative transmembrane domains TM1–TM7 are underlined. The potential sites of N-linked glycosylation (●: asparagine) and of phosphorylation (◆: serine; ▲: threonine; ✱: tyrosine) are shown. (B) Amino acid alignment of the human and mouse $P2Y_{13}$ receptors with the putative amino acid sequence of the newly cloned rat gene, showing homology between species. Regions corresponding to the hydrophobic putative transmembrane domains TM3–TM6–TM7 are highlighted. Boxed residues indicate the typical amino acid motifs believed to be important for nucleotide binding in TM6 and TM7. The overall percentage identity of the putative rat $P2Y_{13}$ receptor is 79 and 87 with human and mouse $P2Y_{13}$ receptors, respectively. (*) Amino acid present in all aligned sequences; (:) presence of conservative substitutions; (·) presence in 50–75% of aligned sequences. Sequences were aligned using ClustalX 1.8.

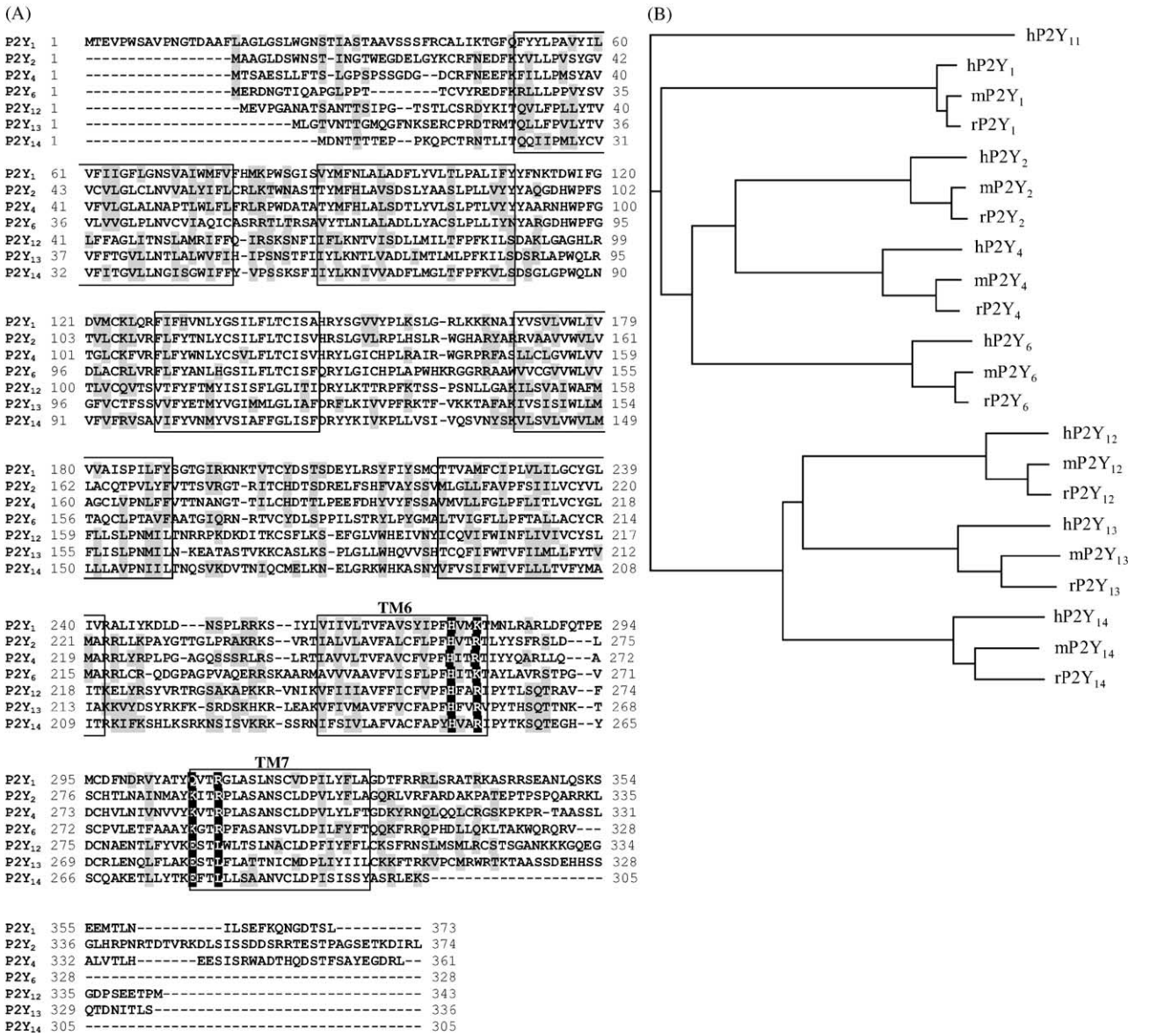

Fig. 3. (A) Amino acid alignment of the six recognised members of the rat P2Y receptor family (rP2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{12}$, P2Y$_{14}$) and the deduced amino acid sequence of the putative rP2Y$_{13}$ receptor. Boxed regions indicate the putative hydrophobic transmembrane domains TM1–TM7. The typical amino acid motifs believed to be important for nucleotide binding in TM6 and TM7 are highlighted in white. Amino acid present in 50–75% of aligned sequences are highlighted in gray. (B) Phylogenetic tree (dendrogram) showing the relationships among all P2Y receptors cloned from man (h), mouse (m) and rat (r), including the putative rat P2Y$_{13}$ receptor cloned in this study.

subtypes (i.e. hP2Y$_1$ and hP2Y$_4$) induces the appearance of functional responses to extracellular nucleotides (i.e. ADP and ATP/UTP, respectively) (data not shown).

The ability of exogenously-added nucleotides to activate the newly cloned receptor in rP2Y$_{13}$-expressing 1321N1 cells has been evaluated by measuring [^{35}S]GTPγS binding. It is known that, upon activation by their cognate ligands, GPCRs couple to G-proteins, and this results in increases of binding of GTP to G-proteins, which can, in turn, be easily determined by quantifying the binding of [^{35}S]GTPγS to membranes [15]. As shown in Fig. 5B, in 1321N1 cells transiently transfected with the rP2Y$_{13}$ receptor, ADP and 2MeSADP (two typical agonists of human and mouse P2Y$_{13}$ receptors) stimulated [^{35}S]GTPγS binding in a concentration-dependent manner, with an EC$_{50}$ value of 442 ± 33 nM and 1170 ± 99 nM, respectively (Table 1). Co-transfection of rat P2Y$_{13}$ receptor with the Gα$_{16}$ subunit of G-proteins induced a significant increase in ADP and 2MeSADP potency, as demonstrated by significant reductions of their EC$_{50}$ values (128 ± 11.2 nM and 677 ± 51 nM, respectively, Fig. 5C, Table 1). The same adenine di-phospho-nucleotides did not activate G-proteins in 1321N1 cells transiently transfected with the empty plasmid (Fig. 5A).

Other adenine or uridine nucleotides, such as ATP, UTP, αβMeATP, βγMeATP, (10–100 μM), UDP-glucose

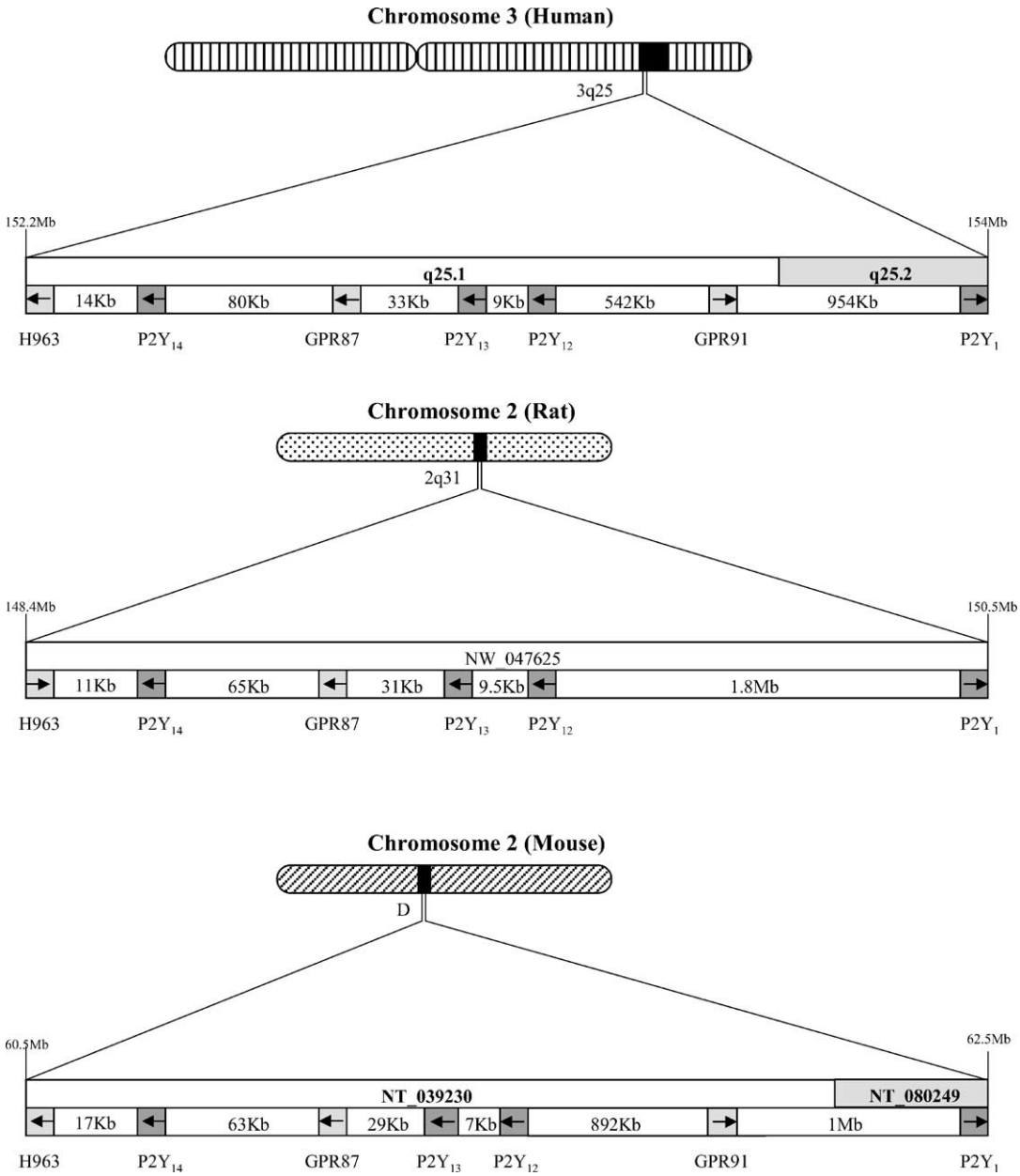

Fig. 4. The cluster of P2Y receptors and related genes on human chromosome 3 and rat mouse chromosomes 2. The schematic representations was reproduced from an Ensembl database search using Enterez Map View and NCBI Sequence Viewer. The chromosomal bands, the size of the intergenic regions and the corresponding genomic contigs (for mouse and rat) are reported. The arrows on the genes represent their orientation on the chromosomes. The exact locations of four known P2Y receptors ($P2Y_1$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) and of three (two for the rat) "P2Y-like" orphan receptor genes are shown (see text for more details).

(50 μM) were not able to induce any increases of [^{35}S]GTPγS specific binding in cells transfected with rat $P2Y_{13}$ receptor in the absence or presence of $G\alpha_{16}$ (Table 1).

AR-C69931MX, that has been reported to act as a selective antagonist at both human $P2Y_{12}$ [30] and $P2Y_{13}$ receptors [15], concentration-dependently inhibited the [^{35}S]GTPγS binding stimulated by 1 μM ADP in 1321N1 cells transfected with $rP2Y_{13}$ receptor both in the absence and presence of the $G\alpha_{16}$ subunit (Fig. 6A). Inhibition of ADP-stimulated binding was complete between 10 and 100 nM AR-C69931MX, with IC_{50} values of 26 ± 1.9 nM and 1.2 ± 0.06 nM, respectively (Fig. 6A, Table 1). In contrast, the selective $P2Y_1$ receptor antagonist MRS2179 had no effect on the [^{35}S]GTPγS binding stimulated by 1 μM ADP in 1321N1 cells transfected with $rP2Y_{13}$ receptor both in the absence and presence of the $G\alpha_{16}$ subunit (Table 1). The human [13] and mouse [14], $P2Y_{13}$ receptors have been shown to selectively couple to G-proteins of the Gi subfamily. To evaluate the implications of Gi proteins in the effects mediated by the recombinant rat $P2Y_{13}$ receptor, we preincubated 1321N1 cells transfected with $rP2Y_{13}$ in the absence or presence of the $G\alpha_{16}$ subunit with PTX (100 ng/ml for 18 h), which is known to ADP-rybosylate and inactivate Gi

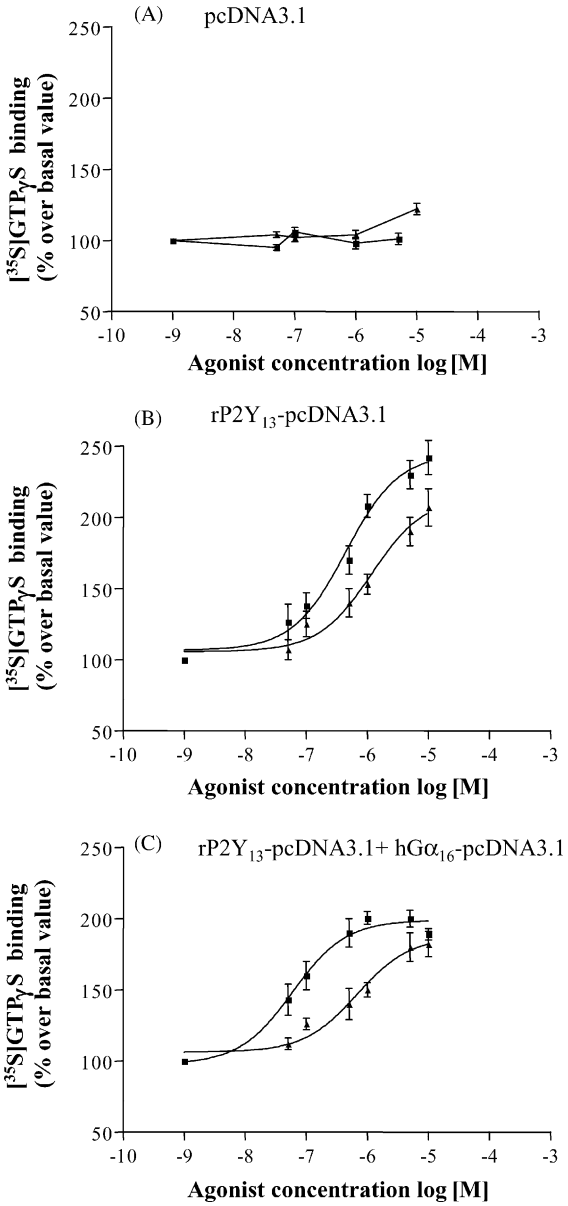

Fig. 5. ADP and 2MeSADP-mediated G-protein activation in 1321N1 cells transiently transfected with the putative rP2Y$_{13}$ receptor in the absence (B) or presence of Gα_{16} protein subunit (C). (A) Agonist concentration–response curve in 1321N1 cells transfected with the empty plasmid (pcDNA3.1) alone. Cell membrane fractions were treated for 15 min at room temperature with the indicated concentrations of ADP (■) or 2MeSADP (▲). Data, expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%, represent the mean ± S.E.M. of determinations obtained in one experiment representative of three.

proteins, prior to membrane preparation and evaluation of [^{35}S]GTPγS binding. PTX treatment resulted in strong inhibition of [^{35}S]GTPγS binding stimulated by 10 μM ADP (Fig. 6B).

3.3. Tissue distribution of the rP2Y$_{13}$ receptor

RT–PCR studies revealed a wide distribution of the rP2Y$_{13}$ receptor (Fig. 7). The highest expression levels were found in the spleen, followed by total brain (with particularly high levels in the cerebral cortex and *C. striatum*, Fig. 7A), liver, kidney and aorta (Fig. 7B and C). Lower but significant levels were also detected in intestine, stomach, skeletal muscle, testis, heart and lung. For a more immediate analysis of rP2Y$_{13}$ receptor distribution, expression levels in the analysed rat tissues are reported in Fig. 7C as percentage of spleen signal.

Table 1
Potency of various purinergic ligands for rat P2Y$_{13}$ receptor in 1321N1 cells

Agonist or antagonist	1321N1 cells expressing rat P2Y$_{13}$ EC_{50} or IC_{50} ± S.E.M.	1321N1 cells expressing rat P2Y$_{13}$ and Gα_{16} EC_{50} or IC_{50} ± S.E.M.
ADP (agonist) (nM)	442 ± 33	128 ± 11.2
2MeSADP (agonist) (nM)	1170 ± 99	677 ± 51
10 μM ATP (agonist) (%)	106 ± 9.3	103 ± 8.4
100 μM αβMeATP (agonist) (%)	99 ± 8.6	97 ± 8.2
100 μM βγMeATP (agonist) (%)	101 ± 9.1	103 ± 5.4
50 μM UDP-glucose (agonist) (%)	100 ± 4.3	101 ± 6.1
50 μM UTP (agonist) (%)	102 ± 7.1	103 ± 4.4
AR-C69931MX (antagonist) (nM)	26 ± 1.9	1.2 ± 0.06
MRS2179 (antagonist) (%)	98 ± 5.5[a]	96 ± 7.3[a]

Results are expressed as EC_{50}/IC_{50} values (means ± S.E.M.; $n = 3$) or as percentage of [^{35}S]GTPγS binding stimulation with respect to basal value (set to 100%).

[a] Percentage of [^{35}S]GTPγS binding with respect to 10 μM ADP (set to 100%). MRS2179 has been tested at 1, 10 and 100 μM concentrations; shown is the percentage of binding stimulation at the highest concentration.

4. Discussion

In the present study, we report, for the first time, the cloning and pharmacological characterisation of the P2Y$_{13}$ receptor from rat tissues. The newly-cloned rat receptor is a 336 amino acid protein with a 79 and 87% sequence identity to the human and mouse P2Y$_{13}$ receptors, respectively, and is 23–49% identical to the other six cloned rat P2Y receptors. As also found for human [3] and mouse P2Y receptors, the newly cloned rat receptor shows highest identity with rP2Y$_{12}$ and rP2Y$_{14}$ (49 and 43%, respectively), and together with these receptors constitutes a phylogenetically- and structurally-distint subgroup within the P2Y receptor family. The newly cloned receptor is on chromosome 2 in close proximity with other P2Y and P2Y-like receptors, in a similar way to what previously reported for human P2Y$_{13}$ receptor (see also below). As expected, when heterologously expressed in 1321N1 cells, rat P2Y$_{13}$ receptor is activated in a concentration-dependent manner by adenine diphospho-nucleotides (although with some important differences, see also below). We also report, for the first time, the tissue distribution of this receptor in the rat. In agreement with the data on the human and mouse receptors [13,14], the highest levels of rat P2Y$_{13}$ receptor

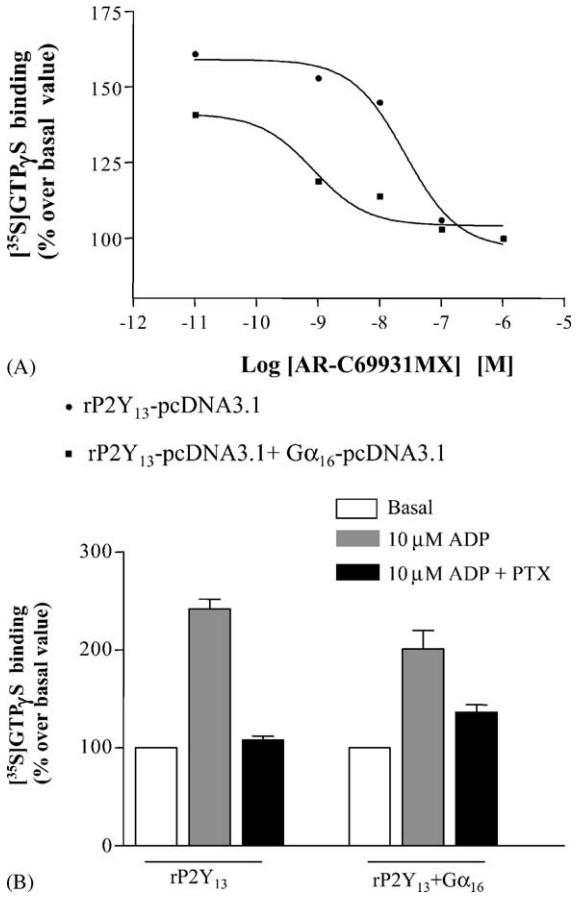

Fig. 6. (A) Effect of AR-C69931MX on stimulation of [^{35}S]GTPγS binding mediated by 1 μM ADP in 1321N1 cells transiently transfected with the putative rP2Y$_{13}$ receptor in the absence or presence of the Gα$_{16}$ protein subunit, as indicated. Cell membrane fractions were treated for 15 min at room temperature with 1 μM ADP, in the absence or presence of the indicated concentrations of AR-C69931MX. Data, expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%, represent the mean ± S.E.M. of determinations obtained in one experiment representative of three. EC$_{50}$ values of AR-C69931MX: 26 nM in rP2Y$_{13}$-pcDNA3.1, and 1.2 nM in rP2Y$_{13}$-pcDNA3.1 + hGα$_{16}$-pcDNA3.1. (B) Effect of PTX on ADP-stimulated [^{35}S] binding in 1321N1 cells transfected with the putative rP2Y$_{13}$ receptor in the absence or presence of the Gα$_{16}$ protein subunit, as indicated. After transfection, cells were pre-incubated with PTX (100 ng/ml for 18 h) prior to membrane preparation and evaluation of [^{35}S]GTPγS binding in the absence (basal) or presence of 10 μM ADP. The increase of [^{35}S]GTPγS binding induced by 10 μM ADP was markedly inhibited by PTX.

were found in the spleen, followed by brain and liver, confirming that this receptor may subserve particularly important pathophysiological roles in the immune and nervous systems. Within the brain, significant levels of expression were found in rat cerebral cortex and *C. striatum*: these data are consistent with the remarkably high concentration of P2Y$_{13}$ receptor in human basal ganglia (caudate, putamen and substantia nigra [14]), suggesting that the rat animal model may appropriately be utilised to study the role of this receptor subtype in the extrapyrimidal system and in regulation of motor behaviour. Regarding the cellular distribution of this receptor within brain cells, in the present study we found that primary rat astrocytes express the P2Y$_{13}$ transcript at levels similar to those of the other cloned P2Y receptors, which may indicate a role for the newly-cloned receptor in reactive astrogliosis [17,18]. In agreement with what found in man [14], high levels of expression were also found in rat small intestine, confirming the rat as an adequate model to assess the role of P2Y$_{13}$ receptor in this tissue. In contrast, at variance from both the human and mouse receptors [14], P2Y$_{13}$ receptor was also expressed at significant levels in rat aorta, heart, kidney, skeletal muscle and stomach (Fig. 7). These differences will have of course to be taken into consideration when using the rat in assessing the pathophysiological roles of the P2Y$_{13}$ receptor.

Since its initial cloning, the pharmacology of the human P2Y$_{13}$ receptor has been characterised in various cell lines (e.g. 1321N1, AG-32, CH0-K1 and HEK293-EBNA cells) by means of inositol phosphate, cAMP, Fluorometric Imaging Plate Reader (FLIPR) assays [13,14], and, more recently, binding of [^{32}P]2MeSADP and of [^{35}S]GTPγS [15]. Globally, results show that the relative potencies of 2MeSADP and ADP (the typical agonists at the P2Y$_{13}$ receptor) at the human receptor are critically dependent on the experimental conditions. In particular, while in some assays (including [^{35}S]GTPγS binding, see also below), 2MeSADP was significantly more potent than ADP, in other experimental assays these two agonists were almost equipotent (ibidem), suggesting that *human* P2Y$_{13}$ receptor could exist in multiple active conformations characterised by "differences in affinity for either agonist, kinetics and preference for G-proteins" (see [15], Section 4). In the only study where the pharmacology of the *murine* P2Y$_{13}$ receptor was characterised, this receptor was found to equally respond to ADP and 2MeSADP, with EC$_{50}$ values of 4.1 and 6.6 nM, respectively, in the FLIPR assay [14]. In the present study, by utilising the [^{35}S]GTPγS binding assay in 1321N1 cells expressing the *rat* receptor, ADP was found to more potently activate P2Y$_{13}$ receptor with respect to 2MeSADP. Co-transfection of these same cells with both the rat receptor and the Gα$_{16}$ subunit of G-proteins induced a significant increase in stimulation of [^{35}S]GTPγS binding by ADP and 2MeSADP, with no changes in their relative potency, suggesting that, in a similar way to *human* P2Y$_{13}$ receptor [13], the *rat* receptor can also couple to this G-protein. However, at variance from *human* P2Y$_{13}$ receptor [15], in this assay 2MeSADP is more potent that ADP at the *rat* receptor (the present results). The present data also show that 10 μM ATP did not activate the *rat* receptor. This is in line with what previously shown for *human* P2Y$_{13}$ receptor, at which ATP and 2MeSATP behaved as weak partial agonists [15]. However, this finding is apparently in contrast with that reported for the *murine* receptor, which was activated by ATP, although with an EC$_{50}$ value approximately 60-fold lower with respect to adenine diphospho-nucleotides [14]. Rat P2Y$_{13}$ receptor was not activated by αβMeATP,

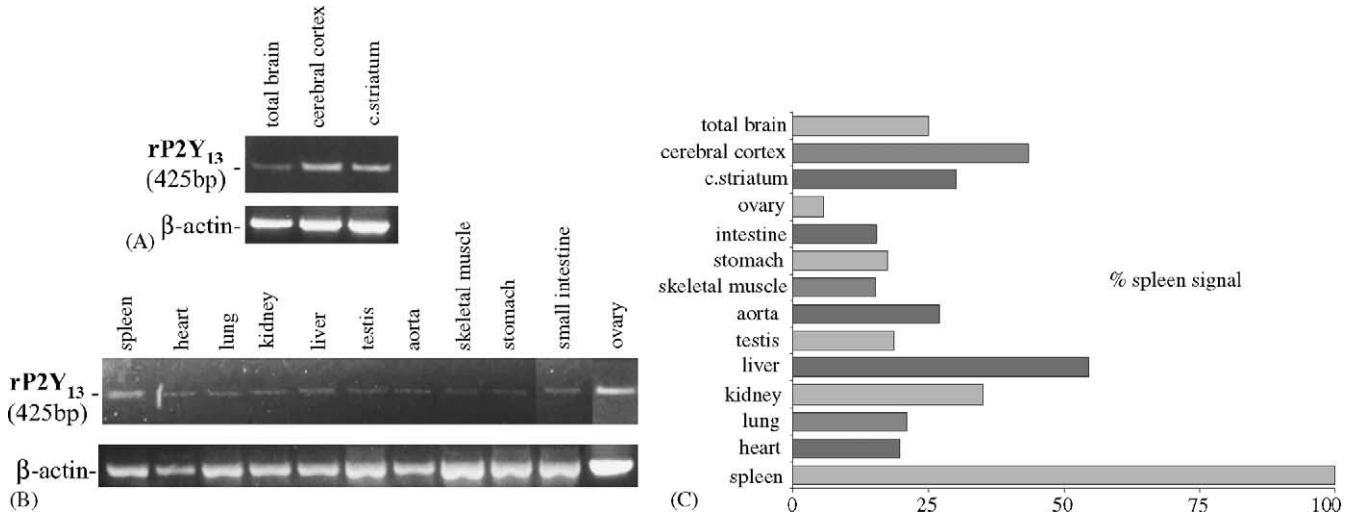

Fig. 7. Expression of $P2Y_{13}$ receptor in rat tissues. In these experiments, $P2Y_{13}$ mRNA was detected by RT–PCR as a specific amplification product of 425 bp (see Section 2). (A) Presence of $P2Y_{13}$ receptor in total rat brain and in selected rat brain areas. (B) Presence of $P2Y_{13}$ receptor in the indicated rat tissues. For data reported in (A) and (B), receptor expression has been evaluated in parallel with the house-keeping gene β-actin. No signals were detected in parallel RNA samples that had not undergone retro-trascription (−RT, data not shown; see Section 2 for more detail). (C) Relative quantitation of the $P2Y_{13}$ expression data shown in (A) and (B), here reported as percentage of signal detected in the spleen, the rat tissue showing the highest $P2Y_{13}$ receptor expression. For all other tissues, receptor expression has been normalised to the corresponding β-actin signal, and then reported as percentage of the spleen signal set to 100%.

βγMeATP, UTP or by the typical $P2Y_{14}$ receptor agonist UDP-glucose. However, in this case no comparison with the behaviour of human and mouse $P2Y_{13}$ receptor is possible, since these nucleotides have been not tested on these orthologues. The AR-C69931MX, which has been previously reported to act as a very potent antagonist of human $P2Y_{12}$ and $P2Y_{13}$ receptors, with comparable IC_{50} values in the low nanoMolar range [15–30] also antagonised the actions mediated by ADP at rat $P2Y_{13}$ receptor with comparable potency (the present results). In a similar way to the human receptor [15], $rP2Y_{13}$ receptor was not antagonised by the selective $P2Y_1$ receptor antagonist MRS2179.

Globally, these results suggest that *rat* $P2Y_{13}$ receptor shares important similarities with its *human* and *mouse* orthologues, but also displays important pharmacological differences which will have to be taken into account when dissecting the effects mediated by this (and other P2Y receptors) in the rat animal models (see for example [31]). Our results also confirm the conclusions of Marteau et al. [15] that the $P2Y_{13}$ receptor is even more similar to the $P2Y_{12}$ than initially believed, as also suggested by antagonism by AR-C69931MX. These similarities seem logical when considering the high degree of aminoacid identity between $P2Y_{12}$ and $P2Y_{13}$ (47.7 and 49% for the human and rat receptors, respectively), and the short distance between these two genes in both the human and rat genome. In this respect, a remarkable clustering of four known P2Y receptor genes and three closely-related GPCRs was recently found on the human chromosome 3q24–3q25 ([26]; Fig. 4). Three of these genes ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) are branched together in the dendrogram of P2Y receptors ([3]; see also Section 1) and are in close proximity with each other (Fig. 4; [27]). The fourth gene is the distantly-related $P2Y_1$ receptor which is approximately 1 Mb away. Three ‘‘orphan’’ GPCRs (i.e. GPR87, GPR91 and H963) are also present in this region. H963 and GPR87 (which are structurally more similar to the $P2Y_{12}$–$P2Y_{14}$ series, and also display the typical amino acid motifs in TM6 and TM7 important for ligand recognition) are in very close proximity with the $P2Y_{14}$, $P2Y_{13}$ and $P2Y_{12}$ genes. The third orphan GPR91 is at approximately 500 kb away, toward the $P2Y_1$ gene (Fig. 4; [27]). This chromosomal localisation suggests that these receptors may have arisen from a common ancestral nucleotide receptor by gene duplication. During evolution the sequences of these receptors have diverged, leading to altered, but still related, ligand preferences [26]. As also confirmed by our Blast analysis, this clustering has been remarkably conserved in both mouse and rat, with the only exception of GPR91 that does not seem to be present in the rat genome (Fig. 4). Besides confirming that the newly cloned rat protein reported here is indeed the rat ortholog of the human $P2Y_{13}$ receptor, such an high conservation of chromosomal localisation suggests that the rat and human receptors may subserve similar physiological functions.

With the rat ortholog of $P2Y_{13}$ in hand, we can now begin to use the rat as model organism to fully characterise the pathophysiological roles of this novel P2Y receptor subtype.

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References

[1] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.
[2] Jacobson KA, Jarvis MF, Williams M. Purine and pyrimidine (P2) receptors as drug targets. J Med Chem 2002;45:4057–93.
[3] Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, et al. Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. Trends Pharm Sci 2003;24:52–5.
[4] Communi D, Robaye B, Boeynaem JM. Pharmacological characterization of the human $P2Y_{11}$ receptor. Br J Pharmacol 1999;128: 1199–206.
[5] Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR. Effect of loss of P2Y(2) receptor gene expression on nucleotide regulation of murine epithelial Cl(−) transport. J Biol Chem 1999;274(37):26461–8.
[6] Robaye B, Ghanem E, Wilkin F, Fokan D, Van Driessche W, Schurmans S, et al. Loss of nucleotide regulation of epithelial chloride transport in the jejunum of $P2Y_4$-null mice. Mol Pharmacol 2003;63(4):777–83.
[7] Dubyak GR. Knock-out mice reveal tissue-specific roles of P2Y receptor subtypes in different epithelia. Mol Pharmacol 2003;63(4): 773–6.
[8] Turner NA, Moake JL, Mclntire LV. Blockade of adenosine diphosphate receptors $P2Y_{12}$ and $P2Y_1$ is required to inhibit platelet aggregation in whole blood under flow. Blood 2001;98:3340–5.
[9] Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest 2001;107:1591–8.
[10] Boeynaems JM, Wilkin F, Marteu F, Duhant X, Savi P, Gonzalez NS, et al. P2Y receptors: new subtypes, new functions. Drug Dev Res 2003;59:30–5.
[11] Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, et al. A G protein-coupled receptor for UDP-glucose. J Biol Chem 2000;275:10767–71.
[12] Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001;409(6817):202–7.
[13] Communi D, Gonzalez NS, Detheux M, Brézillon S, Lannoy V, Parmentier M, et al. Identification of a novel human ADP receptor coupled to Gi. J Biol Chem 2001;276:41479–85.
[14] Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, et al. P2Y(13): identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. J Pharmacol Exp Ther 2002;301(2):705–13.
[15] Marteau F, Le Poul E, Communi D, Labouret C, Savi P, Boeynaems JM, et al. Pharmacological characterization of the human $P2Y_{13}$ receptor. Mol Pharmacol 2003;64:104–12.
[16] Abbracchio MP, Williams M. Purinergic and pyrimidinergic signalling I, II (Handbook of experimental pharmacology 151/I, II). Berlin, Heidelberg: Springer-Verlag; 2001.
[17] Brambilla R, Abbracchio MP. Modulation of cyclooxygenase-2 and brain reactive astrogliosis by purinergic P2 receptors. Ann NY Acad Sci 2001;939:54–62.
[18] Brambilla R, Neary JT, Cattabeni F, Cottini L, D'Ippolito G, Schiller PC, et al. Induction of COX-2 and reactive gliosis by P2Y receptors in rat cortical astrocytes is dependent on ERK1/2 but independent of calcium signalling. J Neurochem 2002;83:1285–96.
[19] Brambilla R, Cottini L, Fumagalli M, Ceruti S, Abbracchio MP. Blockade of A2A adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes. Glia 2003;43(2):190–4.
[20] Fumagalli M, Brambilla R, D'Ambrosi N, Volonte C, Matteoli M, Verderio C, et al. Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. Glia 2003;43: 218–30.
[21] Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J Biol Chem 1997;19:31969–73.
[22] Gilman AG. G proteins: transducers of receptor-generated signals. Ann Rev Biochem 1987;56:615–49.
[23] Strader CD, Fong TM, Graziano MP, Tota MR. The family of G-protein-coupled receptors. FASEB J 1995;9:745–54.
[24] Erb L, Garrad R, Wang Y, Quinn T, Turner JT, Weisman GA. Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. J Biol Chem 1995;270:4185–8.
[25] Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, et al. A mutation analysis of residues essential for ligand recognition at the human $P2Y_1$ receptor. Mol Pharmacol 1997;52:499–507.
[26] Wittenberger T, Schaller HC, Hellebrand S. An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J Mol Biol 2001;307:799–813.
[27] Simon J, Barnard EA. The P2Y nucleotide receptors in the human genome. Acta Biologica Hungarica 2003;54:191–201.
[28] Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. Biochem Biophys Res Commun 1996;222:303–8.
[29] Filtz TM, Li Q, Boyer JL, Nicholas RA, Harden TK. Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. Mol Pharmacol 1994;46:8–14.
[30] Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, Mclnally JI, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 1999;42:213–20.
[31] Queiroz G, Talaia C, Goncalves J. ATP modulates noradrenaline release by activation of inhibitory P2Y receptors and facilitatory P2X receptors in the rat vas deferens. J Pharmacol Exp Ther 2003;307:809–15.