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Molecular cloning and characterization of the mouse $P2Y_4$ nucleotide receptor

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Abstract

To isolate the mouse $P2Y_4$ receptor gene, a mouse genomic library was screened with a human $P2Y_4$ probe. An open reading frame encoding a protein of 361 amino acids was isolated. This protein showed 82% and 95% amino acid identity with the human and rat $P2Y_4$ receptors, respectively. By reverse transcription and polymerase chain reaction (RT-PCR), the $P2Y_4$ messenger RNA was detected in mouse liver, intestine, stomach, bladder and lung among the 16 mouse tissues tested. In 1321N1 transfected cells, the mouse $P2Y_4$ receptor was equally activated by UTP and ATP, and was antagonized by pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and Reactive Blue 2, and not by suramin. Moreover, when expressed in 1321N1 cells, the rat $P2Y_4$ is also antagonized by PPADS. Thus, when compared in the same expression system, the mouse $P2Y_4$ is closer to the rat ortholog in terms of agonist stimulation, while in terms of antagonist profile, the three $P2Y_4$ receptor orthologs are similar.

Keywords: $P2Y_4$ receptor; ATP; UTP; P2 receptor antagonist; 1321N1 astrocytoma cell line

1. Introduction

Despite strong conservation in amino acid sequence, human and rat $P2Y_4$ receptors do not exhibit full conservation in terms of agonist specificity. Indeed, the human $P2Y_4$ is activated preferentially by UTP, and ATP behaved like a partial agonist (Communi et al., 1995) or an antagonist (Kennedy et al., 2000), while the rat ortholog of this receptor is equipotently activated by ATP and UTP (Bogdanov et al., 1998; Webb et al., 1998). The human and rat $P2Y_4$ receptors exhibit also different sensitivity to various P2 receptor antagonists: the rank order of potency being pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) > Reactive Blue 2 ≫ suramin = 0 for the human $P2Y_4$ receptor expressed in 1321N1 cells (Communi et al., 1996) and Reactive Blue 2 ≫ suramin > PPADS = 0 for the rat $P2Y_4$ receptor expressed in *Xenopus* oocytes (Bogdanov et al., 1998).

In the present report, we present data on the cloning, the pharmacological characterization and the tissue distribution of the mouse ortholog of $P2Y_4$.

2. Materials and methods

2.1. Materials

Trypsin was from Flow laboratories (Bioggio, Switzerland). The culture media, the fetal calf serum, G418, Platinum® *Pfx* DNA polymerase, Superscript™ II preamplification system and restriction enzyme were purchased from Gibco (Merelbeke, Belgium). Taq polymerase was from Qiagen (Valencia, CA, USA). Myo-D-2-[^{3}H]inositol (17.7 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) were supplied by Amersham (Ghent, Belgium). FuGENE™ 6 Transfection Reagent was from Boehringer Mannheim. pEFIN3 is an expression vector developed by EUROSCREEN (Brussels, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Nazareth Eke, Bel-

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gium). ATP and UTP were obtained from Sigma (St Louis, MO, USA). Suramin, Reactive Blue 2 and pyridoxal-phosphate-6 azophenyl-2′,4′-disulphonic acid (PPADS) were from Research Biochemicals (Natick, MA, USA)

2.2. Cloning and sequencing

A mouse genomic 129/SVJ DNA library in Lambda FIX II (Stratagene, La Jolla, CA) was screened with a

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mP2Y4   MTSADSLLFTSLGPSPSSGDG----DCKFNEEFKFILLP
rP2Y4   MTSAESLLFTSLGPSPSSGDG----DCRFNEEFKFILLP
hP2Y4   MASTESSLLRSLGLSPGPGSSEVELDCWFDEDFKFILLP
        *:*::* *: *** **  *      ** *:*:*******

mP2Y4   LSYAVVFVLGLALNAPTLWLFLFRLRPWDATATYMFHLA
rP2Y4   MSYAVVFVLGLALNAPTLWLFLFRLRPWDATATYMFHLA
hP2Y4   VSYAVVFVLGLGLNAPTLWLFIFRLRPWDATATYMFHLA
        :********** *********:******************

mP2Y4   LSDTLYVLSLPTLVYYYAARNHWPFGTGFCKFVRFLFYW
rP2Y4   LSDTLYVLSLPTLVYYYAARNHWPFGTGLCKFVRFLFYW
hP2Y4   LSDTLYVLSLPTLIYYYAAHNHWPFGTEICKFVRFLFYW
        *************:*****:******* :**********

mP2Y4   NLYCSVLFLTCISVHRYMGICHPLRAIRWGRPRFAGLLC
rP2Y4   NLYCSVLFLTCISVHRYLGICHPLRAIRWGRPRFASLLC
hP2Y4   NLYCSVLFLTCISVHRYLGICHPLRALRWGRPRLAGLLC
        ******************:********:******:* ***

mP2Y4   LGVWLVVAGCLVPNLFFVTTNANGTTILCHDTTLPEEFD
rP2Y4   LGVWLVVAGCLVPNLFFVTTNANGTTILCHDTTLPEEFD
hP2Y4   LAVWLVVAGCLVPNLFFVTTSNKGTTVLCHDTTRPEEFD
        * ******************  :***:****** *****

mP2Y4   HYVYFSSTIMVLLFGFPFLITLVCYGLMARRLYRPLPGA
rP2Y4   HYVYFSSAVMVLLFGLPFLITLVCYGLMARRLYRPLPGA
hP2Y4   HYVHFSSAVMGLLFGVPCLVTLVCYGLMARRLYQPLPGS
        ***:***::* **** * *:*************:****:

mP2Y4   GQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYLARL
rP2Y4   GQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYQARL
hP2Y4   AQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYLARL
         ********************************** ***

mP2Y4   LNAECRVLNIVNVVYKVTRPLASANSCLDPVLYLFTGDK
rP2Y4   LQADCHVLNIVNVVYKVTRPLASANSCLDPVLYLFTGDK
hP2Y4   LEADCRVLNIVNVVYKVTRPLASANSCLDPVLYLLTGDK
        *:*:*:*****************************:****

mP2Y4   YRNQLQQLCRGSTPKRRTTASSLALVTLHEESISRWADI
rP2Y4   YRNQLQQLCRGSKPKPRTAASSLALVTLHEESISRWADT
hP2Y4   YRRQLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAAT
        ** **:*** *  *: **:*******:* *:*  ***

mP2Y4   HQDSIFPAYEGDRL 361
rP2Y4   HQDSTFSAYEGDRL 361
hP2Y4   PQDSSCSTPRADRL 365
         ***    :  ***
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Fig. 1. Alignment of the three cloned $P2Y_4$ sequences. The amino acid sequences of rat $P2Y_4$ (361aa; Y14705), mouse $P2Y_4$ (361aa; AJ277752) and human $P2Y_4$ (365aa; X91852) are aligned. Hydrophobic regions (transmembrane domains) are indicated by bars. Identical amino acids are indicated by (*), conserved amino acids by (:). Gaps (-) were introduced to maximize the alignment.

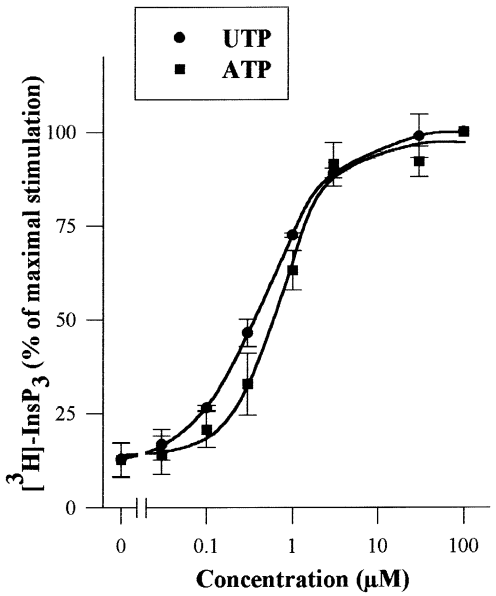

Fig. 2. Concentration–action curves of UTP and ATP on the $InsP_3$ accumulation in mouse $P2Y_4$ receptor-transfected 1321N1 cells. The cells were incubated 30 s in the presence of various UTP and ATP concentrations (0.03, 0.1, 0.3, 1, 3, 30 and 100 μM). The data represent the mean ± S.E.M. from three independent experiments each in triplicate. The EC_{50} values are: EC_{50} UTP = 0.4 ± 0.08 μM and EC_{50} ATP = 0.7 ± 0.3 μM (mean ± S.D. of EC_{50} values obtained from three independent experiments).

radiolabelled human $P2Y_4$ cDNA (600 bp located between transmembrane domains 3 and 7) as a probe. Hybridizations were performed at 42°C in 35% formamide, 5 mM EDTA, 6 × SSC (sodium chloride/sodium citrate) and 0.25% nonfat dry milk for 16 h and the final wash conditions were 0.5 × SSC, 0.12% sodium dodecyl sulfate (SDS) at 55°C. The positive clones isolated were sequenced using an Applied Biosystems Model 370A sequencer.

2.3. Cell culture and transfection

The complete receptor coding sequence was amplified using the Platinum® *Pfx* DNA polymerase and specific mouse $P2Y_4$ primers, and subcloned between the *Bam*HI and *Spe*I sites of the pEFIN3 expression vector. Three micrograms of recombinant plasmid were transfected into 1321N1 astrocytoma cells using the FuGENE™ 6 reagent. Transfected cells were selected with G418 (400 μg/ml) and maintained in Dulbecco's modified Eagle's medium (DMEM) culture medium (10% fetal calf serum, 5% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B) containing

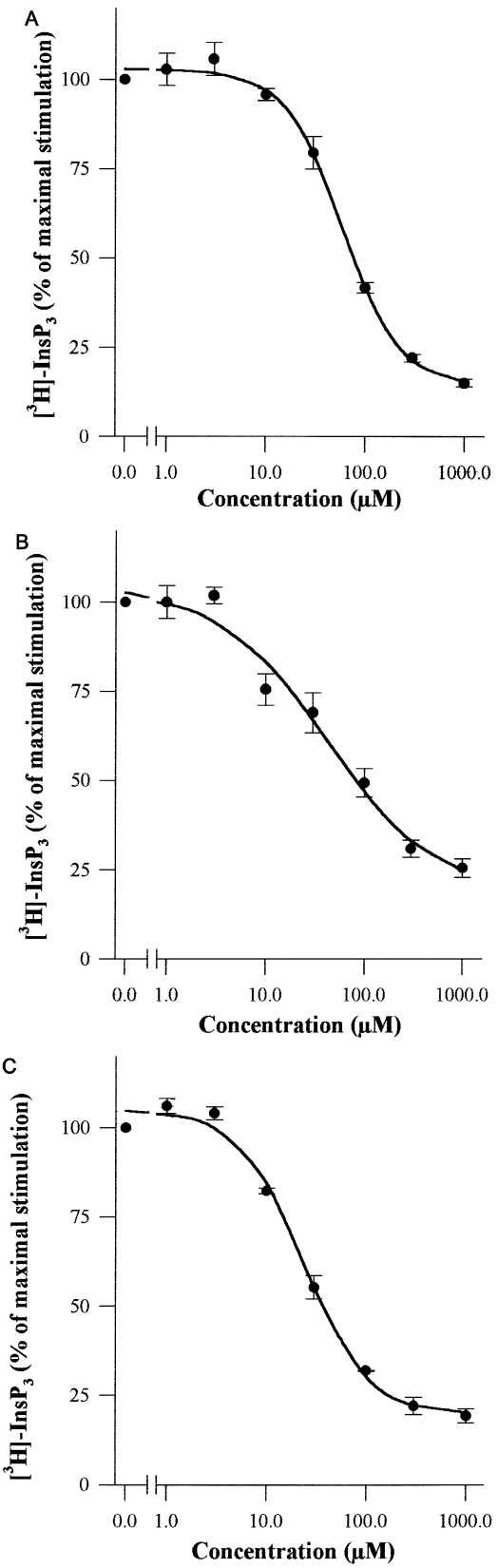

Fig. 3. Concentration-dependence of PPADS and Reactive Blue 2 inhibition of the UTP response in mouse (A, B) and rat $P2Y_4$ (C) receptor-transfected 1321N1 cells. The cells were exposed to various concentrations (1, 3, 10, 30, 100, 300 μM and 1 mM) of PPADS (A, C) and Reactive Blue 2 (B) for 20 min. UTP at a concentration of 1 μM was then added for 30 s. For the mouse $P2Y_4$ receptor, the IC_{50} PPADS = 45 ± 15 μM and IC_{50} Reactive Blue 2 = 47 ± 12.5 μM. For the rat $P2Y_4$ receptor, the IC_{50} PPADS = 25 ± 3.9 μM. In (A) and (B), data are means ± S.E.M. from three separate experiments each in triplicate. In (C), data are means ± range from two separate experiments each in triplicate.

G418 (400 μg/ml). At confluency, cells were trypsinized (1 mg/ml). The 1321N1 cells transfected with the rat $P2Y_4$ receptor were a generous gift from Kendall Harden, University of North Carolina (Kennedy et al., 2000).

2.4. Measurement of inositol triphosphate ($InsP_3$) production

1321N1 cells were seeded in 35 mm Petri dishes in DMEM culture medium (at 2×10^5 cells/dish). The next day, they were labelled 24 h with [^{3}H]inositol (10 μCi/ml) in 5% fetal calf serum DMEM culture medium. Cells were then rinsed twice and placed in KRH (Krebs–Ringer HEPES) buffer (124 mM NaCl; 5 mM KCl; 1.25 mM $MgSO_4$; 1.45 mM$CaCl_2$; 1.25 mM KH_2PO_4; 25 mM HEPES pH 7.4 and 8 mM glucose). After 3 h, tested agonists were added in this same medium for 30 s. When tested, antagonists were added 20 min before stimulation. The incubation was stopped by removing medium and by adding 3% ice-cold perchloric acid solution. Inositol phosphates were separated and extracted on Dowex columns as described previously (Communi et al., 1995). The figures were realized using Sigma plot 2.0. The EC_{50} and IC_{50} values were determined by curve fitting (Sigma plot version 2.0).

2.5. Reverse transcription and polymerase chain reaction (RT-PCR) analysis

Total mouse RNA was extracted from different BALB/C organs by TCG/phenol/chloroform extraction as described by Chomczynski and Sacchi (1987). One microgram of total RNA was submitted to reverse transcription using the Superscript™ II Preamplification system with random hexamers primers (Gibco). Briefly, cDNA was synthesized from 1 μg of each total RNA preparation in a 21 μl reaction volume, in the presence of 200 units of Superscript™ II reverse transcriptase according to the manufacturer's recommendations. Oligonucleotide amplification primers (22- and 20-mers) were designed from the mouse $P2Y_4$ sequence: sense primer 5′-AGCCCAAGTTCTGGAGATGGTG-3′; antisense primer 5′-GGTGGTTCCATTGGCATTGG-3′. PCR was performed on 2 μl of the RT reaction, under the following conditions: 60 s at 94°C, 60 s at 61°C, 60 s at 72°C for 30 cycles. PCR reactions that included each cDNA synthesis reagent except reverse transcriptase were set up in parallel as control for genomic DNA contamination. Each PCR reaction was performed on two independent RT reactions. Amplification

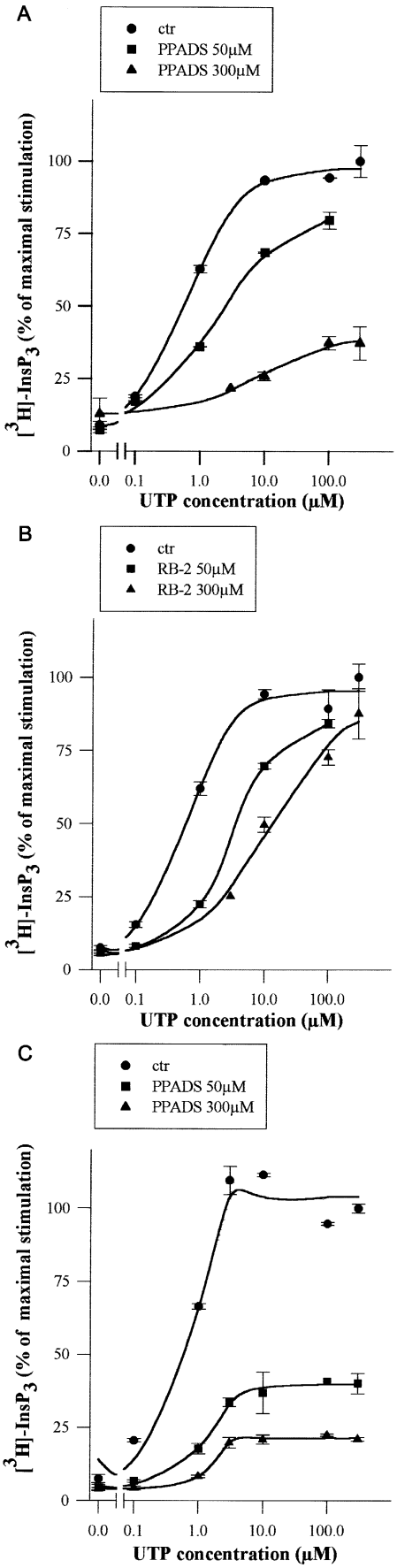

Fig. 4. Effect of PPADS and Reactive Blue 2 on the UTP stimulation of $InsP_3$ in 1321N1-transfected cells. The mouse $P2Y_4$-transfected cells (A and B) and rat $P2Y_4$-transfected cells (C) were incubated for 20 min in the presence or the absence of two concentrations (50 and 300 μM) of PPADS (A, C) or Reactive Blue 2 (B), and then exposed to various concentrations of UTP for 30 s. The data represent the mean ± range from two separate experiments each in triplicate.

products were resolved on a 1.5% (w/v) agarose gel by electrophoresis.

3. Results

3.1. Cloning of the mouse $P2Y_4$ receptor

Four clones were isolated by the screening of a mouse genomic library with a human $P2Y_4$ probe. Analysis by restriction and Southern blotting revealed that each clone contained an identical 16 kb insert. Subcloned fragments of this insert were sequenced and an intronless open reading frame of 361 codons was identified (accession number AJ277752; Fig. 1). The comparison of deduced amino acid sequences of rat $P2Y_4$ and mouse $P2Y_4$ revealed that they were 95% identical, while human $P2Y_4$ and mouse $P2Y_4$ exhibited 82% of identity in amino acids (Fig. 1).

3.2. Functional characterization of the mouse $P2Y_4$ receptor in the 1321N1 cells

The open reading frame of the $P2Y_4$ was amplified by PCR reaction, sequenced and subcloned in the pEFIN3 expression vector. After transfection with the recombinant vector, the 1321N1 astrocytoma cells were selected and characterized for their response to different nucleotides. As shown in Fig. 2, UTP and ATP were equiactive and equipotent on the murine $P2Y_4$ receptor (EC_{50} UTP = 0.4 $\pm$ 0.08 μM; EC_{50} ATP = 0.7 $\pm$ 0.3 μM). The ability of suramin, PPADS and Reactive Blue 2 to inhibit the UTP response was tested. At 100 μM, suramin had no effect on the $InsP_3$ accumulation induced by 1 μM UTP in transfected cells (data not shown). At the same concentration, PPADS and Reactive Blue 2 inhibited this response by 69.8 $\pm$ 3.4% and 60.3 $\pm$ 6.6%, respectively (data not shown). The potencies of PPADS (IC_{50} = 45 $\pm$ 15 μM; Fig. 3A) and Reactive Blue 2 (IC_{50} = 47 $\pm$ 12.5 μM; Fig. 3B) were comparable. These antagonists increased the EC_{50} for UTP (Fig. 4A and B). However, this shift in the concentration–effect curve induced by PPADS was accompanied by a significant decrease in the maximal stimulation of $InsP_3$ produced by UTP (Fig. 4A). This indicated that the inhibitory effect of PPADS on the UTP response does not occur by competitive inhibition.

A different sensitivity for PPADS has been described between the human and the rat $P2Y_4$ receptor orthologs (Communi et al., 1996; Bogdanov et al., 1998). However, these two receptors were not compared in the same expression system. We observed here that, when expressed in 1321N1 cells, the rat $P2Y_4$ is antagonized by PPADS with a similar potency as the human and the mouse orthologs (IC_{50} = 25 $\pm$ 3.9 μM; Fig. 3C). Like for the other $P2Y_4$ receptors, this inhibition was not competitive (Fig. 4C).

3.3. Tissue distribution of the mouse $P2Y_4$ transcript

The tissue distribution of the $P2Y_4$ receptor was investigated by RT-PCR, using specific primers, on total RNA extracted from 16 different mouse tissues (brain, heart, lung, thymus, spleen, kidney, liver, pancreas, muscle, intestine, stomach, salivary glands, bladder, testis, ovary and uterus). As shown in Fig. 5, the mouse $P2Y_4$ transcript was strongly amplified from liver, intestine and stomach extracts. It was also detected in lung and bladder but with a lower intensity. The band observed with the heart is nonspecific since its size is at least 100 bp above that of the 498 bp product expected.

4. Discussion

We have isolated and cloned the mouse ortholog of the $P2Y_4$ receptor. The predicted amino acid sequences of the human, rat and mouse $P2Y_4$ receptors are highly conserved (Fig. 1). The agonist profile for the mouse $P2Y_4$ expressed in the 1321N1 cell line matches the profile described for the rat $P2Y_4$ receptor. Both are activated by UTP and ATP with almost the same potency (EC_{50} around 1 μM) (Bogdanov et al., 1998; Webb et al., 1998). In the $P2Y_2$ receptor, which is also activated by UTP and ATP, three conserved amino acid residues (H^{262}, R^{265} and R^{296}) have

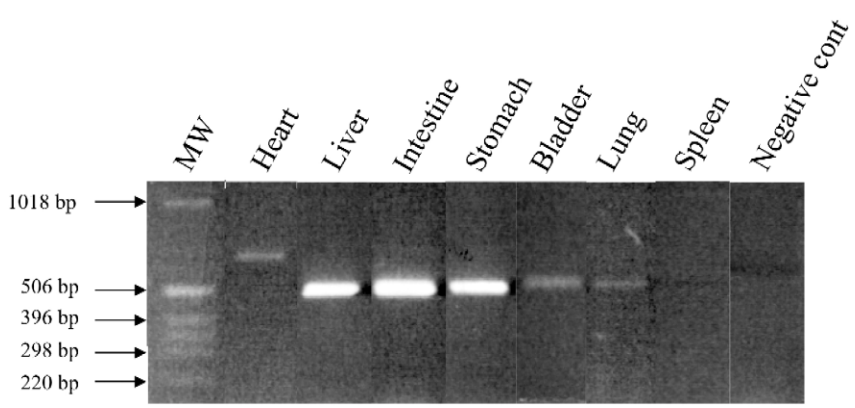

Fig. 5. Detection of mouse $P2Y_4$ mRNA in mouse tissues by RT-PCR experiments. The extraction of RNA and the reverse transcription were performed as described under Materials and methods. PCR products of 498 bp are visualized after electrophoresis on a 1.5% agarose gel and ethidium bromide coloration. Only 7 of the 16 tissues tested are shown.

been shown to be crucial for the binding of both agonists (Erb et al., 1995). These amino acids are also conserved in the three $P2Y_4$ orthologs (Fig. 1) although ATP is only a partial agonist or an antagonist for the human $P2Y_4$ (Communi et al., 1995; Kennedy et al., 2000).

The antagonist profile of the mouse $P2Y_4$ receptor is similar to the one described for the human $P2Y_4$. Indeed, PPADS was the most active antagonist of this receptor with an IC_{50} value around 50 μM, followed by Reactive Blue 2, while suramin was inactive. This rank of activity is similar to the one determined for the human $P2Y_4$ (Communi et al., 1996). In another study, PPADS was found to be only a weak antagonist of the human $P2Y_4$ receptor (Charlton et al., 1996). These authors even described an increase of UTP stimulation by PPADS at lower concentration. The experimental conditions used by these authors were not exactly the same, but no precise explanation of the discrepancy can be provided. Bogdanov et al. (1998) reported, without actually showing the data, that PPADS had no activity on the rat $P2Y_4$ receptor expressed in *Xenopus* oocytes. We show here that when the rat $P2Y_4$ receptor is expressed in 1321N1 cells, PPADS is able to inhibit the UTP-stimulation of this receptor. The exact reason for the discrepancy is unclear, but might obviously be related to the different expression system used. Like for the human and the rat orthologs, Reactive Blue 2 is also a potent inhibitor of the mouse $P2Y_4$, with an IC_{50} value similar to the one measured for PPADS. Like for the human $P2Y_4$, suramin was inactive on the mouse ortholog. We have also observed that when the rat $P2Y_4$ is expressed in 1321N1 cells, suramin at 100 μM was unable to inhibit the UTP-responses (data not shown), while in *Xenopus* oocytes suramin weakly antagonized UTP-responses (Bogdanov et al., 1998). A direct effect of suramin on the particular G protein coupled to $P2Y_4$ receptor in *Xenopus* oocytes cannot be excluded. In conclusion, when compared in the same experimental model, the mouse and rat $P2Y_4$ are equivalent in terms of agonists and antagonists profile.

Concerning the tissue distribution of the $P2Y_4$, little is known about the human ortholog, the messenger RNA of which has been detected in the placenta, peripheral blood leukocytes and several human lung cell lines (Communi et al., 1995; Jin et al., 1998; Communi et al., 1999). The rat $P2Y_4$ receptor messenger distribution has been characterized by RT-PCR analysis (Webb et al., 1998). The transcript was detected in brain, spinal cord, heart and a variety of other peripheral organs. In this study, we detected, by RT-PCR, a strong signal corresponding to the mouse $P2Y_4$ messenger in extracts of liver, stomach and intestine. These last observations are in accordance with recent data suggesting that the UTP and ATP Cl^- secretory response could be mediated by the $P2Y_4$ receptor in the mouse jejunum (Cressman et al., 1999). The mouse $P2Y_4$ messenger has also been detected in the lung tissue, like the human ortholog (Communi et al., 1999). This raises the possibility that the $P2Y_4$ receptor could mediate the residual effect of ATP and UTP on the Cl^- transport by the tracheal epithelium of $P2Y_2 -/-$ mice (Cressman et al., 1999). Contrary to what is observed for the rat receptor, the mouse $P2Y_4$ messenger was not detected in the brain or in the heart (Webb et al., 1998).

In conclusion, in terms of agonist stimulation, the mouse $P2Y_4$ is activated by UTP and ATP and is closer to the rat ortholog, while in terms of antagonist profile, the three $P2Y_4$ receptors are similar.

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