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SPECIAL REPORT

Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor

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An intronless open reading frame encoding a protein (361aa in length) was isolated from a rat genomic library probed with a DNA fragment from rat heart. This protein showed 83% sequence identity with the human $P2Y_4$ ($hP2Y_4$) receptor and represents a homologue of the human pyrimidinoceptor. However, the $rP2Y_4$ receptor is not selective for uridine nucleotides and, instead, shows an agonist potency order of ITP = ATP = ADP(pure) = UTP = ATPγS = 2-MeSATP = Ap_4A > UDP(pure). ADP, ATPγS, 2-MeSATP and UDP are partial agonists. Thus, in terms of agonist profile, $rP2Y_4$ is more like the P2U receptor subtype. The $rP2Y_4$ receptor was reversibly antagonized by Reactive blue 2 but not by suramin which, otherwise, inhibits the $hP2Y_2$ receptor (a known P2U receptor). Thus, $rP2Y_4$ and the $P2Y_2$ subtype appear to be structurally distinct forms of the P2U receptor (where ATP and UTP are equi-active) but can be distinguished as suramin-insensitive and suramin-sensitive P2U receptors, respectively.

Keywords: $P2Y_4$ receptor; nucleotide receptor; pyrimidinoceptor; P2U receptor; ATP; UTP; *Xenopus* oocytes

Introduction The human $P2Y_4$ ($hP2Y_4$) receptor is selective for pyrimidine-based nucleotides (i.e., UTP and UDP) while purine-based nucleotides (e.g., ATP, ADP and ITP) are either weak partial agonists (Communi *et al*., 1995) or seemingly inert (Nguyen *et al*., 1995). Accordingly, the view has been expressed (Communi & Boeynaems, 1997) that $hP2Y_4$ represents an example of the UTP-selective pyrimidinoceptor first proposed by Seifert & Schultz (1989). $hP2Y_4$ receptor-like transcripts have been found in rat neonatal cardiac fibroblasts and cardiac myocytes (Webb *et al*., 1996). Neither the sequence nor the pharmacological profile for this rat $P2Y_4$ ($rP2Y_4$) receptor has been identified. Here, we present data on the isolation, protein sequence and pharmacological properties of the rat homologue of $hP2Y_4$.

Methods Total RNA was isolated from rat heart (Sprague-Dawley, killed by CO_2 inhalation) and cDNA was synthesized using an oligo(dT)-primer and SuperScript reverse transcriptase (Gibco BRL, U.K.). One cDNA fragment was 83% homologous to the previously identified cDNA of the $hP2Y_4$ receptor and was used to screen a rat genomic library (Clontech). The subsequent $rP2Y_4$ clone was purified and subcloned in the *pRN3.lin* transcription vector. Defolliculated *Xenopus* oocytes were injected cytosolically with rat $P2Y_4$ cRNA (40 nl, 1 μg ml^{-1}), incubated for 48 h at 18°C in Barth's solution and kept for up to 7 days at 4°C before the electrophysiological experiments. Nucleotide-evoked membrane currents ($I_{Cl,Ca}$) were recorded from cRNA-injected oocytes using the twin-electrode voltage-clamp technique. Oocytes were superfused with a Ringer solution (5 ml min^{-1}, at 18°C) containing (mM): $NaCl_2$ 110, KCl 2.5, HEPES 5, $CaCl_2$ 1.8, adjusted to pH 7.5. Nucleotides added to the superfusate were applied for 120 s or until currents reached a peak, then washed off with Ringer solution for 60–90 min. Nucleotide responses were normalized to the maximum current (I_{max}) evoked by UTP (100 μM). The concentration that evoked 50% of this maximum response (EC_{50}) was taken from Hill plots. The amount of contaminating ATP in ADP stocks was determined by the method of bioluminescence, using the conversion of luciferin to oxyluciferin catalyzed by firefly luciferase. Contaminating ATP was removed from ADP stocks by adding hexokinase (50 u ml^{-1}) and glucose (25 mM) for 2 h before the assays (see Lazarowski *et al*., 1997). UDP solutions were treated with hexokinase (250 u ml^{-1}) and glucose (25 mM) to remove UTP. P2 antagonists were added to the superfusate in cumulative concentrations, each concentration being applied 60 min before the addition of submaximal concentrations (EC_{70}) of UTP. The antagonist concentration that reduced UTP-responses by 50% (IC_{50}) was taken from inhibition curves.

Results RT-PCR was used to amplify P2Y-like sequences from total RNA isolated from the rat heart. A DNA fragment was isolated, subcloned into the *pCR2.1* vector and 20 independent clones were subjected to partial sequencing analysis. One clone was purified, labelled to high specific activity and used to screen 10^6 independent clones of a rat genomic library. A genomic clone ($rP2Y_4$) was purified and subjected to detailed restriction and hybridization analysis. An intronless open reading frame of 1083bp encoded a protein of 361 amino acids (Figure 1). Hydropathy analysis of the predicted $rP2Y_4$ receptor protein revealed the presence of seven transmembrane spanning domains. $rP2Y_4$ was closely related to the human homologue $hP2Y_4$ (83% identical at the amino acid level) and less related to $rP2Y_2$ (51%). A comparison of the amino acid sequences of $rP2Y_4$, $hP2Y_4$ and $rP2Y_2$ is shown (Figure 1).

Both ATP (EC_{50}, 1.8 ± 0.2 μM; slope, 0.9 ± 0.11; n = 5) and UTP (EC_{50}, 2.6 ± 0.4 μM, slope, 0.9 ± 0.05; n = 5) were equi-active and equi-potent agonists at the recombinant $rP2Y_4$ receptor (Figure 2A). ATP and UTP caused cross-desensitization at the $rP2Y_4$ receptor when superfused successively (each

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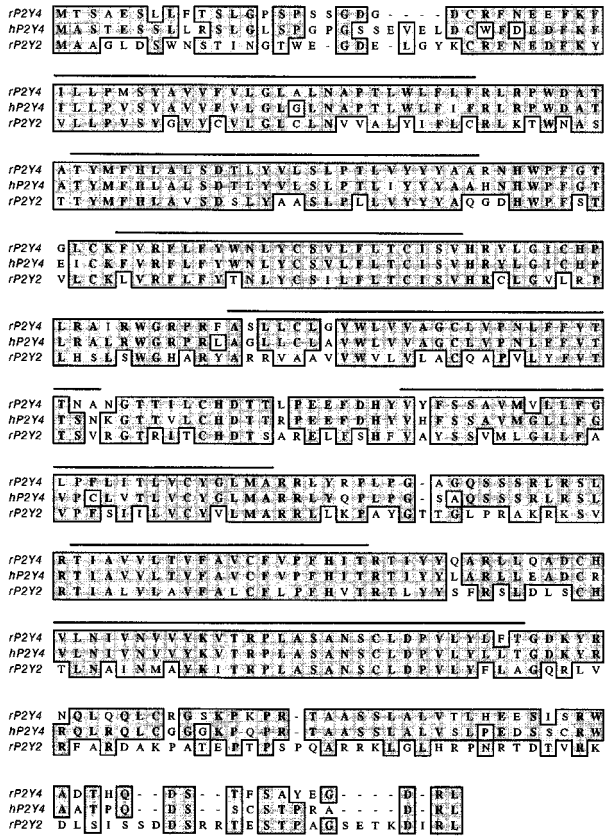

Figure 1 Alignment of P2Y sequences. The amino acid alignment of $rP2Y_4$ (361aa; Y14705), $hP2Y_4$ (365aa; X91852) and $rP2Y_2$ (374aa; L46825). Matches are heavily shaded, positives are lightly shaded. Gaps (-) were introduced to maximize the alignment. Hydrophobic (transmembrane spanning) regions are shown by horizontal bars.

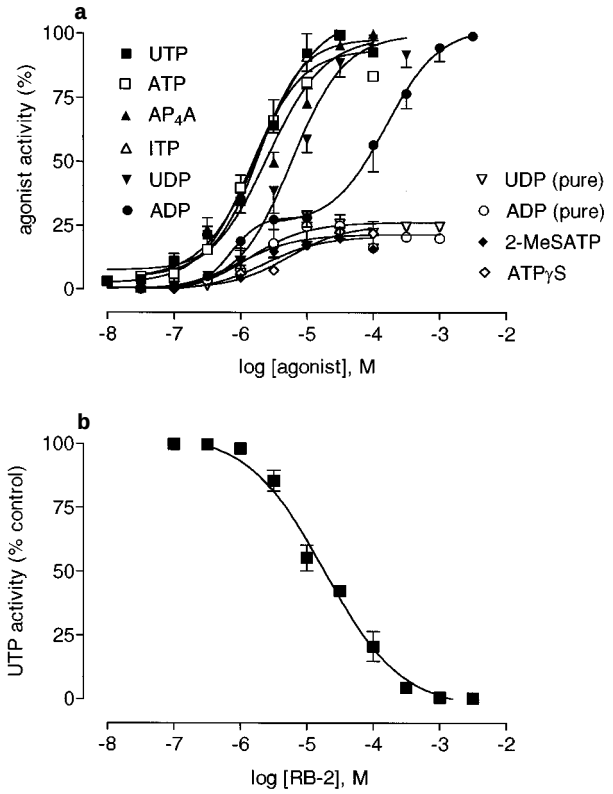

Figure 2 Agonism and antagonism at $rP2Y_4$ receptor. In (a) concentration-response relationships for agonists at recombinant $rP2Y_4$ receptor. Data were normalized to responses to a supramaximal concentration (100 μM) of UTP. In (b) the concentration-dependence of RB-2 inhibition of responses to UTP (3 μM) at the $rP2Y_4$ receptor. Curves fitted by Prism v2.0 (GraphPad). Data are means with vertical lines showing s.e.mean, $n=4$ or 5.

at 3 μM, for 120 s). Membrane currents to supramaximal concentrations (100 μM) of ATP and UTP applied simultaneously were not additive. UDP was approximately 3 fold less potent (EC_{50}, 6.3 ± 1.7 μM; slope, 1.2 ± 0.1; $n=5$) than ATP and UTP, but gave maximal responses similar to these two agonists. Hexokinase pretreatment of UDP stocks failed to affect UDP potency (EC_{50}, 4.2 ± 0.1 μM; $n=4$) but decreased its maximal activity. Thus, UDP stocks were significantly contaminated with UTP. The concentration-response curve for ADP was complex and fitted best by a biphasic curve. The first phase (EC_{50}, 0.67 ± 0.08 μM; slope, 1.6 ± 0.24; $n=5$) reached a plateau at 25% maximal activity (at 10 μM) while the second phase (EC_{50}, 127 ± 27 μM; slope, 1.7 ± 0.1; $n=5$) peaked at the same maximal activity as ATP and UTP. Measurement by the luciferin-luciferase ATP-assay revealed that the ATP content was $4.4\pm0.4\%$ ($n=4$) of ADP solutions. The second phase of the ADP curve was abolished after hexokinase treatment, suggesting it was due to contamination of ADP stocks by ATP. The first phase of the ADP-curve was displaced slightly to the right by hexokinase treatment (EC_{50}, 1.9 ± 0.6 μM; $n=4$). Both ITP (EC_{50}, 1.4 ± 0.5 μM; slope, 0.9 ± 0.2; $n=4$) and Ap_4A (EC_{50}, 3.0 ± 0.7 μM; slope, 0.8 ± 0.1; $n=4$) were as potent and active as ATP and UTP. Both ATPγS (EC_{50}, 2.1 ± 0.5 μM, $n=4$) and 2-MeSATP (EC_{50}, 2.1 ± 0.3 μM, $n=4$) were as potent and active as ADP after hexokinase treatment. The potency order of agonists at $rP2Y_4$ was (by EC_{50} values): ITP = ATP = ADP(pure) = UTP = ATPγS = 2-MeSATP = Ap_4A > UDP(pure). However, ADP, ATPγS, 2-MeSATP and UDP are partial agonists at $rP2Y_4$.

Suramin, PPADS and Reactive blue 2 (RB-2) (0.1 – 100 μM) were tested at $rP2Y4_4$ against UTP-mediated inward currents. At 100 μM, suramin weakly antagonized (by $\sim$10%) UTP-responses, PPADS had no effect while RB-2 almost abolished UTP-responses (data not shown). The inhibitory action of RB-2 was concentration-dependent and, in further experiments, revealed an IC_{50} value of 21.1 ± 2.9 μM ($n=4$) and slope of 0.8 ± 0.1 ($n=4$) against UTP-responses (Figure 2b). The blocking effect of RB-2 was reversed after washout (60 min).

Discussion A clone isolated from a rat genomic library is structurally related to the human $P2Y_4$ receptor gene and mostly likely encodes the rat homologue. The predicted amino acid sequences for the $rP2Y_4$ and $hP2Y_4$ receptors are highly conserved (90%) over the seven hydrophobic (transmembrane spanning) regions and cytoplasmic/extracellular loops but show more diversity at the levels of the N- and C-termini (overall sequence identity was 83%). N- and C-termini of P2Y receptors are unlikely to form the ligand binding domain and, hence, unlikely to affect agonist selectivity (van Rhee *et al*., 1995). The agonist profile for $rP2Y_4$ expressed in *Xenopus* oocytes did not match the profile for $hP2Y_4$ but, instead, was closer to the profile of $rP2Y_2$. The $hP2Y_4$ receptor shows a marked preference for pyrimidine nucleotides (UTP and UDP) and is weakly activated (if at all) by purine nucleotides (Nguyen *et al*., 1995; Communi *et al*., 1995). The $rP2Y_2$ receptor shows no such preference and is stimulated equally by ATP and UTP, as well as by ATPγS, ITP and diadenosine

tetraphosphate (Ap_4A) (Chen *et al*., 1996; Filippov *et al*., 1997). These nucleotides, except for ATPγS, were potent full agonists of $rP2Y_4$ and, accordingly, this new clone is virtually indistinguishable from $rP2Y_2$ on the basis of agonist activity. Another new clone isolated from a turkey blood cDNA library (Boyer *et al*., 1997) is also related to $hP2Y_4$ (56%) yet has an agonist profile (ATP = UTP > Ap_4A > ATPγS > UDP) similar to the profile of $rP2Y_4$ shown here.

$rP2Y_4$ showed a preference for the P2 receptor antagonist Reactive blue 2 (RB-2) but was only slightly inhibited by suramin (at 100 μM). This antagonist profile (RB-2 > > suramin, pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) inactive) for $rP2Y_4$ did not match the ranking activity of these substances at $hP2Y_4$ receptors where only PPADS (30 μM and greater) significantly inhibits while RB-2 is a weak antagonist (Communi *et al*., 1996). Charlton *et al*. (1996) has shown that suramin ($pA_2 = 5.77$) is more effective than PPADS ($pA_2 = 4.32$) at $hP2Y_2$ receptors. Thus, $rP2Y_4$ and $hP2Y_2$ represent two functional types of the P2U receptor which, respectively, are suramin-insensitive and suramin-sensitive. It is interesting that P2U receptors in vascular tissues can be subdivided into two subpopulations on the basis of suramin sensitivity (Dainty *et al*., 1994).

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