Molecular cloning and functional expression of a novel rat heart P2X purinoceptor

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Abstract Here we describe a novel purinergic receptor, the P2X5 receptor, cloned from rat heart. The full-length cDNA encodes a protein 455 amino acids long which shares an overall identity of 40–47% with other members of the P2X purinergic receptor family. P2X5 mRNA transcripts are found predominantly in rat heart but are also present in brain, spinal cord and adrenal gland. Functional expression of the recombinant receptor in HEK-293 cells shows a current that resembles mostly the P2X2 phenotype: the ATP-activated current reveals little agonist desensitization, is not activated by α,β-meATP and is completely blocked by suramin and PPADS.

Key words: Purine receptor; ATP receptor; PPADS; Suramin; Heart; CNS

1. Introduction

The physiological actions of extracellular ATP have been comprehensively described and include contractile regulation of cardiac, vascular and visceral smooth muscle, excitatory and inhibitory effects on neurons from the central and peripheral nervous system and activation of neuroendocrine secretion (for reviews see [1–3]). All these effects are apparently mediated by a miscellaneous group of G-protein coupled receptors (P2Y) and ligand-gated channels (P2X) [4]. To date, despite the numerous functions attributed to extracellular ATP, only four P2X receptors have been identified: P2X1 [5], P2X2 [6], P2X3 [7,8] and P2X4 [9–13]. Analysis of the amino acid sequences provides evidence of a novel family of membrane proteins consisting of two short intracellular domains, two transmembrane-spanning motifs, and a large extracellular loop comprising 10 conserved cysteines. These receptors are structurally unrelated to other ligand-gated channels [14].

Analysis of P2X mRNA expression reveals a broad distribution of P2X1, P2X2 and P2X4 transcripts throughout the major tissues of the body [5,6,9,12,13,15], indicating a role of these proteins in many different physiological actions. In contrast, P2X3 transcripts have a localization limited to nociceptive neurons corresponding to C-fibre afferents of dorsal root ganglia [8]. In addition to expressing P2X3 receptors, these neurons also hold transcripts of all P2X1 to 4 isoforms. Indeed, P2X2/P2X3 heteromultimeric receptors have been proposed to occur in vivo [7]. This raises the fascinating possibility that ATP is involved in the generation of pain signals [7,8,16].

Heterologous expression of each recombinant P2X isoform results in ATP-activated cation-selective channels with diverse pharmacological and desensitization phenotypes [5–13,17]. The main pharmacological distinction is their the relative sensitivities to the agonist α,β-meATP and the antagonists suramin and PPADS. Thus, α,β-meATP is a full agonist for P2X1 and P2X3 receptors [5,7,8,17], but is not effective on the P2X2 and P2X4 forms [9–13,17]. On the other hand, P2X4 is the only purinergic receptor cloned so far that is insensitive to the established P2X blockers suramin and PPADS [9,10,12,13].

The weak reciprocity between the phenotypes reported for heterologous expressed recombinant P2X receptors and the responses observed in native systems indicates that additional subunits might occur. In this paper we describe the cloning and functional expression of a new rat receptor, P2X5, belonging to the P2X ATP-gated channel family.

2. Materials and methods

2.1. Isolation of the P2X5 cDNA

A partial cDNA sequence for the P2X5 receptor (~400 bp) was obtained from rat heart cDNA by degenerate PCR. The PCR thermal profile was: 5 min at 94°C/5 cycles of 1 min at 94°C, 45°C followed by 30 cycles of 1 min at 94, 50 and 72°C. The forward primer was: 5′-TG(G/T)CIG(C/G)ITGGTGCCC(G/T)GT(G/A)GAG-3′; the reverse primer was: 5′-TAC(C/T)TGGCAAA(T/C)CTGAA(A/G)TTG(A/T)AGC-3′. To isolate the unknown 5′ and 3′ sequences we used the Marathon cDNA amplification kit (Clontech) based on a modification of the ligation-anchored PCR amplification technique [18]. Specific oligonucleotides targeted to the P2X5 sequence were extended by nested PCR (Expand Long Template PCR system, Boehringer-Mannheim) towards the 5′ and 3′ mRNA ends, using a library of adaptor-ligated cDNA (constructed from rat brain polyA$^+$ mRNA) and following the manufacturer's recommendations. Finally, to obtain a full-length P2X5 coding sequence, specific oligonucleotides were used to amplify by PCR a rat heart cDNA. To avoid Taq DNA polymerase random mutations, DNA fragments from independent PCR reactions were isolated and cloned. Sequences from both strands were determined.

2.2. Transient transfection of HEK 293 cells

The full-length P2X5 cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Human embryonic kidney cells (HEK-293 cells, ATCC CRL 1573) were transfected (200–500 ng/dish) using a modified calcium phosphate precipitation technique [19]. Transfected cells were incubated for ~40 h before electrophysiological experiments.

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Abbreviations: α,β-meATP, α,β-methylene-ATP; β,γ-meATP, β,γ-methylene-ATP; PPADS, pyridoxal phosphate 6-azophenyl-2′,4′-disulphonic acid; TEA-Cl, tetraethylammonium chloride; 2MeSATP, 2-methylthio-ATP; EC_{50}, half-maximal effective concentration; n_H, Hill coefficient; *N*, number of determinations or cells; bp, base pairs

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2.3. Electrophysiological recordings

Transfected cells were continuously perfused at room temperature with an extracellular bath solution containing (mM): 150 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 KCl, 10 HEPES, 12 glucose (pH 7.3). Patch pipettes were made from borosilicate glass and filled with an intracellular solution containing (mM): 120 CsCl, 20 TEA-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 11 EGTA, 10 HEPES (pH 7.2). Membrane currents were recorded from cells using an EPC-9 amplifier (HEKA Electronik, Lambrecht). Whole-cell currents were sampled at 20 Hz and stored on disk. The membrane potential was clamped at −70 mV in all experiments. Pipette resistance was 6–8 MΩ and the series resistance was compensated by up to 70%. Agonist and antagonist were applied in bath solution with a microcapillary application system. Wild-type HEK-293 cells did not show significant endogenous ATP responses within the range of concentrations used. To ensure complete recovery from desensitization between sucesive agonist applications, we used wash periods of 1 min. Dose-response curves of ATP-activated whole-cell currents were fitted with the sigmoidal function $I = I_{max}/(1 + (EC_{50}/C)^{n_H})$, where I_{max} is the maximal current, C the agonist concentration, EC_{50} the half-maximal efective concentration and n_H the Hill coefficient. Results are expressed as mean ± S.D.

2.4. P2X5 mRNA tissue distribution analysis by RT-PCR

Total RNA was prepared from freshly dissociated tissues by the single-step method [20]. First strand cDNA syntheses (Superscript II reverse transcriptase, GibcoBRL) were independently primed with oligo(dT) and random hexamers (5 μg total RNA, in 20 μl reaction volume). The PCR reactions were performed in 50 μl final volumes containing 1 μl of cDNA, 0.5 μM each of pairs of P2X5 and transferrin receptor (TFR)-specific primers (ref), 200 μM dNTPs, 2.5 units Taq DNA polymerase, and 1×Taq DNA polymerase reaction buffer (Promega). The PCR thermal profile was 5 min at 94°C and 35 cycles of 40–60 s at 94, 59 and 72°C. The P2X5 primers were 5′-CGCTGGGGAGTCTGTTGTAG-3′ (forward, F1) and 5′-TCTCGGTAAAACTCACTC-3′ (reverse, R1). The PCR fragment length was 698 bp. Southern blot analysis of PCR products was performed at high stringency with appropriate α-[^{32}P]dCTP random-primed probes for the TFR and P2X5 genes. An identical result was obtained using the same PCR conditions and an antisense primer to the 3′ untranslated region of the P2X5 gene: 5′-TGGAGTT-GAGGGGCTTTTCT-3′ (reverse, R2). The PCR fragment size was 1083 bp.

To rule out the possibility of genomic DNA contamination of the cDNAs, we performed PCR control reactions under the same conditions but using 0.5 μg of rat genomic DNA. No bands were detected (Fig. 2B), suggesting that the P2X5 oligonucleotides flank intronic structures. The integrity of the genomic DNA control was assessed (data not shown) by amplification with specific primers for the Kv1.4 potassium channel (a gene with no introns).

2.5. Drugs

ATP (disodium salt), CTP (sodium salt), UTP (sodium salt), α,β-MeATP (lithium salt), β,γ-MeATP (sodium salt), dATP (sodium salt) and AMP (sodium salt) were obtained from Sigma; GTP (disodium salt) was purchased from Fluka; ADP (free acid) was obtained from Boehringer Mannheim; PPADS (tetrasodium salt) and 2MeSATP (tetrasodium salt) were obtained from Research Biochemicals Inc.; suramin was purchased from Calbiochem.

3. Results and discussion

3.1. Cloning of the P2X5 receptor

Using PCR techniques we have isolated a new cDNA from rat heart. The deduced amino acid sequence revealed a new protein with high structural homology to the previously described P2X receptors. Following the accepted nomenclature and classification of purinoceptors we designate this protein as P2X5. The new cDNA encodes a 455 amino acid protein (Fig. 1) that contains the structural patterns previously described for the P2X receptor family [14]. Two hydrophobic amino acid stretches could constitute a pair of transmembrane segments, connecting a large extracellular loop (comprising 10 cysteine residues) to the cytosolic N- and C-terminal segments. The amino acid identity of the P2X5 protein with the other members of the P2X family is: P2X1 (41%), P2X2 (45%), P2X3 (43%) and P2X4 (47%). Like all the cloned P2X receptors, the P2X5 isoform exhibits maximum amino acid homology within the central core of the protein (bracketed by the two putative transmembrane segments) but any kind of resemblance is lost at the C-terminal segment (Fig. 1). The P2X5 receptor contains three *N*-glycosylation consensus sites, N-X-(ST), located in the putative extracellular loop but none of them is conserved throughout all the members of the P2X family. Additionally, the new P2X isoform holds four protein

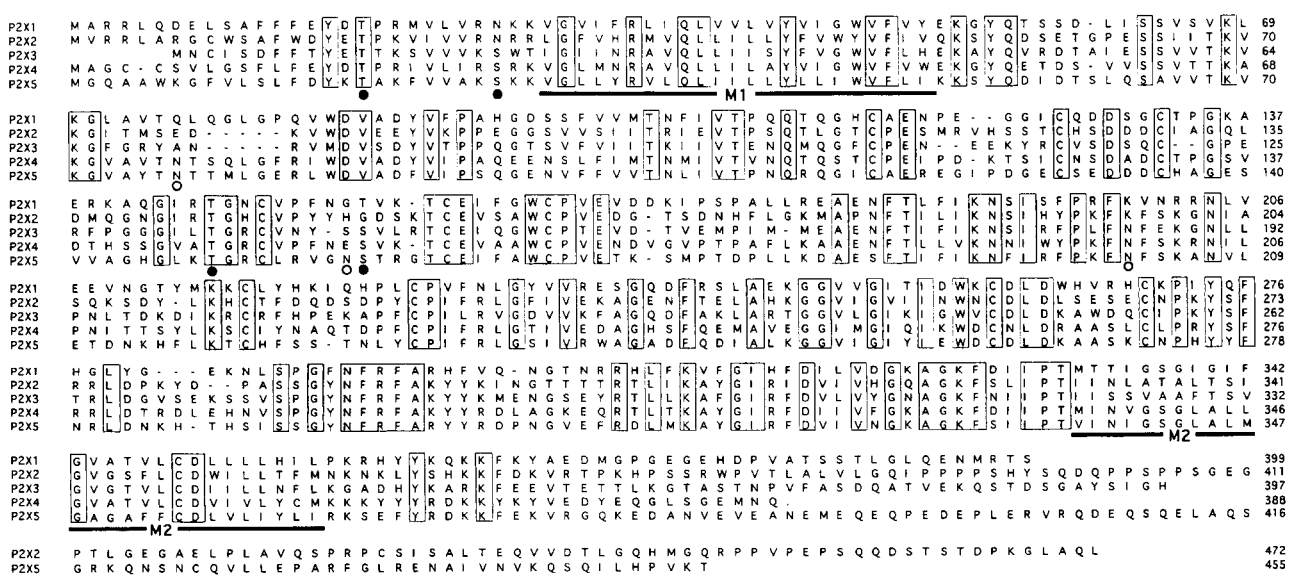

Fig. 1. Aligment of deduced amino acid sequences for the cloned rat P2X receptors. Residues conserved in all five sequences are denoted by open boxes. The two presumed transmembrane-spanning segments are underlined, the putative sites for P2X5 *N*-linked glycosylation are indicated by an open circle, and the protein kinase C consensus sequences by a closed circle. The sequence for the P2X5 cDNA has been submitted to the EMBL Nucleotide Sequence Database with the following accession number: X97328.

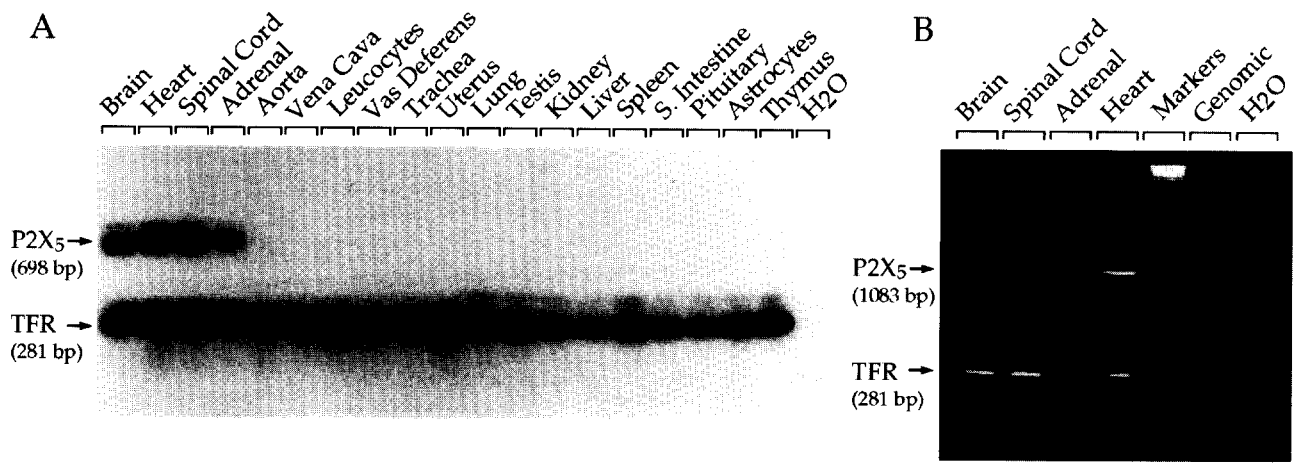

Fig. 2. (A) Analysis of P2X5 mRNA tissue expression by RT-PCR (oligonucleotides S1, R1) and Southern blot autoradiography using a P2X5-specific probe (P2X5 arrow) and a rat transferrin receptor probe (TFR arrow). H_2O: control samples without cDNA. (B) Ethidium bromide stained agarose gel of RT-PCR products (5 μl, oligonucleotides S1, R2) for brain, spinal cord, adrenal gland and heart. The PCR products from a genomic DNA amplification (0.5 μg) are shown. Note that no relevant fragments were amplified, indicating that the P2X5 oligonucleotides did specifically amplify the cDNA. Southern blot analysis of the genomic DNA sample revealed no hybridization signals (data not shown). We obtained an equivalent result using the oligonucleotides S1 and R1. H_2O: control sample without cDNA.

kinase C (PKC) consensus sites, (ST)-X-(KR), but only two of them are positioned in the N-terminal domain, presumably facing the cytosol. Interestingly, one of these PKC sites is conserved throughout all the P2X isoforms (Fig. 1).

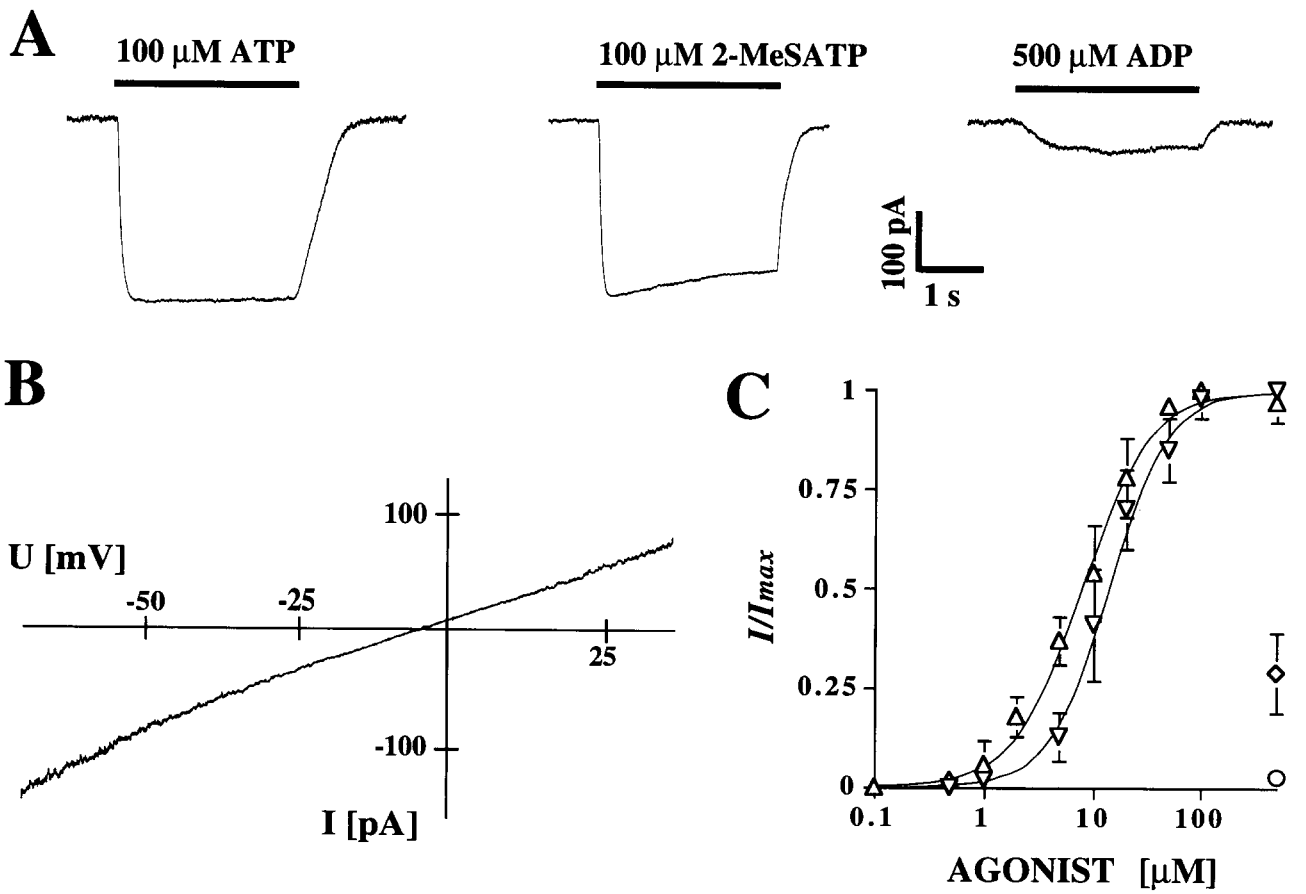

Fig. 3. (A) Inward currents evoked by ATP, 2MeSATP and ADP in HEK-293 cells transfected with P2X5 cDNA (B) Current-voltage relationship obtained through voltage ramps. (C) Dose-response curves for ATP (open triangles) and 2MeSATP (inverted open triangle). Responses to ADP (open diamond) and αβ-meATP (open circle) are indicated.

Table 1
P2X5 responses and EC_{50} achieved upon the application of various compounds

Compound	μM/EC_{50} (μM)	% response	n_H	N
ATP	500/EC_{50} = 8 ± 2	100	1.3 ± 2	6
2MeS ATP	500/EC_{50} = 15 ± 4	105 ± 5	1.4 ± 2	3
αβ-meATP	500	< 3		8
β,γ-meATP	500	9 ± 5		8
ADP	500	29 ± 10		5
AMP	500	41 ± 14		8
Adenosine	500	< 5		5
dATP	500	13 ± 4		3
CTP	500	< 2		5
GTP	500	< 6		3
UTP	500	< 3		3

The currents are expressed as the percentage (mean ± S.D.) of the maximal response obtained with 500 μM ATP.

3.2. Tissue distribution of P2X5 mRNA expression

Tissue distribution of P2X5 transcripts was assessed by RT-PCR and the resulting PCR products (oligonucleotides S1, R1) were analyzed by Southern blotting (Fig. 2A). This P2X isoform showed a more restricted mRNA expression pattern than those observed for P2X1, P2X2 and P2X4 receptors [5,6,9,12,13]. Transcripts were detected only in a few major tissues, namely brain, heart, spinal cord and adrenal gland. To guarantee the specificity of the RT-PCR assay, we repeated the PCR reactions using an antisense oligonucleotide targeted to the P2X5 3′ untranslated region (oligonucleotide R2), and obtained an identical result. Although we did not attempt quantitative analysis of P2X5 transcript expression levels, a visual estimation of the PCR product yield (stained with ethi-

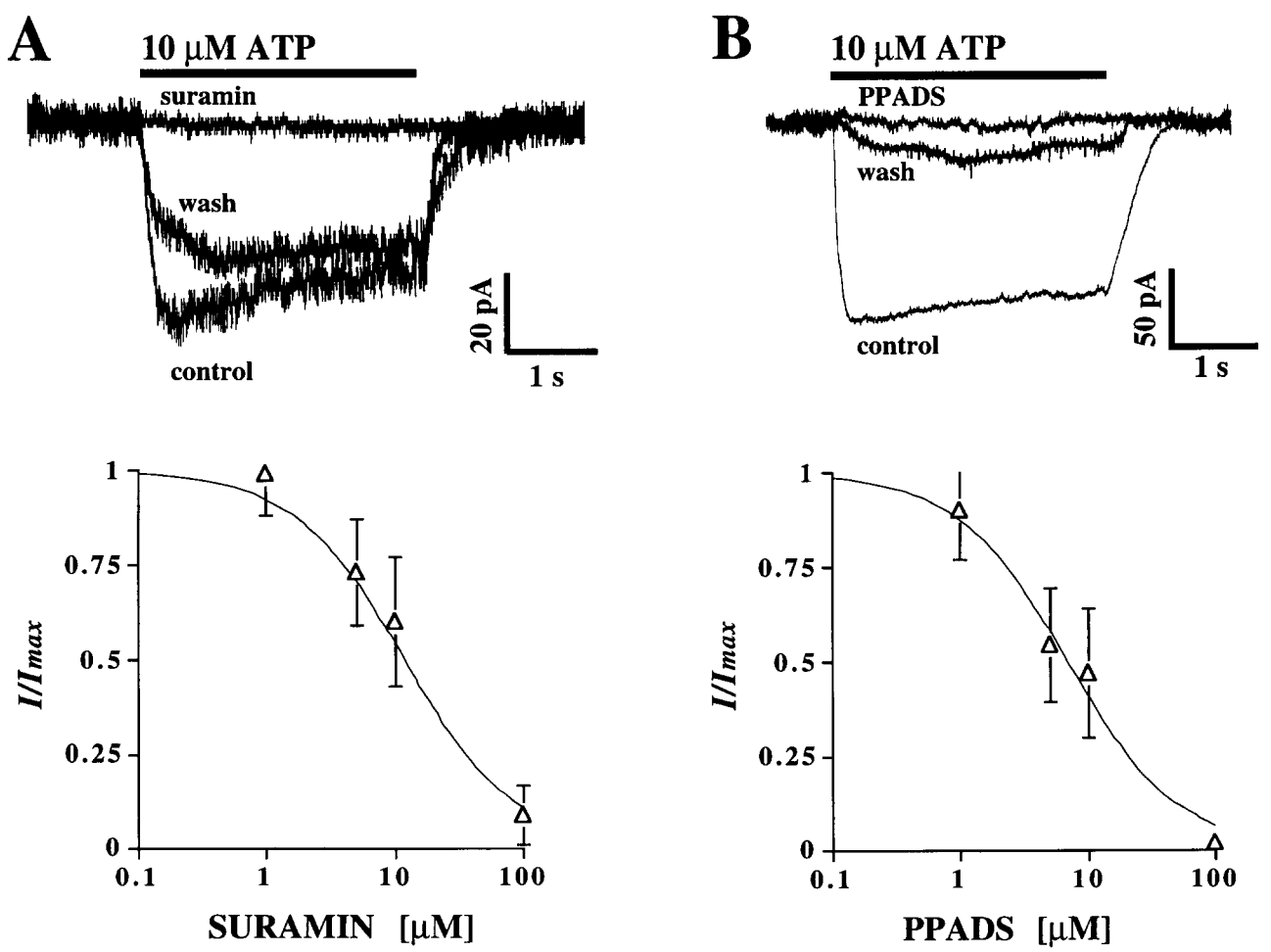

Fig. 4. (A) Effects of suramin when co-applied with 10 μM ATP. Superimposed currents are shown for control trace, suramin block (100 μM), and after 5 min of wash-out. The panel shows the relative maximal currents obtained in the presence of increasing concentrations of suramin. The continuous line is the fit to the data as described in Section 2 (IC_{50} = 13 ± 7, N = 3). (B) PPADS inhibition of ATP-evoked currents (10 μM ATP). The antagonist was preincubated for up to 1 min and co-applied afterwards with the agonist. Superimposed currents are shown for control trace, PPADS block (100 μM) and after 5 min wash-out. The panel represents the relative maximal current obtained in the presence of increasing concentrations of PPADS. The continuous line is the fit to the data as described in Section 2 (IC_{50} = 7 ± 4, N = 3).

dium bromide) consistently showed much greater mRNA expression in heart than in the other tissues (Fig. 2B). The expression of the P2X5 receptor in the cardiovascular system seems to be limited to the heart, with no detectable mRNA levels in blood vessel cDNA (aorta and vena cava). The P2X4 receptor is the only one that has been shown previously to occur in rat heart [12,13] and therefore a major question will be to assess whether both P2X4 and P2X5 isoforms co-assemble into heteromultimeric receptors.

3.3. Expression of recombinant P2X5 receptors in HEK293 cells

Transient transfection of HEK-293 cells with a P2X5 full-length cDNA resulted in the formation of functional homomeric ATP-gated channels. Fast application of ATP (100 μM) to cells at −70 mV holding potential induce a rapid inward current (~200–400 pA), which exhibited no voltage rectification and reverted at ~−5 mV. The channel showed little desensitization in the continuous presence of the agonist (Fig. 3A,B). The apparent concentration for half-maximal response (EC_{50}) to ATP was 8 ± 2 μM. The P2X5 receptor was also fully activated by 2MeSATP (EC_{50} = 15 ± 4 μM) (Fig. 3C). The rank order of potency (determined as the magnitude of current elicited at 500 μM) for ATP and other analogs was: ATP≥2MeSATP > AMP ≈ ADP > dATP ≈ β,γ-meATP. No significant current was recorded after application (500 μM) of α,β-meATP, CTP, GTP, UTP or adenosine (Table 1).

The P2X5 ATP-elicited currents (10 μM) were readily blocked in a fully reversible manner (< 3 min for complete recovery) when co-applied with suramin (EC_{50} = 13 ± 7 μM) (Fig. 4A), but the inhibition observed for PPADS (EC_{50} = 7 ± 4 μM) displayed only a partial reversibility (Fig. 4B). In this respect, it is noteworthy that the P2X5 receptor has a lysine residue (K251) which has been shown to be crucial for the affinity and slow wash-out kinetics of PPADS inhibition for the P2X1 and P2X2 receptors [10].

The P2X5 isoform displays particular pharmacological and kinetic features which provide the basis for its classification with respect to the other P2X receptors. On the basis of the lack of effect of α,β-meATP, the P2X5 receptor can be grouped together with the P2X2 and P2X4 isoforms [6,9–13,17]. The P2X4 channel remains the only receptor cloned so far that is insensitive to suramin and PPADS [9,10,12,13], while the P2X5 isoform shares, mutually with the P2X1 and P2X2 channels, a similar slow rate for the onset and washout of PPADS block [17].

Taking all these pharmacological data into account, the P2X5 receptor exhibits a remarkable similarity to the P2X2 channel. This resemblance is extended even to the rate of desensitization: only the P2X2 and P2X5 receptors experience a very low run down of the current in the continuous presence of the agonist [6,17]. However, the fact that P2X2 mRNA was not detected in rat heart [6], an organ in which P2X5 transcripts are predominantly expressed, suggests that both proteins do not fulfil the same physiological functions in native systems.

In conclusion, we have cloned a rat cDNA, that encodes a novel ATP-gated ion channel, P2X5. This receptor is not activated by α,β-meATP and is blocked by the P2X antagonists suramin and PPADS. P2X5 mRNA was found primarily in rat heart but is also located in the central nervous system (brain and spinal cord) as well as in the adrenal gland.

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Note added in proof

When this paper was under revision, a sequence virtually identical to the one presented here was published, Collo et al., (1996) J. Neurosci. 16, 2495–2507. A comparison of the protein sequences reveals two differences: F191 and Q396 are S191 and R396 in the protein described by Collo et al.