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Desensitization of the $P2X_2$ receptor controlled by alternative splicing

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Abstract P2X receptors are ion channels gated by extracellular ATP. We report here cloning of a $P2X_2$ receptor splice variant ($P2X_{2-2}$) carrying a 207 bp deletion in the intracellular C-terminus and the analysis of the corresponding genomic structure of the $P2X_2$ gene. $P2X_{2-2}$ is as highly expressed as the original $P2X_2$ sequence in various tissues. ATP-activated currents mediated by heterologous expressed $P2X_2$ or $P2X_{2-2}$ receptors showed significant differences in desensitization time constants and steady-state currents in the continuous presence of ATP. These results imply functional differences between cells differentially expressing these $P2X_2$ isoforms.

Key words: P2X receptor; Genomic sequence; Alternative splicing; Desensitization

1. Introduction

P2X receptors are ion channels gated by extracellular ATP. They are expressed in a broad variety of tissues and known to mediate fast synaptic transmission [7,8] and synaptic transmission between sympathetic nerves and smooth muscle [9,19]. By now 7 P2X receptor subunits ($P2X_1$–$P2X_7$) have been cloned which share a putative two-transmembrane segment structure with intracellular C- and N-termini and an extracellular loop [2,3,5,6,14,20,21]. Heterologous expression of each subunit results in the formation of non-selective cation channels with differential sensitivities for the agonists ATP and α,β-methylene-ATP and the antagonists pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin. They also differ in their time constant of desensitization: $P2X_1$ and $P2X_3$ are fast desensitizing while $P2X_2$ and $P2X_4$ to $P2X_7$ show a much slower desensitization.

Here we report the cloning of a splice variant ($P2X_{2-2}$) of the $P2X_2$ receptor subtype and the identification of the corresponding rat genomic sequence. We used heterologous expression of the $P2X_2$ and $P2X_{2-2}$ receptor to look for functional differences between both isoforms.

2. Materials and methods

2.1. RT-PCR analysis

Total RNA from brain, spleen, kidney, intestine and organ of Corti was isolated from adult Wistar rats (Charles River, Germany) with the RNeasy Total RNA-Kit (Qiagen, Chatsworth, CA). First strand cDNA was synthesized from 1 μg total RNA using an oligo d(T) primed 20 μl reaction by the Superscript RNase H-Reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) under standard conditions [1].

PCR amplification of a $P2X_2$-specific fragment was performed using the following primers: sense primer (up2: 5′-ATTCGCACAGGGCACTGTGTACC-3′, positions 424–446) and antisense primer (lo2: 5′-TTAGCAGCCAAAGCTGTCTCT-3′, positions 1460–1480) resulting in a 1056 bp fragment. Nested PCR: sense primer (up3: 5′-GCCTGGTGCCCGGTGGAG-3′, positions 484–501), antisense primer (lo2) resulting in a 955 bp fragment.

The PCR amplifications were performed on a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer, Norwalk, CT) under standard conditions [18] using 1 μl of the first strand cDNA in a final volume of 50 μl, 1 U AmpliTaq (Perkin Elmer) and a primer concentration of 40 ng/μl. Reaction conditions for the first and second round of amplification were 45 cycles of 30 s 94°C, 30 s 60°C and 30 s 72°C starting with 3 min at 94°C and ending with an extension of 5 min at 72°C. The reaction products were at first analyzed by gel electrophoresis. Identity of the PCR products was tested by Southern blot analysis using a 165 bp PCR fragment of the $P2X_2$ receptor sequence as a hybridization probe (positions 519–684). In addition PCR products were gel-isolated and, after blunting with kinase, subcloned in an *Eco*RV cut, dephosphorylated pBluescript vector (Stratagene, La Jolla, CA). Sequences of the recombinant fragments were determined on both strands by fluorescent DNA sequencing (Applied Biosystems Inc., Foster City, CA).

2.2. Genomic organization of the $P2X_2$ receptor gene

Genomic DNA was isolated from rat liver using the QIAamp Tissue Kit (Qiagen, Chatsworth, CA). To identify the genomic $P2X_2$ receptor sequence two overlapping fragments were amplified from the start codon to 61 bp downstream of the stop codon (3′UT region) of the published $P2X_2$ receptor sequence [2]. The primer pair for the amplification of the 5′ fragment was: sense primer (up1: 5′-GCCATGGTCCGGCGCTT-3′, bp −3–14) and antisense primer (lo1: 5′-GGCTGACACCTCGCAGGTCTT-3′, bp 466–486); to amplify the second fragment the primer pair up2/lo2 (as described in Section 2.1) was used. The PCR conditions were 45 cycles of 1 min 94°C, 1 min 60°C and 1 min 72°C starting with 3 min at 94°C and ending with an extension of 5 min at 72°C. The fragments were subcloned as described in Section 2.1. The recombinant cloned DNA was denatured by NaOH and sequenced on both strands using Sequenase Version 2.0 Kit (Amersham/USB, Cleveland, OH). For both fragments 10 independent clones were analyzed, showing identical sequences.

2.3. Expression of $P2X_2$ isoforms in Xenopus oocytes and HEK293 cells

For functional expression a full-length $P2X_{2-2}$ receptor clone was constructed. The *Hpa*I/*Xho*I restriction fragment of the original $P2X_2$ receptor clone [2] in pcDNA 3 (Invitrogen, San Diego, CA) was substituted by the corresponding fragment of the subcloned isoform carrying the deletion. The identity of the resulting $P2X_{2-2}$ clone was verified by sequencing.

Capped cRNAs specific for $P2X_2$ and $P2X_{2-2}$ were synthesized in vitro using T7 polymerase (Stratagene capping kit) and injected into *Xenopus* oocytes.

Handling and injection of *Xenopus* oocytes has been described previously [10]. 24 h after injection oocytes were treated with collagenase

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type II (Sigma, 0.5 mg/ml) for 45 min. After collagenase treatment the follicle cell layer was removed manually with fine forceps. Oocytes were defolliculated carefully as follicular oocytes are known to have intrinsic P2 receptors [12]. Not injected defolliculated oocytes from every donor animal were tested for endogenous ATP-activated currents which have been described previously to be activated at agonist concentrations >100 μA [12,13]. Similar to the observations of King et al. [12] we found these endogenous ATP-activated currents in only one batch of oocytes excised from one of 20 donor frogs. This batch of oocytes was not used. Electrophysiological recordings were performed 3–7 days after injection using a two-microelectrode voltage clamp. Current and voltage electrodes were pulled from thick-walled borosilicate glass. They were filled with 3 M KCl and had resistances between 0.1 and 0.5 MΩ. Currents were recorded with a TurboTec 01C amplifier (npi, Tamm, Germany), digitized at 30 Hz (ITC16, HEKA, Lamprecht, Germany) and stored on the hard disk. The bath chamber was made up as a narrow canal to achieve complete solution exchanges in less than 3 s. The extracellular solution was composed of (in mM): NaCl 115, KCl 2.5, $MgCl_2$ 1.8, HEPES 10; pH adjusted to 7.3 with NaOH. All experiments were performed at room temperature (approximately 23°C). To exclude that the decline of current during ATP application is due to a channel block mediated by extracellular divalent cations, we elicited ATP-activated currents in an extracellular solution with the divalent cations substituted by polyvinylpyrrolidone (Boehringer Mannheim) [16]. Under these conditions the ATP-activated currents declined with the same time course as in extracellular solutions containing Ca^{2+} or Mg^{2+}.

The HEK293 cells were transiently transfected with the cDNA encoding $P2X_2$ or $P2X_{2-2}$ using lipofectin (Life Technologies Inc.) as previously described [14]. Conventional whole-cell recording and fast-flow U-tube agonist application methods were used. Internal pipette solution was (mM): KCl, K-aspartate or Cs-aspartate 140, NaCl 20, EGTA 10 and HEPES 5. Extracellular solution was (mM): NaCl 147, KCl_2 2, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 12; pH was adjusted to 7.35 with NaOH. ATP (magnesium salt), α,β-methylene-ATP (lithium salt) and PPADS were purchased from Sigma (St. Louis, MO) and suramin from Bayer (Leverkusen, Germany).

3. Results

3.1. Detection and cloning of the $P2X_{2-2}$ receptor isoform

Various rat tissues were tested for $P2X_2$ receptor expression with PCR using a $P2X_2$-specific primer pair (up2/lo2) (Fig. 1A). The PCR products resulted in three distinct bands of 995 bp, 910 bp and 790 bp length in the gel electrophoresis. All three bands showed hybridization with a $P2X_2$-specific probe in Southern blot analysis. For identification of the sequences the fragments were isolated, subcloned and sequenced. The largest band with 995 bp yielded the original $P2X_2$ sequence [2].

The smallest band with 790 bp revealed a 207 bp in-frame deletion in the C-terminal region at nucleotide positions 1111–1317 of the original $P2X_2$ sequence (Fig. 2).

Similarly, a RT-PCR reaction on brain RNA with primers (up1/lo2) covering the whole coding region of $P2X_2$, besides the expected 1483 bp fragment, yielded an approximately 200 bp shorter fragment (not shown). This suggests that the 207 bp deletion is a naturally occurring variant of the $P2X_2$ receptor possibly generated by alternative splicing.

Subcloning and sequencing of the intermediate band yielded either the sequence of the original or that of the 207 bp deleted form. Control PCRs with cloned DNA of the original $P2X_2$ sequence and the 207 bp deletion mixed in a 1:1 relation also resulted in three bands in gel electrophoresis. Therefore we interpret this band as a PCR artefact (Fig. 1B; see Section 4).

3.2. Genomic structure of the $P2X_2$ receptor gene

To obtain the genomic organization and putative splice sites

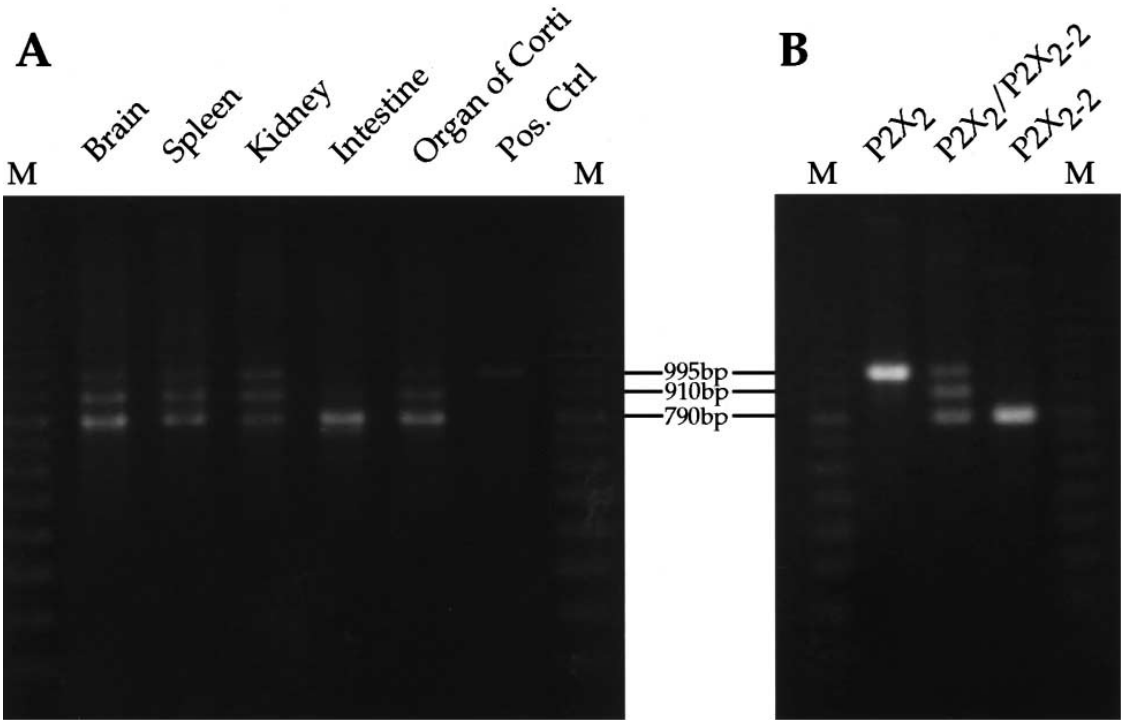
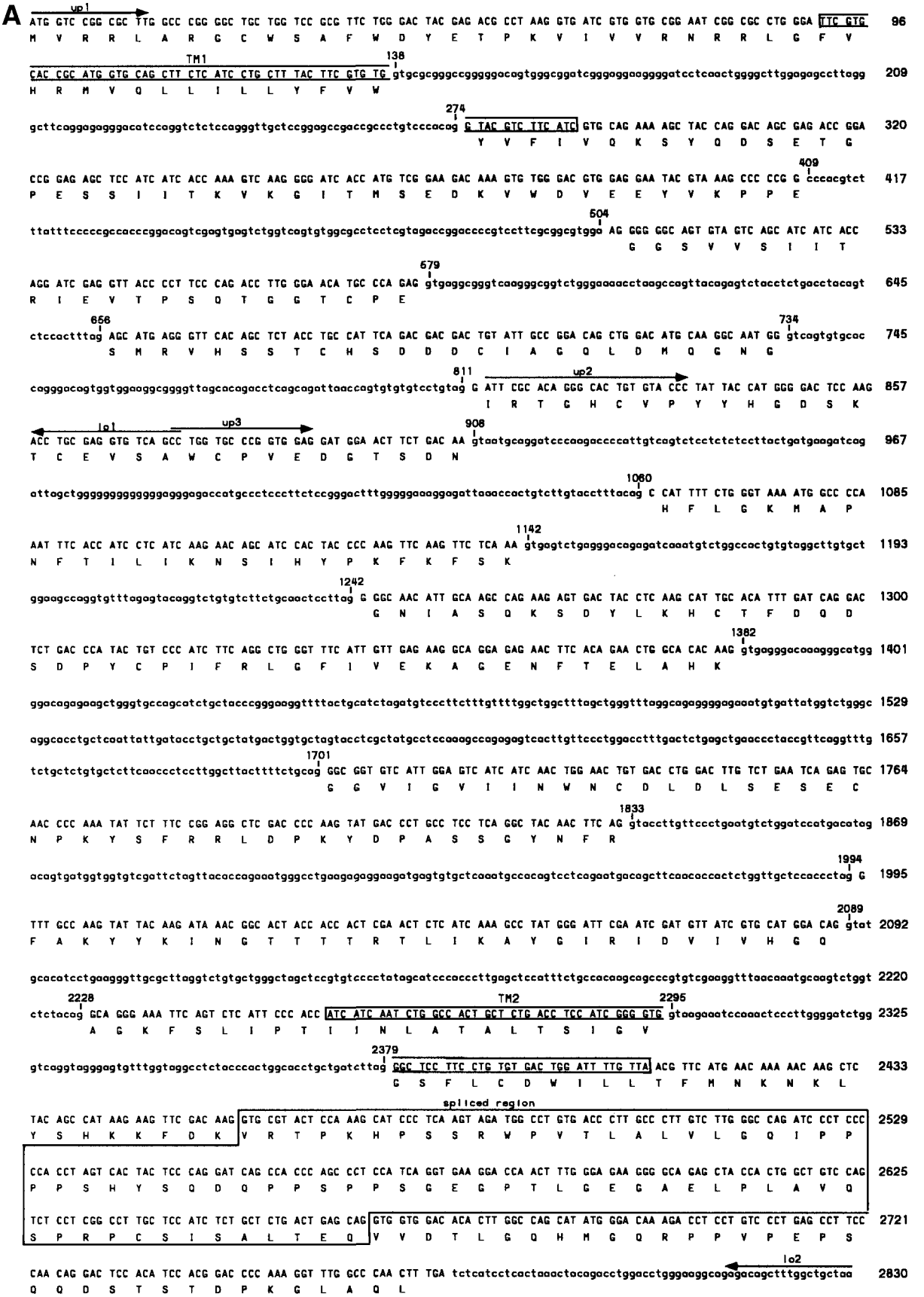


Fig. 1. A: Tissue distribution of $P2X_2$ isoforms detected by RT-PCR. The PCR yielded three products with 995 bp, 910 bp and 790 bp fragment length, respectively. The positive control was cloned $P2X_2$ DNA. As marker a 100 bp ladder was used. B: PCR using a mixture of the cloned $P2X_2$ sequence and the 207 bp deleted isoform resulted in the same 3 fragments as in A.


A
up1
TM1
up2
up3
lo1
lo2
TM2
spliced region

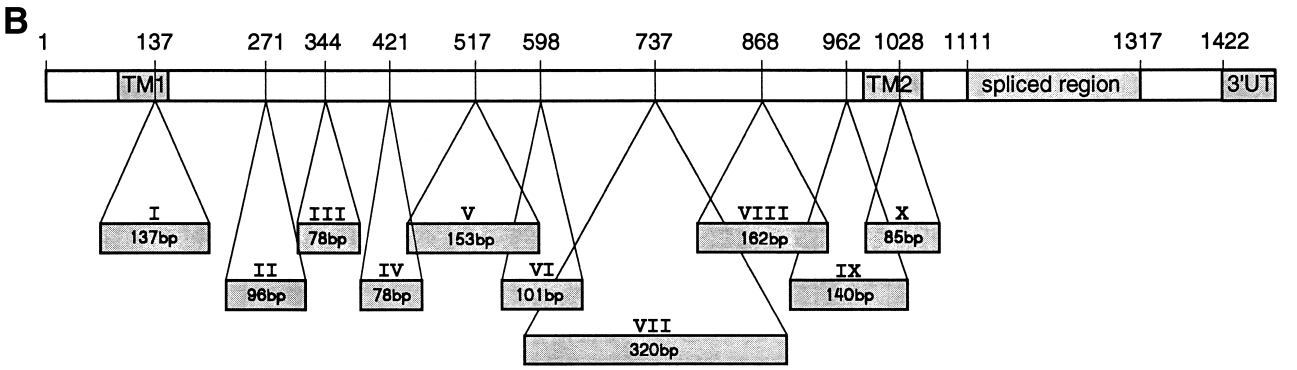


Fig. 2. A: Genomic structure of the rat $P2X_2$ gene. Eleven exons are shown with deduced amino acid sequences indicated below. The exon-intron borders are indicated by the corresponding base pair numbers, the 10 intron sequences are written in small letters. The transmembrane domains TM1 and TM2 and the spliced region of the $P2X_{2-2}$ isoform are shown in frames. The positions of primers are indicated by arrows. The sequence has been deposited in the EMBL data base under accession number Y09910. B: Organization of the $P2X_2$ receptor gene. The position of the introns (I–X) is indicated by the nucleotide number of the $P2X_2$ receptor coding region (A of ATG = 1). The putative transmembrane domains (TM1 and TM2) and the deleted region of the $P2X_{2-2}$ isoform are indicated.

of the $P2X_2$ receptor gene we amplified two overlapping PCR fragments covering the coding region of the rat $P2X_2$ receptor gene (Fig. 2A). The resulting genomic sequence with 2830 bp compared to 1481 bp of the $P2X_2$ receptor cDNA includes 10 introns ranging in size from 78 to 320 bp, most of them in the 100 bp range (Fig. 2B). Interestingly, the 207 bp deletion of the $P2X_2$ receptor isoform, which we detected in various tissues coexpressed with the $P2X_2$ receptor, is not flanked by introns but by consensus sequences for splice sites which induce splicing of a fragment directly out of an exon [15].

3.3. *Functional expression of the $P2X_{2-2}$ receptor*

Both isoforms of the $P2X_2$ receptor were expressed in *Xenopus* oocytes and HEK293 cells. Fig. 3 shows ATP-activated currents investigated in defolliculated oocytes with the two-microelectrode voltage clamp technique at a holding potential of −70 mV.

300 μM ATP applied for 1 min elicited inward currents with amplitudes of 2.1 ± 1.0 μA (mean ± S.D.; $n = 8$) in $P2X_2$ injected oocytes and of 5.5 ± 3.1 μA ($n = 12$) in $P2X_{2-2}$ injected oocytes. Comparing the ATP-activated currents of both clones we found a significantly faster desensitization of the $P2X_{2-2}$ receptor with desensitization time constants of $\tau = 12 \pm 1$ s ($n = 12$) compared to the $P2X_2$ receptor with $\tau = 56 \pm 6$ s ($n = 8$) ($P < 10^{-7}$ revealed by Student's *t*-test).

Steady-state current amplitudes of the ATP-activated currents (I_s; measured as remaining current amplitude compared to the maximum current amplitude) were evaluated during application of 300 μM ATP for 5–15 min. $P2X_2$ receptor currents desensitized to steady-state currents of $I_s = 7.4 \pm 3.4\%$ ($n = 9$) whereas $P2X_{2-2}$ receptor currents desensitized to significantly smaller current amplitudes of $I_s = 0.9 \pm 0.3\%$ ($n = 8$) ($P < 0.0005$).

The pharmacological properties of the $P2X_{2-2}$ receptor were measured in HEK293 cells using whole-cell recording at a holding potential of −75 mV. There was no obvious difference compared with the $P2X_2$ receptor. ATP induced concentration-dependent inward currents with a maximal response observed at 300 μM; half-maximal concentration (EC_{50} value) was 7.8 ± 2.1 μM and Hill slope was 1.7 ± 0.12 (mean ± S.E.M.; $n = 9$). α,β-Methylene-ATP (10–300 μM) was virtually inactive as an agonist at this receptor. Currents induced by 30 μM ATP were inhibited nearly completely by suramin (1–10 μM) and PPADS (10 μM).

4. Discussion

In this paper we describe cloning and functional expression of the splice variant $P2X_{2-2}$ of the $P2X_2$ receptor characterized by a 207 bp deletion in the C-terminus. We also report the cloning of the $P2X_2$ receptor genomic sequence which consists of 11 exons comprising the published cDNA sequence [2] and 10 introns (Fig. 2B). Analysis of splice donor and acceptor sequences showed that all intronic sequences except that of intron II fit to the extended GT-AG rule with the consensus sequences $^{C}/_{A}AG|GT^{A}/_{G}AGT$ (for splice donor sequence) and $(^{T}/_{C})_{n}N^{C}/_{T}AG|G$ (for splice acceptor sequence) [15]. In the $P2X_2$ receptor genomic sequence the 207 bp deletion in the C-terminus is not flanked by any intronic sequence but the splice junction sites of the deleted fragment are flanked by sequences fitting to the extended GT-AG rules. The 207 bp deletion is spliced out of a genomic region which codes for an exon of the $P2X_2$ receptor.

A recently published truncated form of the $P2X_2$ receptor [11] carries a 85 bp insertion containing a stop codon which is identical to intron X of the genomic sequence. This isoform was detected in pituitary gland and stria vascularis of the inner ear, but no functional expression has been shown. However, the large number of exon-intron borders suggests the existence of further $P2X_2$ receptor splice variants.

$P2X_2$-specific RT-PCR yielded the $P2X_2$ and $P2X_{2-2}$ fragments and a third band of 910 bp length besides. Neither subcloning nor direct sequencing of this band repeated for several times resulted in any sequence information with the expected size. The 910 bp band could not be eliminated by changing PCR conditions. Different annealing temperatures, numbers of PCR cycles and primer concentrations have been tested. Control PCRs using a mixture of cloned $P2X_2$ and $P2X_{2-2}$ DNA also resulted in three bands (Fig. 1B). Therefore we suggest that the 910 bp band may represent an artefact created by an interaction of the $P2X_2$ isoforms.

All tissues tested in this study using RT-PCR showed an expression of both isoforms (Fig. 1A) with a tendency to a stronger expression of the $P2X_{2-2}$ receptor.

Functional comparison of the $P2X_2$ and $P2X_{2-2}$ variants

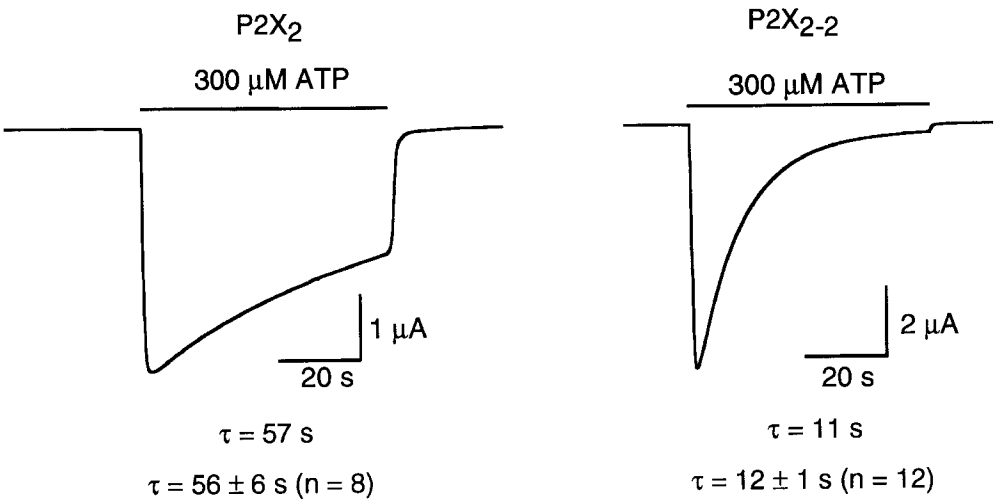


Fig. 3. Functional expression of the $P2X_{2-2}$ receptor and comparison with $P2X_2$. 300 μM ATP elicited inward currents which differed significantly in time constants of desensitization (mean ± S.D.) between the two isoforms. The recordings were made in *Xenopus* oocytes with two-electrode voltage clamp at a holding potential of −70 mV.

revealed significant differences. ATP-activated currents mediated by the $P2X_{2-2}$ receptor desensitized faster than $P2X_2$-mediated currents. Desensitization was nearly complete for the $P2X_{2-2}$ receptor (0.9 ± 0.3%) whereas the $P2X_2$ receptor desensitized to a steady-state level of 7.4 ± 3.4%. Thus $P2X_2$ rather than $P2X_{2-2}$ receptors mediate a steady Ca^{2+} influx in the presence of ATP. This may represent a remarkable functional difference between cells expressing either of the two $P2X_2$ isoforms. Differential expression of the $P2X_2$ and $P2X_{2-2}$ variants needs further investigation since in earlier studies probes for in situ hybridization, Northern blot analysis or antibody staining did not discriminate between them [2,11,14].

Besides the large difference in desensitization observed between the two splice variants we observed smaller but also significant differences between different batches of oocytes. This might be explained by a different level of second messenger regulation of the P2X receptors in different oocyte batches. There are several putative PKA/PKC regulation sites in the N-terminal and C-terminal regions of the P2X receptor sequence which might account for these effects. Some of these plus one proline-rich region with an SH3 binding motif for the association to cytoskeletal proteins [17] are localized within the 207 bp domain spliced out in $P2X_{2-2}$ which is located in the C-terminal intracellular region of the protein.

Coexpression of the $P2X_2$ and the $P2X_3$ receptor has been shown in dorsal root ganglion sensory neurons which transmit nociception [4,14]. Intermediate time courses of desensitization in ATP-activated currents measured in sensory neurons have been explained by heteromultimerization of $P2X_2$ and $P2X_3$. Such time courses might also be explained by an involvement of the $P2X_{2-2}$ receptor. Therefore, to understand the basis of important physiological processes like nociception the expression pattern, possible heteromultimerization and regulation of the $P2X_2$ receptor isoforms need further detailed investigation.

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