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Research report

Molecular cloning, functional characterization and possible cooperativity between the murine $P2X_4$ and $P2X_{4a}$ receptors [1]

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Abstract

We have cloned and functionally characterised the mouse orthologue of the $P2X_4$ receptor, $mP2X_4$, and a splice variant of this receptor, $mP2X_{4a}$. $mP2X_4$ is 388 amino acids in length and shares 94% and 87% identity with the rat and human $P2X_4$ receptors, respectively, while $mP2X_{4a}$ is 361 amino acids in length and lacks a 27-amino acid region in the extracellular domain corresponding to exon 6 of the known P2X receptor gene structures. When expressed in *Xenopus laevis* oocytes, $mP2X_4$ produces a rapid inward current in response to ATP with an EC_{50} of 1.68 ± 0.2 μM, consistent with the affinity of the rat and human $P2X_4$ receptors for ATP. This agonist response is potentiated by the P2X receptor antagonists suramin, Reactive blue 2 and, over a limited concentration range, by PPADS. Although $mP2X_{4a}$ forms a poorly functional homomeric receptor, it appears able to interact with the full-length $mP2X_4$ subunit to result in a functional channel with a reduced affinity for ATP. These results suggest a possible role for splice variants of P2X receptors in the formation of functional heteromeric ion channels.

Keywords: P2X receptor; $P2X_4$; Ion channel; Alternative splicing; ATP; Oocyte

1. Introduction

P2X receptors are ATP-activated intrinsic ion channels, permeable to monovalent and divalent cations, that allow physiologically significant levels of calcium influx into cells at voltages near the resting membrane potential. To date, seven subunits of P2X receptors have been cloned and the pharmacology of the recombinant homomeric receptor subunits determined in heterologous expression systems (for reviews, see Refs. [4,12,18]). At least three of the P2X receptor subunits, $P2X_2$, $P2X_4$ and $P2X_5$, have been shown to undergo alternative splicing [2,6,9,13,21]. Although localization studies have revealed co-expression of P2X receptor subunits in various neuronal and non-neuronal tissues (reviewed in Ref. [12]), there has been only one report describing a native P2X channel in sensory neurons, where the functional properties could be explained by a specific heteropolymerization of recombinant P2X receptor subunits [17]. The diversity of P2X receptor subunits and the additional complexity imposed by the presence of alternatively spliced variants, together with the potential for heteropolymerization between different P2X subunits, are among the variables that act in concert to determine the phenotypic response of any given cell type activated by extracellular ATP.

Of the known P2X receptor subunits, $P2X_4$ is the most ubiquitous and is represented in both neuronal and non-neuronal tissues. cDNAs encoding $P2X_4$ receptors have been isolated from hippocampus [1], brain [20,22], superior cervical ganglia [3], pancreatic islets in rat [25] and from brain [7] and stomach in human [6]. The human $P2X_4$ receptor maps to chromosome 12q24.32 [7]. Transcripts of approximately 2.0 kb in size for both rat and human $P2X_4$ are detected by northern analysis in all tissues examined with the exception of skeletal muscle in rat; this expression has been confirmed by RT-PCR studies. In situ hybridization studies reveal extensive distribution of $P2X_4$ mRNA in rat brain with particularly strong expression in the

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[1] The cDNA sequences encoding $mP2X_4$ and $mP2X_{4a}$ have been assigned Genbank accession numbers AF089751 ($mP2X_4$) and AF089752 ($mP2X_{4a}$).

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Purkinje cells of the cerebellum [1,5,20,22,24]. Strong expression of $P2X_4$ is also seen in spinal cord motoneurons [1,22], in the seminiferous tubules of the testis and the crypts of the colonic mucosal layer [24]. $P2X_4$ immunoreactivity in rat brain is detected with variable intensity throughout the neuraxis and, in Western blot analysis, the anti-$P2X_4$ antibody detects a native 57-kDa protein in different microdissected regions of rat brain and a 56-kDa protein where the recombinant $P2X_4$ receptor is expressed in human embryonic kidney (HEK) 293 cells [14].

In this paper, we report the cloning and functional characterization of the murine $P2X_4$ orthologue and a splice variant of this receptor, $mP2X_{4a}$, isolated from mouse brain. The full length $mP2X_4$ receptor encodes a functional receptor, whose response to ATP is potentiated by prototypical P2X receptor antagonists whereas the splice variant $mP2X_{4a}$ is weakly activated by maximal concentrations of ATP, insensitive to all other agonists tested, and functions poorly.

2. Materials and methods

A mouse brain cDNA library was purchased from Stratagene, UK. Nucleotide and nucleoside analogues, Reactive blue 2 and collagenase were obtained from Sigma with the exception of ATP, which was purchased from Boehringer Mannheim, UK. DNA sequencing reagents were purchased, along with the radiochemicals and Hybond N + membranes, from Amersham, UK. The $m^7G(5')ppp(5')G$ cap analogue (Ambion) was obtained from AMS Biotechnology, UK, Superscript II from Life Technologies, UK, and AGS*Gold*™ DNA polymerase from Hybaid, UK. Reagents for the isolation of total RNA were purchased from 5 Prime → 3 Prime (CP Laboratories, UK). Synthetic oligonucleotides were obtained from Life Technologies, UK and from Genosys, UK. All other molecular biological enzymes and reagents were obtained from Promega, UK. MetaPhor® agarose was purchased from FMC (Flowgen, UK). The rat $P2X_2$, human $P2X_4$ and rat $P2X_4$ cDNAs were generous gifts from D. Julius, W. Stühmer and X. Bo, respectively. We are grateful to G. Lambrecht for providing PPADS and to Bayer UK for providing suramin.

2.1. Library screening and generation of a full length $mP2X_4$ cDNA clone

A mouse brain cDNA library (cat. no. 937314) was screened at low stringency using the rat $P2X_2$ cDNA (Genbank accession no. U14414) as a probe. Approximately 300,000 plaque-forming units were transferred to Hybond N + membranes (Amersham, UK) and screened with a 985-bp *Ssp*I–*Not*I fragment which comprises the cDNA sequence encoding the second half of the extracellular domain, the second transmembrane domain and the C-terminus of the rat $P2X_2$ protein, as well as the entire 3′ non-coding region. Hybridization was performed overnight at 37°C in 40% formamide, 6× SSC, 5× BFP, 0.5% SDS, 50 mM sodium phosphate with 100 μg/ml sheared and denatured salmon sperm DNA and 50 ng of ^{32}P-radiolabelled probe fragment. Final washing of the membranes was at 50°C in 0.5× SSC, 0.1% SDS. Positive clones were purified to homogeneity and the corresponding pBluescript phagemids were in vivo excised from the Uni-ZAP XR vector according to the manufacturer's protocol. The DNA sequence of double-stranded plasmid DNA was obtained using the dideoxy chain termination method and the complete sequence of both strands was determined. A full length cDNA encoding the mouse $P2X_4$ receptor protein was constructed by replacing the 130-bp *Mlu*I–*Bam*HI fragment of mouse $P2X_{4a}$ with the 211-bp *Mlu*I–*Bam*HI fragment of I.M.A.G.E. cDNA clone identification number 789504 (Genbank accession number AA387605). The full length cDNAs encoding the mouse $P2X_4$ and $P2X_{4a}$ receptor proteins were independently subcloned into an appropriate vector for the production of capped cRNA transcripts [16].

2.2. Polymerase chain reaction amplification of $mP2X_4$ and $mP2X_{4a}$

Total RNA was isolated from C57BL/6J 10-week female adult brains and 3 μg of this was reverse-transcribed (RT) using a mouse $P2X_4$-specific primer located at the 3′-end of the coding region (5′-TATGGACGTGTGGGATTCC-3′); one tenth of the reverse-transcribed cDNA was used per PCR reaction. Control DNA was isolated from the mouse brain cDNA library (cat. no. 937314) for use in PCR. Briefly, an aliquot of the library was snap-frozen and thawed five times, phenol–chloroform extracted, ethanol precipitated and resuspended in water. A total of 5×10^6 plaque-forming units of mouse brain cDNA library was used in each PCR reaction. One nanogram of plasmid DNA containing either $mP2X_4$ or $mP2X_{4a}$ was included as a positive control. Template cDNA was amplified in a reaction containing 75 mM Tris–HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgSO_4$, 0.6 μM each of forward (5′-TGGGACTGGAAGGTGTGTTC-3′) and reverse (5′-GACGGAATATGGGGCAGAAG-3′) primers, 300 μM dNTPs and 1 unit of AGS*Gold*™ DNA polymerase. After a 3-min denaturation at 94°C, the reactions were amplified with the following profile: 30 s at 94°C, 30 s at 48°C and 30 s at 68°C for 35 cycles. One tenth of each reaction was reamplified under the above conditions. Reaction products were electrophoresed on a 2.75% MetaPhor® agarose gel.

2.3. In vitro transcription

Capped, in vitro transcribed cRNA was produced from 5 μg of linearised plasmid. The linearised DNA template was removed by incubation with RQ1 DNase. The cRNA was phenol–chloroform extracted and was ethanol precipi-

tated twice before resuspension in sterile water. The concentration of cRNA was determined spectrophotometrically and adjusted to a final concentration of 1 mg/ml. The integrity of the transcribed cRNA was verified by denaturing agarose gel electrophoresis in MOPS formaldehyde buffer.

2.4. Oocyte preparation, oocyte injection and electrophysiology

Stages V and VI *Xenopus laevis* oocytes were removed from the inner ovarian epithelial lining using fine forceps and were stored at 4°C in Barth's solution. In order to eliminate responses from endogenous purinoceptors present in the follicle cells [10,11], oocytes were defolliculated using a two-step process of collagenase treatment (2 mg/ml of type 1A in Ca^{2+}-free Ringer solution) followed by mechanical stripping of the follicle cell layer. Defolliculated oocytes were cytosolically injected with $mP2X_4$, $mP2X_{4a}$ or varying ratios of $mP2X_4$:$mP2X_{4a}$ cRNAs (40 nl at 1 ng/nl) and incubated at 18°C. At least two different cRNA preparations for each receptor were tested. At 48–60 h post-injection, oocytes were studied under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2A)

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                                                          TM I
mP2X4 protein      1 MAGCCSVLRAFLFEYDTPRIVLIRSRKVGLMNRVVQLLILAYVIGWVFVWEKGYQETDSV  60
hP2X4 protein      1 MAGCCSALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGYQETDSV  60
rP2X4 protein      1 MAGCCSVLGSFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGYQETDSV  60
mP2X4a protein     1 MAGCCSVLRAFLFEYDTPRIVLIRSRKVGLMNRVVQLLILAYVIGWVFVWEKGYQETDSV  60
                     ****** *  ********************** *************************

mP2X4 protein     61 VSSVTTKAKGVAVTNTSQLGFRIWDVADYVVPAQEENSLFIMTNMIVTVNQTQGTCPEIP 120
hP2X4 protein     61 VSSVTTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVMTNVILTMNQTQGLCPEIP 120
rP2X4 protein     61 VSSVTTKAKGVAVTNTSQLGFRIWDVADYVIPAQEENSLFIMTNMIVTVNQTQSTCPEIP 120
mP2X4a protein    61 VSSVTTKAKGVAVTNTSQLGFRIWDVADYVVPAQEENSLFIMTNMIVTVNQTQGTCPEIP 120
                     ******* ********* ************ ********* *** * * ****  *****

mP2X4 protein    121 DKTSICDSDANCTLGSSDTHSSGIGTGRCVPFNASVKTCEVAAWCPVENDAGVPTPAFLK 180
hP2X4 protein    121 DATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCEVAAWCPVEDDTHVPQPAFLK 180
rP2X4 protein    121 DKTSICNSDADCTPGSVDTHSSGVATGRCVPFNESVKTCEVAAWCPVENDVGVPTPAFLK 180
mP2X4a protein   121 DKTSICDSDANCTLGSSDTHSSGIGTGRCVPFNASVKTCEVAAWCPVENDAGVPT----- 175
mP2X4 EST          1          ANCTLGSSDTHSSGIGTGRCAPFNASVKTCEVAAWCPVENDAGVPTPAFLK  51
                     * *  * *** ** **  *** *  ****^ ** ************** *  ** *****

mP2X4 protein    176 AAENFTLLVKNNIWYPKFNFSKRNILPNITTSYLKSCIYNARTDPFCPIFRLGQIVADAG 240
hP2X4 protein    181 AAENFTLLVKNNIWYPKFNFSKRNILPNITTTYLKSCIYDAKTDPFCPIFRLGKIVENAG 240
rP2X4 protein    181 AAENFTLLVKNNIWYPKFNFSKRNILPNITTSYLKSCIYNAQTDPFCPIFRLGTIVEDAG 240
mP2X4a protein   176 ----------------------RNILPNITTSYLKSCIYNARTDPFCPIFRLGQIVADAG 213
mP2X4 EST         52 AAENFTLLVKNNIWYPKFNFSKRNILPNITTSYLKSCIYNARTDPFCPIFRLGQIVADAG 111
                     ******************************* ******* * *********** **  **

mP2X4 protein    214 HSFQEMAVEGGIMGIQIKWDCNLDRAASHCLPRYSFRRLDTRDLEHNVSPGYNFRFAKYY 300
hP2X4 protein    241 HSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYY 300
rP2X4 protein    241 HSFQEMAVEGGIMGIQIKWDCNLDRAASLCLPRYSFRRLDTRDLEHNVSPGYNFRFAKYY 300
mP2X4a protein   214 HSFQEMAVEGGIMGIQIKWDCNLDRAASHCLPRYSFRRLDTRDLEHNVSPGYNFRFAKYY 273
mP2X4 EST        112 HSFQEMAVEGGIMGIQIKWDCNLDRAASHCLPRYSFRRLDTRD                 154
                     **** ************ ********** ************** ***************

                                                          TM II
mP2X4 protein    274 RDLAGNEQRTLTKAYGIRFDIIVFGKAGKFDIIPTMINVGSGLALLGVATVLCDVIVLYC 360
hP2X4 protein    301 RDLAGNEQRTLIKAYGIRFDIIVFGKAGKFDIIPTMINIGSGLALLGMATVLCDIIVLYC 360
rP2X4 protein    301 RDLAAKEQRTLTKAYGIRFDIIVFGKAGKFDIIPTMINVGSGLALLGVATVLCDVIVLYC 360
mP2X4a protein   274 RDLAGNEQRTLTKAYGIRFDIIVFGKAGKFDIIPTMINVGSGLALLGVATVLCDVIVLYC 333
                     ****  ***** ************************** ******** ****** *****

mP2X4 protein    334 MKKRYYYRDKKYKYVEDYEQGLSGETDQ 388
hP2X4 protein    361 MKKRLYYREKKYKYVEDYEQGLASELDQ 388
rP2X4 protein    361 MKKKYYYRDKKYKYVEDYEQGLSGEMNQ 388
mP2X4a protein   334 MKKRYYYRDKKYKYVEDYEQGLSGETDQ 361
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Fig. 1. Amino acid sequence alignment of the murine, human and rat $P2X_4$ receptors. Alignment of the amino acid sequences of the murine ($mP2X_4$), human ($hP2X_4$) and rat ($rP2X_4$) orthologues of the $P2X_4$ receptor and of the $mP2X_{4a}$ splice variant and the murine $mP2X_4$ expressed sequence tag (EST). The 27 amino acid deletion in $mP2X_{4a}$ is indicated by dashes. Residues conserved in all three species are indicated by an asterisk (*). The two transmembrane domains are overlined. The single amino acid difference between the protein encoded by the mouse $P2X_4$ EST and those encoded by the mouse brain cDNAs described in this paper, and the rat and human orthologues is indicated by a carat (^). This difference is most probably due to a reverse transcriptase error that arose during the construction of the mouse 11.5 dpc embryo cDNA library from which the EST was derived as a valine residue is present in all proteins except that encoded by the EST and, further, since both the 11.5 dpc embryo and mouse brain cDNA libraries originated from the same *Mus musculus* strain, C57BL/6J.

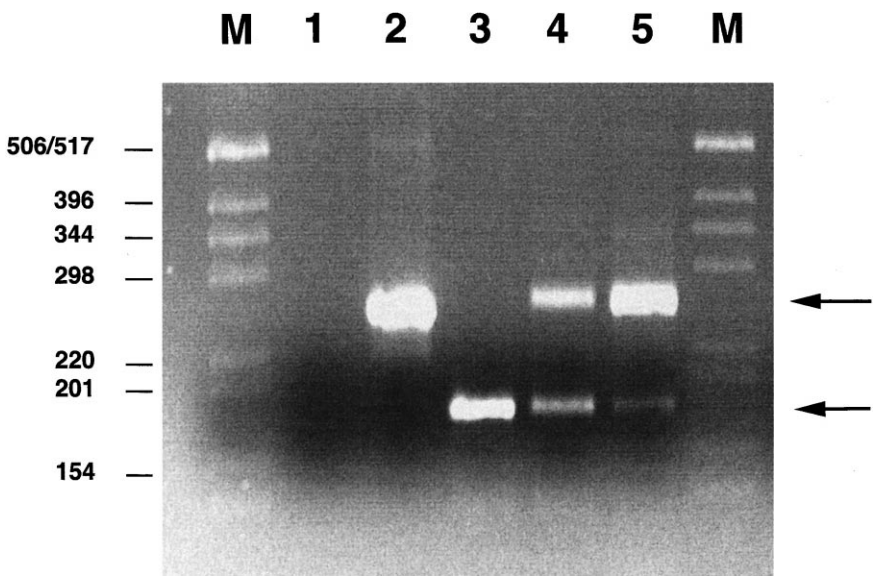

Fig. 2. Polymerase chain reaction amplification of $mP2X_4$ and $mP2X_{4a}$ isoforms. Primers flanking the alternatively spliced region were used in polymerase chain reaction to amplify $mP2X_4$-specific isoforms. M: DNA marker (position and molecular weights in nucleotides are indicated to the left of the figure); lane 1: no template (negative control); lane 2: $mP2X_4$ plasmid (positive control); lane 3: $mP2X_{4a}$ plasmid (positive control); lane 4: mouse brain cDNA library; lane 5: mouse brain cDNA. Arrows indicate the position of the full-length and alternatively-spliced isoforms of the $mP2X_4$ receptor.

and standard electrophysiological conditions. The voltage-recording and current-recording microelectrodes (tip resistance of 1–5 MΩ) were filled with 3.0 M KCl. Oocytes were superfused (at 5 ml/min) with Ringer's solution (pH 7.45) containing 110 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM $BaCl_2$. Inward currents were generated by stimulating the recombinant purinoceptors with superfused ATP (applied for 60–180 s) at the concentrations given in the text, followed by a period of washout of 20 min; the period of washout following antagonist applications was 1 h. Data were recorded on magnetic tape using a DAT recorder (Sony 1000ES) and displayed using a pen recorder (Gould) and were normalised to the maximum current (I_{max}) obtained with a supramaximal concentration (100 μM) of ATP. Dose–response data were transformed by the equation log $(I/I - I_{max})$ to yield Hill plots which were used to determine the EC_{50} values. The Hill coefficient (n_H) was obtained from the slope of these plots. ATP and antagonists were dissolved in Ringer's solution. Experiments were carried out at room temperature (18–20°C). Data are expressed as the mean ± S.E.M., analysed by ANOVA and compared with the Mann–Whitney test (two-tailed) using Instat V2.0 (GraphPad).

3. Results

Two independent cDNAs encoding the same 361 amino acid protein were isolated from the mouse brain cDNA library. The cDNAs were 1863 and 1891 nucleotides in length and were identical in their coding region and 3′-untranslated sequence, with an additional 28 nucleotides present at the 5′-end of the longer cDNA. Although the protein encoded by these cDNAs appeared to be the murine orthologue of $P2X_4$ ($mP2X_4$), a comparison with both the rat and human $P2X_4$ receptor sequences ($rP2X_4$ and $hP2X_4$) revealed that a 27-amino acid region located in the extracellular domain was missing (Fig. 1). This protein was subsequently called mouse $P2X_{4a}$. A search of the Genbank expressed sequence tag database (dbEST) identified a murine sequence (Genbank accession number AA387605) with greater than 99% nucleotide sequence identity with the $mP2X_{4a}$ cDNAs in which the 81 nucleotides encoding the missing 27 amino acids were present (Fig. 1). This EST clone was used to engineer a full-length mouse $P2X_4$ receptor cDNA.

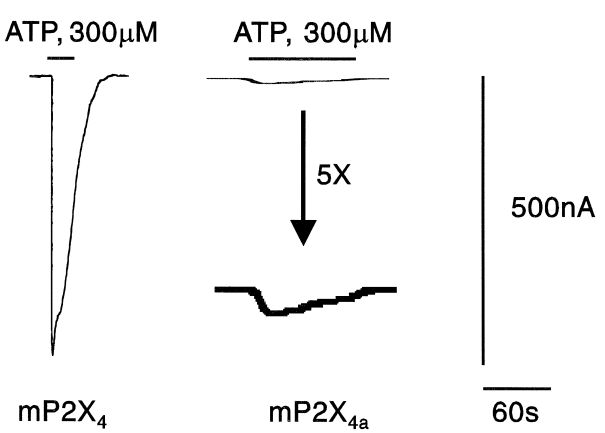

Fig. 3. ATP-sensitivity of homomeric $mP2X_4$ and $mP2X_{4a}$ ion channels. Inward currents to ATP were evoked from defolliculated *Xenopus* oocytes expressing either $mP2X_4$ or $mP2X_{4a}$ receptors. Whereas $mP2X_4$ was maximally activated by 300 μM ATP, the splice variant $mP2X_{4a}$ responded with a much smaller inward current. This response is shown at a five times magnification in the figure. Holding potential $v_H = -60$ mV.

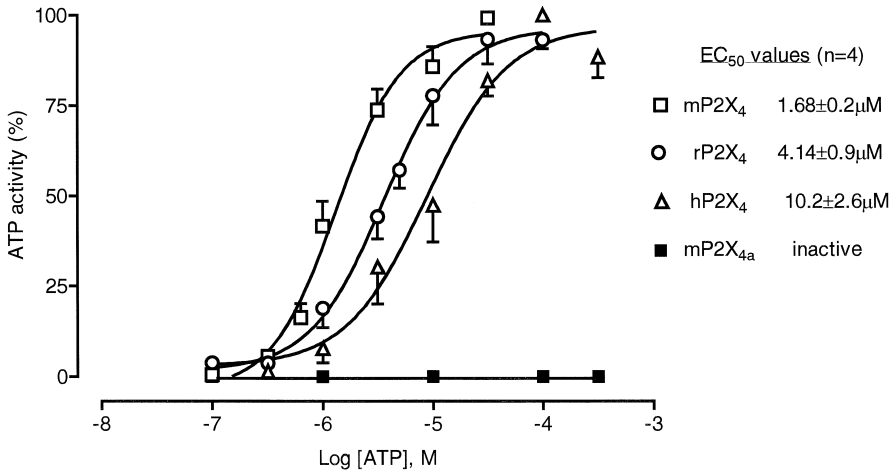

Fig. 4. Concentration response curves for ATP at $P2X_4$ receptors. Concentration response curves for ATP at the murine, rat and human $P2X_4$ receptors and at the mouse $P2X_{4a}$ splice variant. Responses were normalised to the maximum current obtained with a supramaximal concentration (100 μM) of ATP. For $mP2X_{4a}$, high concentrations of ATP (< 300 μM) gave rise to small inward currents of 1–10 nA. These small responses were normalised to the mean maximum current (see Table 1) obtained from $mP2X_4$ evoked by ATP (100 μM). Concentration–response data were transformed by the equation $\log(I/I - I_{max})$ to yield Hill plots which were used to determine the EC_{50} values which are expressed as the mean ± S.E.M. ($n = 4$).

Since a full-length $mP2X_4$ cDNA was not found amongst the 300,000 brain cDNA library recombinants screened, primers flanking the alternatively spliced region were used in polymerase chain reaction to determine whether both the full-length and deleted region were present in the same tissue. Fig. 2 reveals that both mouse brain cDNA and the cDNA pool from the brain library contain the two different forms of the $P2X_4$ receptor. There appears to be a greater proportion of the smaller fragment present in the cDNA library than in brain tissue, which would enhance the probability of isolating the deleted form of this receptor in a library screen.

The $P2X_4$ receptor protein encoded by this full-length mouse cDNA is 388 amino acids long and has 94% and 87% identity with the rat and human $P2X_4$ receptor proteins, respectively, each of which is also 388 amino acids in length. The 10 conserved cysteine residues located in the extracellular domain of members of the P2X receptor family are present in the $mP2X_4$ protein. Like the rat receptor, the mouse $P2X_4$ protein has a glutamine residue at position 78 and, like both the rat and human $P2X_4$ receptors, has a glutamic acid residue at position 249. Of the 51 positions where amino acid differences are observed between $mP2X_4$ and either $rP2X_4$ or $hP2X_4$, only four

Table 1
Comparison of activity indices at $mP2X_4$ and $mP2X_{4+4a}$

ATP activity	$mP2X_4$ (40 ng/oocyte)	$mP2X_4$ (20 ng/oocyte) + $mP2X_{4a}$ (20 ng/oocyte)	$mP2X_4$ (8 ng/oocyte) + $mP2X_{4a}$ (32 ng/oocyte)	$mP2X_4$ (8 ng/oocyte)	*N*
EC_{50}	1.68 ± 0.2 μM	4.49 ± 1.0 μM*	6.22 ± 1.8 μM*	2.25 ± 0.4 μM	4
n_H	1.55 ± 0.25	1.29 ± 0.24	1.32 ± 0.11	1.27 ± 0.13	4
E_{rev}	−3.5 ± 1.5 mV	−4.5 ± 1.9 mV	−0.9 ± 0.7 mV	−3.8 ± 2.1 mV	4
I_{max}[a]	817 ± 138 nA	575 ± 102 nA	453 ± 83 nA	103 ± 14 nA**	12
I_{max} (range)	(134–1700 nA)	(230–1394 nA)	(86–1158 nA)	(28–187 nA)	12
Rise time[b]	7.2 ± 1.2 s	5.9 ± 0.6 s	6.1 ± 0.7 s	6.8 ± 0.4 s	12
Inactivation (1/e)[c]	22.5 ± 2.8 s	28.3 ± 4.2 s	28.9 ± 4.6 s	23.3 ± 4.2 s	12
Potentiation[d]	212 ± 95%	318 ± 46%	326 ± 125%	230 ± 56%	4

Comparison of activity indices at $mP2X_4$ and at co-expressed $mP2X_4$ and $mP2X_{4a}$ receptors. $mP2X_4$ cRNA was injected at either 40 or 8 ng/oocyte (first and fourth data columns, respectively). A total of 20 ng of $mP2X_4$ and 20 ng of $mP2X_{4a}$ cRNAs (second data column) or 8 ng of $mP2X_4$ and 32 ng of $mP2X_{4a}$ cRNAs (third data column) were co-injected per oocyte. Seven different indices of ATP activity were examined for each of the four different injection compositions. Concentration–response data were transformed by the equation $\log(I/I - I_{max})$ to yield Hill plots which were used to determine the EC_{50} values which are expressed as the mean ± S.E.M.; the Hill coefficient (n_H) was obtained from the slope of these plots.

[a] I_{max} was calculated as the amplitude of the response to 100 μM ATP.

[b] Rise time for activation in response to 100 μM ATP.

[c] Time for the response to decay by 1/e in the presence of 100 μM ATP.

[d] Potentiation of the response to 0.6 μM ATP by 150 μM suramin.

Statistical significance (using Mann–Whitney test) at * $P < 0.05$ and ** $P < 0.01$ when the value for each activity index in data columns 2, 3 or 4 is compared with the corresponding value of that index in data column 1.

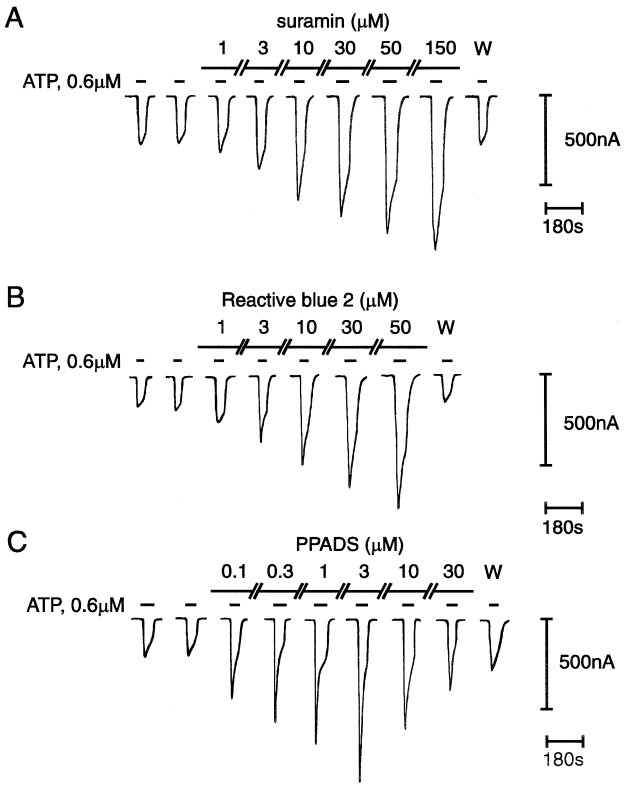

Fig. 5. Antagonist activity at $mP2X_4$ receptors. Potentiation of the response of $mP2X_4$ to ATP by suramin, Reactive blue 2 (RB-2) and PPADS. The response of $mP2X_4$ to a submaximal concentration of ATP (0.6 μM) approximately equal to the EC_{20} was potentiated by the cumulative addition of suramin, Reactive blue 2 or PPADS at the concentrations shown in the figure. Each concentration of antagonist was applied for 20 min. Washout (W) was for 60 min following the last application of antagonist. The change from potentiation to inhibition by PPADS at concentrations greater than 3 μM may not be due to competition with ATP at the receptor but, rather, may be due to the chemical degradation of the ligand binding site by PPADS which is considered to be a Schiff's base.

occur in the transmembrane regions and all are conservative substitutions of hydrophobic residues. The seven consensus sites for N-linked glycosylation (NXS/T) present in the extracellular domain of the rat and human $P2X_4$ receptors are conserved in $mP2X_4$; an eighth such site is present in the murine receptor. A number of potential sites for modification by protein kinases are also present in the amino and carboxyl termini of $mP2X_4$.

The ability of ATP to activate the full length $mP2X_4$ receptor and the truncated $mP2X_{4a}$ receptor was determined in the *Xenopus* oocyte expression system (Figs. 3 and 4). The $mP2X_{4a}$ receptor appears to form a poorly functioning ion channel since high concentrations of ATP (< 300 μM) gave rise to small currents (1–10 nA), indicating that the probability of channel opening was exceedingly low (Fig. 3). In order to determine whether $mP2X_{4a}$ responded better to other naturally-occurring nucleotide and nucleoside analogues, CTP, GTP, ITP, UTP, ADP, AMP, adenosine, UDP, UMP and uridine were tested individually, each at a concentration of 200 μM. The ApnA series ($n = 2$–6), each at a concentration of 20 μM, was also tested. No response to any of these ligands was observed (data not shown). In contrast, $mP2X_4$ produced a rapid inward current of up to 1.7 μA (see I_{max} range in Table 1), similar to those observed for the rat and human orthologues, in response to ATP. The EC_{50} value for ATP at $mP2X_4$ was 1.68 ± 0.2 μM, which is similar to the EC_{50} values we observed for ATP at both the rat and human $P2X_4$ receptors (Fig. 4). The response of $mP2X_4$ to ATP was potentiated by cumulative applications of the P2 antagonists suramin, Reactive blue 2 or PPADS, with a PPADS-specific inhibition of this effect at higher concentrations of this antagonist (Figs. 5 and 6).

To determine whether it was possible to observe any interaction between the $mP2X_4$ and $mP2X_{4a}$ receptors, we co-injected varying ratios of the two cRNAs into oocytes and examined the electrophysiological properties of the resulting receptor proteins (Table 1). When $mP2X_4$ and $mP2X_{4a}$ were co-injected in a 1:1 or a 1:4 ratio, respectively, there was no observable difference in the Hill coefficient, the reversal potential, the rise time for activation or the inactivation kinetics of the expressed channels, nor was there any difference in the potentiation of the ATP response by the antagonist suramin when compared with the properties of the homomeric $mP2X_4$ receptor. There were, however, slight but statistically significant ($P < 0.05$)

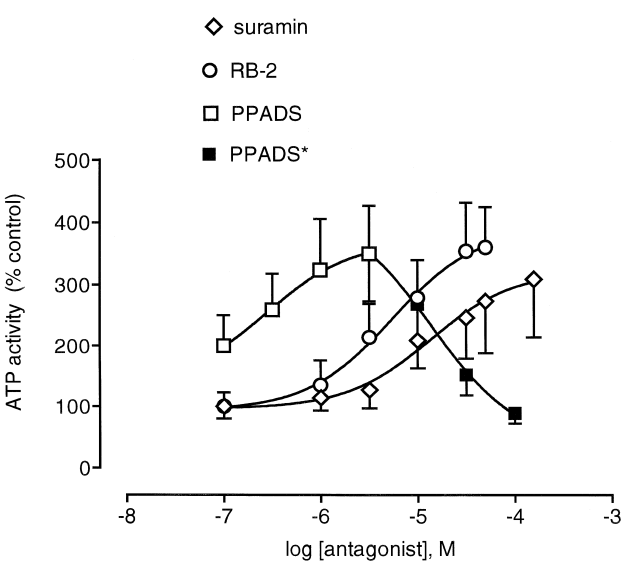

Fig. 6. Comparison of antagonist activity at the $mP2X_4$ receptor. The response of $mP2X_4$ to a submaximal concentration of ATP (0.6 μM) was maximized to 100% and the potentiation and/or inhibition of ATP responses by increasing, additive, concentrations of suramin, RB-2 or PPADS was plotted. PPADS, at concentrations greater than 3 μM, showed an inhibition of ATP responses, with an estimated IC_{50} value equal to 21 ± 4 μM ($n = 4$). This IC_{50} is based on the inhibition of the maximal ATP response in the presence of PPADS. No such inhibition was seen for either suramin or RB-2, up to a concentration of 150 μM, which potentiated the response of $mP2X_4$ to ATP at all concentrations tested.

differences seen in the EC_{50} for ATP. There was no significant reduction in I_{max} of the resultant ATP-gated ion channel when $mP2X_4$ and $mP2X_{4a}$ cRNAs were co-injected in 1:1 and 1:4 ratios. However, the I_{max} value fell significantly when $mP2X_4$ cRNA was injected alone at a 1:5 dilution in water ($P < 0.01$; see Table 1).

4. Discussion

When oocytes injected with full-length $mP2X_4$ were exposed to ATP, rapid inward currents were elicited. These responses were partially desensitising (by approximately 90% over 200 s) and required lengthy wash periods of 20 min for full recovery. The activity of ATP at $mP2X_4$ is similar to its activity at the human and rat $P2X_4$ orthologues. In our hands, the response of $mP2X_4$ to ATP is potentiated by suramin, Reactive blue 2 and PPADS in a manner similar to, although more pronounced than, that seen at the rat $P2X_4$ receptor stimulated by submaximal concentrations of ATP in the presence of 50 μM antagonist [1]. PPADS, at concentrations greater than 3 μM, showed an inhibition of ATP responses, with an estimated IC_{50} value equal to 21 ± 4 μM ($n = 4$). This IC_{50} is based on the inhibition of the maximal ATP response in the presence of PPADS. Suramin, Reactive blue 2 and PPADS have been shown to exert mild to pronounced, although not full, inhibitory effects on ATP-evoked currents at the rat $P2X_4$ receptor when applied at higher (> 50 μM) concentrations [3,20,22,25] and also at the human $P2X_4$ receptor [6,7], which is particularly sensitive to PPADS [7], although it has been shown that application of PPADS at concentrations greater than 100 μM is able to inhibit currents through other ligand-gated channels [3] which poses difficulties for the use of this compound. A lysine residue at position 78 of the rat $P2X_4$ receptor has been shown to increase suramin affinity [7] and a lysine residue at position 249 is required for full antagonism by PPADS [3]. $mP2X_4$ has a glutamine residue at position 78 and a glutamic acid residue at position 249 which may account for the failure of this receptor to be blocked by these antagonists.

The action of antagonists at $P2X_4$ receptors is not straightforward and appears to be dependent upon a number of factors. Knowing that antagonists were able to potentiate ATP responses at the rat $P2X_4$ receptor, we used a low (0.6 μM) concentration of ATP, corresponding to the EC_{20}, in our experiments to allow for the full potentiating effects of the antagonists to be evinced. However, several studies of antagonist action at $P2X_4$ receptors had been carried out using supramaximal concentrations of agonist [6,20,25]. Under such conditions, antagonists must compete against excess agonist and only inhibitory effects can be detected. A combination of submaximal concentrations of ATP and low concentrations of antagonist is necessary to detect the potentiating effect of P2 receptor antagonists at the $P2X_4$ receptor, whether it be the mouse or rat orthologue.

Application of high concentrations (300 μM) of ATP at $mP2X_{4a}$ yielded small inward currents of less than 1–10 nA. It was not possible to ascertain whether this was due to a significantly decreased affinity of this truncated $P2X_4$ receptor for ATP or if there was some impediment to the formation of a multimeric receptor channel complex. Considering the possibility that $mP2X_{4a}$ might be activated by a ligand other than ATP, we tested a number of naturally-occurring nucleotide and nucleoside analogues and failed to detect a response. This left the possibility that $mP2X_{4a}$, although unable to form an effective homomeric channel, might participate in the formation of a heteromeric channel with $mP2X_4$. We examined the electrophysiological properties of oocytes injected with different ratios of $mP2X_4$ and $mP2X_{4a}$, trying to determine whether there was any discernible change in channel function that could signify an involvement of $mP2X_{4a}$. When decreasing concentrations of $mP2X_4$ cRNA transcripts were injected into oocytes in the presence of $mP2X_{4a}$, there was no significant decrease in the maximal current (I_{max}). Injection of 8 ng of $mP2X_4$ cRNA in the presence of 32 ng $mP2X_{4a}$ cRNA yielded a channel with a significantly greater I_{max} than that seen when 8 ng of $mP2X_4$ cRNA was injected alone in the same injection volume. The presence of $mP2X_{4a}$ subunits appeared to contribute to the formation of functional heteromeric channels to account for the higher level of I_{max} although the amount of cRNA for the active $mP2X_4$ subunit remained the same. We assume that the translation efficiency of each subunit cRNA is identical and, further, that I_{max} gives a qualitative estimate of receptor density. The EC_{50} of ATP at the heteromeric $mP2X_4$:$mP2X_{4a}$ channel was significantly less than at the homomeric $mP2X_4$ channel, whose affinity for ATP appears to be independent of concentration of cRNA injected. These results suggest that there was a subtle interaction between the $mP2X_4$ and $mP2X_{4a}$ subunits and that $mP2X_{4a}$, whilst forming a poorly functional homomeric receptor, is able to interact with a full-length $mP2X_4$ to result in a functional heteromeric ion channel, although this is at the expense of its affinity for ATP. A recent report describing a direct interaction between the rat $P2X_4$ and $P2X_6$ receptor subtypes suggests that the ability of these subunits to interact in a biochemical assay may reflect a native expression of heteromeric $P2X_{4+6}$ channels with unique functional properties [15]. The results of our co-expression studies suggest that the $mP2X_4$ and $mP2X_{4a}$ isoforms may also participate in the formation of a heteromeric channel in native tissues.

The $mP2X_{4a}$ clone isolated from the library screening appears to be a splice variant of the $P2X_4$ receptor subtype. PCR amplification of the region containing the alternatively spliced sequence revealed that both isoforms were expressed in mouse brain and were also present in the

brain library that was screened. The mouse brain cDNA was reverse transcribed from mRNA primed with an oligonucleotide located at the C-terminus of the receptor, suggesting that the full length $mP2X_4$ sequence is expressed in mouse brain and is co-expressed with $mP2X_{4a}$.

While there is no information about the structural organization of the $P2X_4$ gene from any species, the genomic organization of the rat $P2X_2$ [2], the mouse $P2X_3$ [23], and the human $P2X_7$ (Genbank accession numbers Y12851, Y12852, Y12853, Y12854 and Y12855) genes has been elucidated. There is a significant degree of similarity between the genomic structures of the $rP2X_2$, $mP2X_3$ and $hP2X_7$ genes, which consist of 11, 12 and 13 exons, respectively. The position of the exon-intron boundaries of each of the known P2X gene structures is conserved, with eight of the first 10 introns occurring at the same location in each of the three P2X genes. The site of each of the remaining two introns is within one amino acid of the position of the corresponding intron in the other two P2X genes; the position of intron 1 in the human $P2X_7$ gene is found one residue before that of the first intron in both the $rP2X_2$ and $mP2X_3$ genes whereas intron 4 in the $mP2X_3$ gene is located one residue before that of intron 4 in the $rP2X_2$ and $hP2X_7$ genes. This conservation of structure, not unusual in multigene families, suggests that the genomic organization of the $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_6$ genes may be similarly conserved. If this is the case, $mP2X_{4a}$ is encoded by an mRNA in which the exon corresponding to exon 6 of the known P2X genes has been excised along with the flanking introns. It has recently been reported that the human $P2X_4$ gene appears to be alternatively spliced, following the isolation of a cDNA comprised of chaperonin hsp90-like sequence on the N-terminus followed by amino acid residues 95–388 of $hP2X_4$ [6]. Like $mP2X_{4a}$, this cDNA encoded a protein exhibiting little or no ion channel function when challenged with ATP. In this instance, the putative exons 1 and 2 are missing and the $P2X_4$ sequence present in the human splice variant begins at exon 3.

A number of splice variants of $P2X_2$ have been identified from rat pituitary and stria vascularis [9], rat brain [2] and rat cerebellum [21]. $P2X_{2\text{-}1}$ is encoded by an mRNA in which intron 10 sequence is present [9]. The predicted protein is missing the second half of the second transmembrane domain and C-terminus, and, instead contains a translated sequence from the included intron, terminating 29 amino acid residues beyond the junction at exon 10 and intron 10. $P2X_{2\text{-}2}$ [2] was independently identified as $P2X_{2b}$ [21] and corresponds to the excision of an optional intron in exon 11. $P2X_{2c}$ and $P2X_{2d}$ [21] result from the use of cryptic 3′ splice junctions in exon 2 and in intron 1, respectively, affecting the sequence of the second half of the first transmembrane domain. $P2X_{2c}$ and $P2X_{2d}$ have been shown to be non-functional, while $P2X_{2\text{-}2}$ ($P2X_{2b}$) is able to form a homomeric channel in *Xenopus laevis* oocytes and HEK 293 cells that is similar to the channel formed by $P2X_2$, with a more rapid desensitization rate than observed for the full length $P2X_2$ receptor.

A presumptive splice variant of $P2X_5$, missing sequence corresponding to exon 10 of the known P2X genes, has been isolated from human brain and has been shown to encode a receptor unable to form functional homomeric channels [13]. In light of increasing evidence for alternative splicing of P2X subtypes, we have examined the sequence of RP-2, an mRNA associated with programmed cell death in immature rat thymocytes [19]. The sequence of RP-2 from nucleotide 42 to the end of the cDNA is identical to that of rat $P2X_1$. A consensus splice acceptor site is found immediately preceding nucleotide 42 and RP-2 appears to be a splice variant of the rat $P2X_1$ receptor in which intron sequence is present. By inference with the known P2X receptor genes, this intron would appear to be intron 6, with $P2X_1$ sequence commencing at exon 7. An orthologue of RP-2 has also been isolated from mouse thymus [8]. Splice variants have not been reported for either $P2X_3$ or $P2X_7$, the two other genes whose structure has been determined, nor have splice variants of $P2X_6$ been described.

The majority of eukaryotic genes are interrupted by introns which are spliced out of the pre-mRNA to yield a single messenger RNA. In some instances, however, pre-mRNAs are alternatively spliced and a single gene is able to give rise to more than one mRNA sequence. Splicing of pre-mRNAs, therefore, affords a post-transcriptional means of regulating gene expression. Each of the different patterns of alternative splicing observed in the P2X receptor family influences the potential function and functionality of the encoded proteins. Alteration, deletion or addition of critical sequences in P2X subunits produces an altered ATP receptor. While we have been unable to ascribe a specific function to $mP2X_{4a}$, we believe that this splice variant may play a subtle role in P2X channel function, perhaps participating as a ‘silent partner’ in the formation of functional heteromeric channels. The eventual elucidation of the physiological role of the splice variants of P2X receptors will add a new and exciting element to the modulation of neurotransmission by purinoceptors.

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