Neuropharmacology 63 (2012) 394–404

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Retention of a new-defined intron changes pharmacology and kinetics of the full-length P2X2 receptor found in myenteric neurons of the guinea pig

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form
29 March 2012
Accepted 1 April 2012

Keywords:
ATP
P2X2 receptors
Alternative splicing
Intron retention
Myenteric neurons
Guinea pig
P2x2 gene

ABSTRACT

P2X2 plays an important role in ATP signaling in guinea pig myenteric plexus. Here, we cloned and characterized three P2X2 isoforms expressed in myenteric neurons. RT/PCR was used to amplify the cDNA of P2X2 variants. These were expressed in *Xenopus* oocytes, and nucleotide-induced membrane currents were recorded with the two-electrode voltage clamp technique. Three P2X2 cDNAs were identified in myenteric single neurons, named P2X2-1, P2X2-2 and P2X2-4. Based on the analysis of the structural organization of these variants we predicted that P2X2-2 is the fully processed variant, which lead us to propose a new exon-intron arrangement of P2X2 receptor gene with 12 exons and 11 introns. In agreement with this new model, the intron 11 is retained in P2X2-1 and P2X2-4 variants by alternative splicing. Expression of P2X2-1, P2X2-2 and P2X2-4 were found in 92, 42 and 37%, respectively, out of 40 analyzed single neurons. P2X2-4 does not form functional channels, and homomeric channels formed by P2X2-1 and P2X2-2 have different pharmacological profile. Thus, the former receptor is more sensitive to ATP, BzATP, and PPADS, whereas, suramin inhibited both receptors in a biphasic- and monophasic-manner, respectively. α,β-meATP has very low efficacy on either channel. Furthermore, ionic currents mediated by P2X2-1 have slower desensitization than P2X2-2. These results indicate that P2X2-1 was the most common P2X2 transcript in myenteric neurons and displays significant phenotypical changes implicating that retention of the intron 11 plays a major role in ATP signaling in the intestinal myenteric plexus.

1. Introduction

P2X receptors are ionotropic channels activated by extracellular ATP. Seven P2X subunits have been isolated in mammals (P2X1-7) and the crystal of zebra fish P2X4.1 receptor showed that three subunits conform the functional receptor (Kawate et al., 2009). ATP has excitatory effects via activation of P2X channels in central and peripheral neurons (Burnstock, 2006; Surprenant and North, 2009), including enteric neurons (Barajas-Lopez et al., 1994, 1996a; Galligan and Bertrand, 1994). In myenteric neurons of the guinea pig small intestine immunoreactivity for various P2X subunits has been reported, such as P2X2 (Castelucci et al., 2002), P2X3 (Poole et al., 2002) and P2X7 (Hu et al., 2001). In myenteric neurons of mouse small intestine, it has been proposed that homomeric P2X2 channels mediate fast excitatory post-synaptic potentials (Ren et al., 2003).

Membrane currents induced by ATP in myenteric neurons have kinetics and pharmacological properties that resemble (Barajas-Lopez et al., 1996a; Brake et al., 1994; Galligan and Bertrand, 1994) those described for the recombinant rat P2X2 receptor (North, 2002). For instance, they have similar desensitization kinetics, show the same sensitivity to ATP and ATP-γ-S, and are highly resistant to α,β-meATP. Currents with similar properties are found in the great majority of myenteric neurons (>90% of all recorded neurons), which is in agreement with recent findings from our laboratory showing the presence of P2X2-mRNA in about 93% of myenteric neurons (Valdez-Morales et al., 2011). In apparent controversy with such data, strong P2X2 immunoreactivity has been reported in about 30% of the guinea pig myenteric neurons

Abbreviations: α,β-meATP, α,β-methylene ATP; I_{ATP}, ATP-induced currents; PPADS, pyridoxalphophate-6-azophenyl-2′,4′-disulphonic acid; UTRs, untranslated region; BzATP, 2′-3′-O-(4-benzoylbenzoyl)-ATP; ORF, Open Reading Frame; *dreP2rx2*, zebrafish *P2rx2*; cpo*P2rx2*, guinea pig *P2rx2*.

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doi:10.1016/j.neuropharm.2012.04.002

(Castelucci et al., 2002). A simple explanation for this discrepancy is that myenteric neurons express isoforms of the P2X2 subunit, which might not be recognized by the antibody. In support to this hypothesis, splicing variants for P2X2 subunit have been reported in human, rat, and mouse (see Coddou et al., 2011). In the guinea pig organ of *corti* three P2X2 variants were reported: P2X2-1, P2X2-2 and P2X2-3 (Parker et al., 1998). P2X2-1 and P2X2-2 differ in the length of the C-terminal domain being the first isoform 64 amino acids longer. The P2X2-3 variant is similar to P2X2-1 but has additional 27 amino acids at the extracellular loop due to an in frame retained intron. P2X2-1 and P2X2-2 variants are well conserved among their mammalian orthologous (Coddou et al., 2011; Gever et al., 2006; Parker et al., 1998).

In this study, we characterized three forms of guinea pig P2X2 expressed by myenteric neurons in an effort to better understand the functional and pharmacological properties of P2X myenteric channels and their contribution to ATP signaling. Our findings lead us to propose a new exon-intron arrangement of P2X2 receptor gene (*P2rx2*), based on the observation that the full-length spliced form is the P2X2-2 variant instead of P2X2-1. According to this new model, *P2rx2* is constituted by 12 exons and 11 introns and P2X2-1 is generated by retention of intron 11. Our data shows that this intron retention changes the kinetics and the pharmacological profile of P2X2 channels.

2. Materials and methods

2.1. Genomic sequence analysis of P2X receptors

Genomic and cDNA sequences encoding P2X2 receptors were obtained from the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA, http://www.ncbi.nlm.nih.gov) and Ensembl database (http://www.ensembl.org). Exon-intron structure of *P2rx2* was derived from the aligned cDNA/genomic sequence or obtained directly from NCBI.

2.2. Cloning of P2X2 receptor

Tissues extracted from thymus and small intestine, were triturated in a mortar with liquid nitrogen. The RNAqueous RNA isolation kit (Life Technologies, Texas, USA) was used to obtain total RNA according to the manufacturer's protocol. First strand cDNA were synthesized using Superscript reverse transcriptase II (Life Technologies, Texas, USA) in the presence of oligo $(dT)_{18}$ for 1.5 h at 42 °C. PCR was performed using specific guinea pig P2X2 primers designed at the 5′ and 3′ UTRs (untranslated region) regions to amplify the entire coding sequence (F1 and R1, see Table 1). PCR reaction was done using Platinum Pfx Taq DNA Polymerase (Life Technologies, Texas, USA), conditions were as follows: initial denaturation for 2 min at 95 °C, then 40 amplification rounds of denaturation for 15 s at 95 °C, alignment for 20 s at 60 °C, and extension for 1 min 45 s at 68 °C; the final extension was 5 min at 68 °C. PCR products were analyzed by electrophoresis in 0.8% agarose gels stained with 1 μg/ml ethidium bromide. Images were obtained with Gel-Doc 2000 Gel Documentation System (Bio-Rad). PCR products were cloned into the pGEM-T Easy Vector (Promega, Wisconsin, USA) sequencing and subcloned in pCDNA3 vector.

2.3. Primary neuronal cultures

Guinea pigs (100–150 g) of either sex were sacrificed by cervical dislocation and carotid exsanguination. These methods have been approved by the Animal Care Committee of the IPICYT and are in agreement with the published Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society. Methods for dissection, dissociation and culture of myenteric neurons have been published in detail previously (Barajas-Lopez et al., 1996b). Briefly, a segment of 10–15 cm from the proximal jejunum was removed, placed in modified Krebs solution (in: mM: NaCl, 126; NaH2PO4, 1.2; MgCl2, 1.2; CaCl2, 2.5; KCl, 5; NaHCO3, 25; glucose, 11; gassed with 95% O2 and 5% CO2) and opened longitudinally. The mucosa and submucosal layers of this intestinal segment were dissected away. In guinea pig, most circular muscle layer can be removed leaving behind the longitudinal layer with the myenteric plexus embedded with it. This myenteric preparation was dissociated using a sequential treatment with two enzymatic solutions. The first solution contained papain (0.01 mg/ml; activated with 0.4 mg/ml L-cysteine) and the second contained collagenase (1 mg/ml) and dispase (4 mg/ml). Enzymes were removed by washing with L15 medium and the neurons were placed on round coverslips coated with sterile rat tail collagen in culture solution, which was composed of minimum essential medium 97.5% (v/v), 2.5% (v/v) guinea pig serum, 15 mM glucose, 2 mM L-glutamine, antibiotics (10 U/ml penicillin, 10 μg/ml streptomycin), and antimitotics (10 μM cytosine-β-D-arabinofuranoside, 10 μM fluorodeoxyuridine and 10 μM uridine) to prevent the growth of non-neuronal cells.

Table 1
List of primers used to amplify P2X2 variants.

Primer name	Sequence (5′-3′)
F1	GTTCTGGGCACCATGGCTGC
F2	CATCGTGCAGAAGAGCTACCA
F3	TTCACAGAGCTGGCACACAG
R1	TCCTGTCTGCAGACCTGGCGT
R2	GGAGACCCAACAACTTTGCCTG
R3	ATGGTGGGAATCAGGCTGAA

2.4. Single cell PCR

We used single myenteric neurons from primary 3–7 days old cultures, which were harvested under visual control into the glass pipette by applying negative pressure. Neurons were differentiated of other cell types by their round, compact, and bright body from which, long neurites arise (Eclipse TE200OU, Nikon). This pipette had a tip diameter of about 4–6 μm and contained 6 μl of RNase-free RT buffer (with RNase inhibitor, 20U; oligo $(dT)_{18}$, 2.3 μM; dNTPs, 150 μM; dTT 1.2 mM; 10X RT Buffer Superscript III First-Strand Synthesis System; Life Technologies , Texas, USA). The content of the pipette was expelled into a PCR-tube containing 12 μl of RNase-free RT buffer and 0.5 μl of NP40 1% to allow cell membrane solubilization and the reaction was incubated at 65 °C for 2 min. After adding 0.5 μl of reverse transcriptase III, the sample was transferred to 37 °C for 60 min, the reaction was inactivated by heating the sample to 70 °C for 10 min and placed on ice. Negative controls were performed without template; no false amplifications were obtained. PCR was performed using the same pairs of primers described above using Platinum Taq DNA Polymerase (Life Technologies, Texas, USA) and the PCR protocol were as follows: initial denaturation for 3 min at 94 °C, then 30 amplification rounds of denaturation for 15 s at 94 °C, alignment for 15 s at 60 °C, and extension for 1 min 45 s at 72 °C; the final extension was 5 min at 72 °C. Two separated nested PCR reactions were performed with different combination of primers using as a template 1 μl of a 1:10 dilution or 0.3 μl directly of the first PCR. The strategy is detailed in Fig. 1. The first reaction distinguished by length P2X2-1 of P2X2-2 using a set of primers 1: the reverse primer R1 and an internal forward primer F2 (see Table 1). The second combination (set 2) discriminated between P2X2-1 and P2X2-4 using the forward primer F1 and an internal reverse primer R2 (Table 1). Due to the low signal obtained in some of the neurons that difficult their visualization on agarose gels, we performed a third round of amplification changing primers F2 and R2 to F3 and R3, respectively (Table 1). PCR products of 839 (P2X2-1) or 647 bp (P2X2-2) were obtained with set 1 and 1025 (P2X2-1) or 833 bp (P2X2-4) with set 2 (Fig. 1). For these PCR amplifications, we used the same conditions as in the first PCR. PCR products were confirmed to be P2X2 by sequencing analysis.

2.5. Preparation of Xenopus laevis oocytes

Frogs were anesthetized by immersions in a solution of 10 mM Tricaine (3-aminobenzoic acid ethyl ester) (Sigma–Aldrich, MX) and oocytes were removed by dissection. Oocytes stages V and VI were manually defolliculated and placed in a storage saline solution, containing: extracellular solution (NaCl, 88 mM; KCl, 2 mM; $CaCl_2$, 1 mM; $MgCl_2$, 1 mM; and HEPES, 5 mM pH adjusted to 7.2–7.4 with NaOH) supplemented with theophylline (0.5 mM) and piruvic acid (2 mM). Cells were injected with 36 nl of cap and poliA P2X2 mRNA synthesized with T7 mMessage mMachine (Life Technologies, Texas, USA) and incubated at 14 °C for 12–36 h before the electrophysiological experiments.

2.6. Electrophysiological recordings

Membrane currents of oocytes were recorded using the two-electrode voltage clamp. These electrodes consisted in glass pipettes (0.3–0.8 MΩ resistance) filled with 2 M KCl solution containing 10 mM EGTA. ATP-induced currents (I_{ATP}) were recorded at a holding potential of −60 mV and at room temperature (22–24 °C). ATP solutions were freshly prepared and maintained in ice to decreased degradation. ATP and its analogs, α,β-meATP and BzATP, were applied usually for 5–15 s or until the current reached its peak. These nucleotides were washout for at least 3 min between consecutive applications. Suramin, and PPADS were pre-applied 15 s, 4 min and 2 min, respectively to reach the maximal effect (Guerrero-Alba et al., 2010). Concentration–response curves were constructed using oocytes batches from at least two different frogs. During these electrophysiological experiments the recording chamber was continuously superfused with standard external solution at approximately 3.3 ml/min. The rapid exchange of the external solution around the recorded cell was done using an eight-tube device. Each tube was connected to a syringe containing the control or an experimental solution. The tube containing the control solution was placed in front of the cell being recorded and the external