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P2X4 subunits are part of P2X native channels in murine myenteric neurons

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ABSTRACT

The aim of the present study was to investigate if P2X4 receptors are expressed in murine myenteric neurons and if these receptors contribute to form functional channels in the neuronal membrane by using molecular and electrophysiological techniques. The whole-cell recording technique was used to measure membrane currents induced by ATP (I_{ATP}) in myenteric neurons. Compared with recombinant P2X4 receptor-channels (reported by others in a previous study), native myenteric P2X receptors have a relative lower sensitivity for ATP (EC_{50}=102 μM) and α,β methylene ATP (not effect at 30 or 100 μM). BzATP was a weak agonist for native P2X receptors. KN-62 had no effect on myenteric P2X channels whereas PPADS (IC_{50}=0.54 μM) or suramin (IC_{50}=134 μM) were more potent antagonists than on P2X4 homomeric channels. I_{ATP} were potentiated by ivermectin (effect that is specific on P2X4 receptors) and zinc. Western blotting shows the presence of P2X4 protein and RT-PCR the corresponding mRNA transcript in the small intestine. Immunoreactivity for P2X4 receptors was found in most myenteric neurons in culture. Single-cell RT-PCR shows the presence of P2X4 mRNA in 90% of myenteric neurons. Our results indicate that P2X4 receptors are expressed in the majority of myenteric neurons, contribute to the membrane currents activated by ATP, and because most properties of I_{ATP} does not correspond to P2X4 homomeric channels it is proposed that P2X4 are forming heteromeric channels in these neurons. P2X4 subunits have a widespread distribution within the myenteric plexus and would be expected to play an important role in cell signaling.

1. Introduction

P2X receptors are cation nonspecific channels activated by extracellular adenosine 5′ triphosphate (ATP). They are distributed widely along different tissues and are conserved in a large variety of organisms including mammals, birds, and fishes (Fountain and Burnstock, 2009). In mammals there are seven subunits each of them encoded by different genes (P2X1–P2X7) (Coddou et al., 2011; North, 2002). Functional channels are constituted by three P2X subunits (Kawate et al., 2009; Torres et al., 1999). All subunits, except P2X6 (Coddou et al., 2011; North, 2002),can form homomeric channels, and all of them, except P2X7, (however see (Guo et al., 2007)), are able to combine with other subunits to form heteromeric channels with specific biophysical and pharmacological properties (Bo et al., 1995; Brake et al., 1994; Chen et al., 1995; Surprenant et al., 1995; Valera et al., 1994).

Among P2X receptors, the P2X4 subtype is one of the most ubiquitous in its expression along different tissues (Bo et al., 2003; Surprenant and North, 2009). In the mouse (Norenberg et al., 2012), it is the only P2X receptor that is positively modulated by ivermectin, as it has been determined in heterologous expression systems (Coddou et al., 2011; Khakh et al., 1999; Priel and Silberberg, 2004; Sim et al., 2006). Furthermore, this receptor shows either a low sensitivity (IC_{50} > 100 μM) (Buell et al., 1996; Soto et al., 1996) or positive modulation by suramin (Bo et al., 1995; Townsend-Nicholson et al., 1999). Zn^{2+} has also been reported that positively modulates P2X4 receptors, however, this action is not specific on these receptors because this cation has a similar effect on P2X2, P2X3, and P2X5 receptors (Coddou et al., 2011). P2X receptors play different roles in accordance to the tissue where they are expressed. In the enteric nervous system,

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they mediate fast excitatory postsynaptic potentials (Galligan and Bertrand, 1994). In myenteric neurons of mouse small intestine, it has been reported that P2X2 receptors, which are widely distributed in the neurons of this plexus, are essential for fast excitatory neurotransmission (Ren et al., 2003). The presence of P2X7 functional receptors in guinea pig myenteric neurons (Valdez-Morales et al., 2011) and mouse gastrointestinal tract (Gulbransen et al., 2012) is also in agreement with the abundant immunoreactivity for this receptor reported in guinea pig and rodents (Gulbransen et al., 2012; Hu et al., 2001; Vanderwinden et al., 2003). P2X3 immunoreactivity is rather weak (Ruan and Burnstock, 2005), which correlates well with P2X3-like currents recorded in less than 10% of murine myenteric neurons (Bian et al., 2003). Extensive immunoreactivity for P2X5 receptors has also been described and appears to be located mainly on nerve fibers (Ruan and Burnstock, 2005), which makes difficult to quantify the proportion of neurons expressing this receptor. Immunoreactivity for P2X1, P2X6 or P2X4 subunits appears to be absent (Ruan and Burnstock, 2005). However, controversial findings exists, at least for the presence of P2X4 receptor in the rat gut. Thus, immunoreactivity for P2X4 receptors was found to be widely distributed within the rat myenteric plexus (Bo et al.2003). A more recent study, however, locates P2X4 immunoreactivity on macrophages of the gut (Yu et al., 2010). Our aim here, was to investigate if P2X4 receptors are expressed in murine myenteric neurons, if they contribute to form native P2X channels, and if they are co-expressed with the P2X5 receptors. Whole-cell recordings showed that I_{ATP} was potentiated by ivermectin and immunoreactivity for P2X4 receptors is present in the great majority of myenteric neurons in culture. Furthermore, single-cell RT-PCR showed the presence of P2X4 mRNA in 90% of myenteric neurons. Therefore, all together, our results indicate that P2X4 subunits are expressed in most murine myenteric neurons and coexpressed with P2X5 receptors in one fourth of these cells. Our experimental evidence also indicates that P2X4 subunits contribute to I_{ATP}.

2. Materials and methods

2.1. Cultures of myenteric neurons

All the animals protocols used were 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.

C57BL/6J mice (19–25 g) of either sex were killed by cervical dislocation and carotid exsanguination. Immediately after the sacrifice a segment of 10–15 cm of jejunum was removed, placed in modified Krebs solution (in mM: NaCl, 126; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KCl, 5; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2 and 5% CO_2). The *external muscularis*, containing the myenteric plexus, was peel off and used for primary neuronal cultures. Neurons were dissociated using a sequential treatment with two enzymatic solutions as previously reported for our laboratory (Barajas-Lopez et al., 1996). The first one contained papain (0.01 mg/ml; activated with 0.4 mg/ml L-cysteine) and the second one collagenase (1 mg/ml) and dispase (4 mg/ml). Enzymes were removed with L15 medium and dissociated neurons were dispersed in culture solution and placed on round coverslips coated with sterile collagen from rat-tail. Culture medium was prepared with minimum essential medium 97.5% (v/v) and fetal bovine serum 2.5% (v/v), and supplemented with 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.

2.2. Patch clamp recordings

Whole-cell current recordings were made as previously described (Barajas-Lopez et al., 1996) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Currents were filtered at 0.5 or 1 kHz and sampled at 1 or 2 kHz, respectively. Recordings were carried out at room temperature (22–24 °C). Patch pipettes were mounted to a micromanipulator (Sutter, MP-85 Novato, CA, USA) and had resistances typically between 2 and 3 MΩ (internal diameter of the tip was around 2 μm). This low-resistance and a slight suction inside the pipette during current recordings help us to maintain a low access resistance (4.4 ± 0.18 MΩ; $n=9$), which will generate a voltage error of less than 10 mV for currents smaller than 2 nA, therefore, no compensation was intended for this factor. Membrane potential was held at −60 mV during the voltage-clamp experiments and membrane currents were captured in a hard drive of a PC using AXOTAPE (Molecular Devices, Sunnyvale, CA, USA) and analyzed using AXOGRAPH 4.9 software (Molecular Devices, Sunnyvale, CA, USA).

In some experiments, a voltage-ramp protocol was used, which consisted in a constant pre-conditioning voltage step from −60 to +70 mV for 400 ms (to inactivate transient currents), followed by a voltage ramp from +70 to −110 mV during 1100 ms, see inset of Fig. 1B. Membrane currents generated in response to the ramp were recorded before (Control) and then in the presence of ATP. Control currents were then subtracted to those recorded in the presence of ATP to obtain only the currents induced by this agonist. I_{ATP} values were then plotted against the membrane potential to build the current–voltage relationships shown in this study. For ramp experiments currents were stored in a hard drive of a PC using CLAMPEX 6 (Molecular Devices, Sunnyvale, CA, USA). The voltage-ramp experiments were carried out in the presence of 30 mM tetraethyl ammonium (TEA) and 30 μM Cd^{2+} to block most of the voltage-dependent potassium and calcium currents.

During electrophysiological experiments the recording chamber was continuously perfused with standard external solution at around 2 ml/min. A rapid exchange of the external solution around the recorded neurons was obtained using an eight-tube device, in which each tube was connected to a syringe containing the control or a given experimental solution. The tube containing the control solution was positioned in front of the cell being recorded and the external application of substances were done by abruptly moving another tube in front of the cell, which was already draining the same control solution plus the experimental drugs (e.g. ATP). The tube device was mounted and moved with a Water Robot Micromanipulator (WR-88; Narishigue Scientific Instrument Lab, Tokyo Japan). Effects of the substances were terminated by returning to the front of the cell the control solution tube. External solution was applied by gravity and the level of the syringes was frequently adjusted to minimize changes in flow rate. Standard external solution had the following composition (in mM): NaCl, 160; $CaCl_2$, 2; glucose, 10; HEPES, 5 and CsCl, 3; the pH was adjusted to 7.3–7.4 with NaOH. The standard pipette solution contained (in mM): CsCl, 150; EGTA, 10; HEPES, 5; NaCl, 10; ATPMg, 4.5 and GTPNa, 0.1, and was adjusted to pH 7.3–7.4 with CsOH.

2.3. RT-PCR amplification from jejunum

The brain (control) and a 10 cm segment of jejunum were removed from the same mice and triturated in a mortar with liquid nitrogen. Total RNA was isolated using the Ambion RNAqueous RNA isolation kit (Life Technologies, Texas, USA) following the manufacturer's protocol. Then, 5 μg of total RNA was reverse transcribed (RT) to first strand cDNA using oligo $(dT)_{18}$ and SuperScript II Reverse Transcriptase (Life Technologies, Texas, USA), for 1.5 h at 42 °C according to the manufacturer's protocol.