European Journal of Pharmacology 744 (2014) 190–202

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

Subcellular distribution and early signalling events of P2X7 receptors from mouse cerebellar granule neurons

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ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form
20 October 2014
Accepted 22 October 2014
Available online 30 October 2014

Keywords:
Cerebellar granule neurons
P2X7 receptors
Microfluorometry
Electrophysiology
ATP
BzATP

ABSTRACT

The subcellular distribution and early signalling events of P2X7 receptors were studied in mouse cerebellar granule neurons. Whole-cell patch-clamp recordings evidenced inwardly directed non-desensitizing currents following adenosine 5′-triphosphate (ATP; 600 μM) or 2′-3′-o-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP; 100 μM) administration to cells bathed in a medium with no-added divalent cations (Ca^{2+} and Mg^{2+}). Nucleotide-activated currents were inhibited by superfusion of 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+} or 100 nM Brilliant Blue G (BBG), hence indicating the expression of ionotropic P2X7 receptors. Fura-2 calcium imaging showed $[Ca^{2+}]_i$ elevations in response to ATP or BzATP at the somas and at a small number of axodendritic regions of granule neurons. Differential sensitivity of these $[Ca^{2+}]_i$ increases to three different P2X7 receptor antagonists (100 nM BBG, 10 μM 4-[(2*S*)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester, KN-62, and 1 μM 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine hydrochloride hydrate, A-438079) revealed that P2X7 receptors are co-expressed with different P2Y receptors along the plasmalemma of granule neurons. Finally, experiments with the fluorescent dye YO-PRO-1 indicated that prolonged stimulation of P2X7 receptors does not lead to the opening of a membrane pore permeable to large cations. Altogether, our results emphasise the expression of functional P2X7 receptors at both the axodendritic and somatic levels in mouse cerebellar granule neurons, and favour the notion that P2X7 receptors might function in a subcellular localisation-specific manner: presynaptically, by controlling glutamate release, and on the cell somas, by supporting granule neuron survival against glutamate excytotoxicity.

1. Introduction

ATP is a well-recognized signalling molecule that acts as a neurotransmitter and a neuromodulator in both the peripheral and the central nervous system (CNS) (Burnstock et al., 2011; Courjaret et al., 2012). The actions of ATP and other nucleotides are mediated by P2 receptors, which are divided into two groups: metabotropic P2Y receptors, coupled to G proteins, and ionotropic P2X receptors. Seven different P2X subunits (P2X1–7) have been cloned to date from various mammalian species. P2X receptors exhibit high permeability to Ca^{2+} and to a lower extent also to Na^+ and K^+ ions, such that P2X receptor activation induces both Ca^{2+} entry and membrane depolarisation (Bretschneider et al., 1995; Egan et al., 2006). P2X7 subunits share most structural characteristics with other members of the P2X family, but are

Abbreviations: ATP, adenosine 5′-triphosphate; A-438079, 3-(5-(2,3-dichlorophenyl)-1 H-tetrazol-1-yl)methyl pyridine hydrochloride hydrate; BSA, bovine serum albumin; BBG, Brilliant Blue G; BzATP, 2′-3′-o-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; CNS, central nervous system; DAPI, 4′6-diamidino-2-phenylindole; EGTA, ethylene glycol-bis(2-aminoethyl)-N′,N′,N′,N′-tetraacetic acid; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethil)-1-piperazineethanesulfonic acid; KN-62, 4-[(2*S*)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester; NMDG, N-methyl-D-glucamine; PBS, phosphate-buffered saline

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http://dx.doi.org/10.1016/j.ejphar.2014.10.036
0014-2999/

endowed with a unique intracellular C-terminal domain that is considerably larger than that of the other P2X subunits (Sperlagh et al., 2006; Costa-Junior et al., 2011).

P2X7 receptors are expressed in neurons from different CNS regions, including cerebellum (Atkinson et al., 2004; Sanchez-Nogueiro et al., 2005), hippocampus (Armstrong et al., 2002; Atkinson et al., 2004), cerebral cortex (Wirkner et al., 2005), medulla oblongata (Deuchars et al., 2001), and spinal cord (Deuchars et al., 2001; Armstrong et al., 2002; Wang et al., 2004). Functional P2X7 receptors have been reported in midbrain, cortical, and cerebellar synaptic terminals (Miras-Portugal et al., 2003; Gomez-Villafuertes et al., 2007; Alloisio et al., 2008; Marin-Garcia et al., 2008). Activation of P2X7 subunits at the presynaptic area is able to induce calcium-dependent exocytotic release of classical neurotransmitters such as acetylcholine, glutamate or GABA (Deuchars et al., 2001; Leon et al., 2008; Cervetto et al., 2012).

Recently, there have been a growing number of reports involving P2X7 receptors in several other aspects of CNS physiological and pathophysiological functioning (Zimmermann, 2011). In this respect, activation of P2X7 receptors leads to inhibition of glycogen-synthase kinase-3 (GSK-3), this serving as an alternative survival route to classical neurotrophic factors in cerebellar granule neurons (Ortega et al., 2009, 2010). Likewise, activation of P2X7 receptors negatively controls neurite formation and axonal growth in neuroblastoma cells and cultured hippocampal neurons (Diaz-Hernandez et al., 2008; Gomez-Villafuertes et al., 2009). Also, P2X7 receptors have been involved in the pathophysiology of neuropathic pain, Alzheimer, Parkinson and Huntington diseases, spinal cord injury, stroke, multiple sclerosis and cerebral malaria (Diaz-Hernandez et al., 2009, 2012; Marin-Garcia et al., 2009; Skaper et al., 2010; Leon-Otegui et al., 2011). Interestingly, opening of a membrane pore permeable to large molecules (up to 900 kDa) following sustained P2X7 receptor activation is commonly accepted as a pathophysiology-related feature of P2X7 receptor signalling.

Previously, we described the expression of functional P2X7 receptors in mouse cerebellar granule neurons (Sanchez-Nogueiro et al., 2005, 2009). Here, we have confirmed such an expression of P2X7 receptors by using patch-clamp recordings and studied early signalling (cytosolic Ca^{2+} concentration – $[Ca^{2+}]_i$ – increases and membrane pore formation) events of P2X7 receptors at both the somatic and axodendritic levels. Our results indicate a limited contribution of P2X7 receptors to Ca^{2+} signalling in neurites from differentiated granule neurons and the absence of non-specific membrane pores during sustained stimulation. Altogether, these results and those from our previous work suggest that segregation of P2X7 receptors into well-defined subcellular regions would allow a region-specific functioning, by, for instance, regulating neurotransmitter release at synaptic terminals and orchestrating the cellular defence against cytotoxicity at the somatic level.

2. Materials and methods

All experiments were carried out at the Universidad Complutense de Madrid following the guidelines of the International Council for Laboratory Animal Science (ICLAS) and in accordance with European and Spanish regulations (2010/63/UE; RD 53/2013).

2.1. Culture of mouse cerebellar granule neurons

Cerebella from six mouse (C57B1/6 J) pups (7 days old, killed by cervical dislocation) were removed aseptically, washed in Earl's Balanced Salt Solution (Sigma, Barcelona, Spain) and immersed in isolation medium (composition in mM unless otherwise stated: NaCl 130, KCl 4, $MgSO_4$ 1.5, Na_2HPO_4 10, 4-(2-hydroxyethil)-1-piperazineethanesulfonic acid (HEPES) 10, glucose 15, bovine serum albumin (BSA) 0.05, and penicillin/streptomycin 50 U/ml; pH 7.4) before being cut in small pieces. Tissue fragments were then transferred to a screw-cap tube and incubated in 10 ml of isolation medium supplemented with trypsin type I (from bovine pancreas, 0.25 mg/ml; Sigma) at 37 °C for 10 min under mild horizontal shaking. Enzymatic digestion was stopped by addition of 10 ml of isolation medium containing trypsin inhibitor type I-S from soybean (STI) (0.25 mg/ml; Sigma) and deoxyribonuclease I from bovine pancreas (DNase I) (250 U/ml; Sigma) and the tissue suspension was centrifuged 1 min at 66g. The supernatant was then centrifuged at 156g for 10 min. Both pellets were pooled and resuspended in 1 ml of isolation medium containing STI 0.25 mg/ml, DNase I 250 U/ml, and $MgSO_4$ 1.5 mM, and the suspension volume was made up to 50 ml with isolation medium. An additional centrifugation was performed (156g, 10 min) and the resulting pellet was suspended in Neurobasal medium (Gibco, BRL, Paisley, Renfrewshire, UK). Cell number and viability were assessed by the trypan blue exclusion method. Neurons were plated onto coverslips pre-coated with poly-L-lysine (Biochrom AG, Berlin, Germany) in Neurobasal medium supplemented with B27 (Gibco, BRL), penicillin 100 U/ml, streptomycin 0.1 mg/ml and anfotericin 0.25 μg/ml, KCl 21 mM, and glutamine 2 mM (all from Sigma), and were maintained in a humidified incubator at 37 °C in 5% CO_2. Cells were seeded at different densities as follows: 0.5×10^6 cells/ml for calcium imaging, 0.25×10^5 cells/ml for patch-clamp recordings, and 1.5×10^6 cells/ml for YO-PRO-1 uptake experiments. Neurobasal medium was replaced 24 h following cell plating and every 3 days thereafter. Cerebellar granule cells used in calcium imaging or YO-PRO-1 uptake experiments were kept in culture for a minimum of 9 days, such that a profuse axodendritic tree could be developed.

2.2. Immunocytochemistry

Cells attached to coverslips were fixed with cold 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) for 15 min, washed thrice with PBS, and, subsequently, incubated for 1 h in blocking solution (PBS containing 5% donkey serum (v/v), 3% BSA (w/v), and 0.1% Triton X-100 (v/v)). Then, cells were incubated during 1 h at room temperature with mouse anti-glial fibrillary acidic protein (GFAP) IgG (1:200 dilution; Sigma). After three washes in blocking solution, cells were exposed for 1 h at room temperature with anti-mouse FITC-conjugated secondary antibody (1:200 dilution; Sigma), and washed again three times. Cell nuclei were revealed with 4′6-diamidino-2-phenylindole (DAPI, 1 μM, 5 min; Sigma). Coverslips were observed under a NIKON-TE-200 microscope equipped with a Fluor-S 60 × /1.4 oil objective and fluorescein and DAPI filters. Images were obtained using a Kappa camera ACC1 controlled by 206 Kappa image base control software from Kappa optronics GmbH (Germany).

2.3. Electrophysiological experiments

Electrophysiological recordings were performed with an EPC9 patch-clamp amplifier using PatchMaster software (HEKA Electronic, Lambrecht, Germany). Pipettes of approximately 3 MΩ were fabricated from borosilicate glass (Kimax 51, Witz Scientific, Holland, OH, USA) with a Narishige PP830 puller (Tokyo, Japan), and subsequently fire-polished and coated with dental wax. Recording solutions were designed to enhance P2X7 receptor-mediated currents by removing divalents from the bath and dialyzing cells with N-methyl-D-glucamine (NMDG). Hence, the standard extracellular solution (bath solution) had the following composition (in mM): NaCl 140, HEPES 10, glucose 30 (pH 7.2, adjusted with NaOH; 298 mOsm). To examine the permeability of P2X7 receptor activated-channels to large cations, $NMDG^+$ was substituted for Na^+ in some experiments. Likewise, recording pipettes were filled with a solution containing (in mM): NMDG 140, ethylene glycol-