Neuropharmacology 73 (2013) 122–137

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

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

P2X7 receptors at adult neural progenitor cells of the mouse subventricular zone

Nanette Messemer [a], Christin Kunert [a], Marcus Grohmann [a], Helga Sobottka [a], Karen Nieber [b], Herbert Zimmermann [c], Heike Franke [a], Wolfgang Nörenberg [a], Isabelle Straub [a], Michael Schaefer [a], Thomas Riedel [a], Peter Illes [a,*], Patrizia Rubini [a,**]

[a] Rudolf Boehm Institute of Pharmacology and Toxicology, University of Leipzig, Haertelstrasse 16-18, 04107 Leipzig, Germany
[b] Department of Pharmacology for Natural Sciences, Institute of Pharmacy, University of Leipzig, 04103 Leipzig, Germany
[c] Institute of Cell Biology and Neuroscience, Goethe University, 60438 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 1 October 2012
Received in revised form
1 May 2013
Accepted 4 May 2013

Keywords:
Adult neural progenitor cells
Brain subventricular zone
P2X7 receptors
Extracellular ATP

ABSTRACT

Neurogenesis requires the balance between the proliferation of newly formed progenitor cells and subsequent death of surplus cells. RT-PCR and immunocytochemistry demonstrated the presence of P2X7 receptor mRNA and immunoreactivity in cultured neural progenitor cells (NPCs) prepared from the adult mouse subventricular zone (SVZ). Whole-cell patch-clamp recordings showed a marked potentiation of the inward current responses both to ATP and the prototypic P2X7 receptor agonist dibenzoyl-ATP (Bz-ATP) at low Ca^{2+} and zero Mg^{2+} concentrations in the bath medium. The Bz-ATP-induced currents reversed their polarity near 0 mV; in NPCs prepared from P2X7$^{-/-}$ mice, Bz-ATP failed to elicit membrane currents. The general P2X/P2Y receptor antagonist PPADS and the P2X7 selective antagonists Brilliant Blue G and A-438079 strongly depressed the effect of Bz-ATP. Long-lasting application of Bz-ATP induced an initial current, which slowly increased to a steady-state response. In combination with the determination of YO-PRO uptake, these experiments suggest the dilation of a receptor-channel and/or the recruitment of a dye-uptake pathway. Ca^{2+}-imaging by means of Fura-2 revealed that in a Mg^{2+}-deficient bath medium Bz-ATP causes $[Ca^{2+}]_i$ transients fully depending on the presence of external Ca^{2+}. The MTT test indicated a concentration-dependent decrease in cell viability by Bz-ATP treatment. Correspondingly, Bz-ATP led to an increase in active caspase 3 immunoreactivity, indicating a P2X7-controlled apoptosis. In acute SVZ brain slices of transgenic Tg(nestin/EGFP) mice, patch-clamp recordings identified P2X7 receptors at NPCs with pharmacological properties identical to those of their cultured counterparts. We suggest that the apoptotic/necrotic P2X7 receptors at NPCs may be of particular relevance during pathological conditions which lead to increased ATP release and thus could counterbalance the ensuing excessive cell proliferation.

1. Introduction

Besides acting as metabolic intermediates, ATP, ADP or uracil nucleoside tri- and diphosphates (UTP, UDP, UDP-glucose and UDP-galactose) are now recognized to act as key extracellular signalling molecules in various organ systems including the central nervous system (CNS) (Abbracchio and Burnstock, 1994). The cellular effects of these compounds are mediated by seven P2X receptors (P2X1-7 subtypes, which are ligand-gated cation channels; Khakh and North, 2006) and eight G-protein-coupled P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$ receptors; Abbracchio et al., 2006).

Neuroepithelial stem cells in the embryonic nervous system generate most of the neurons and glia in the developing brain (Doetsch, 2003). In addition neurogenesis continues in the adult

Abbreviations: α,β-meATP, α,β-methylene ATP; 5-BDBD, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one; BBG, Brilliant Blue G; Bz-ATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine-5′-triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CNS, central nervous system; E_{max}, maximal effect; EC_{50}, concentration of agonist producing 50% of E_{max}; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; 2-methylthio ATP, 2-MeSATP; MTT, 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazoliumbromid; Msi1, musashi1; NPC, neural progenitor cell; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; SVZ, subventricular zone; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; wt, wild-type; X^2 concentration, divalent cation concentration.

* Corresponding author. Tel.: +49 341 9724614; fax: +49 341 9724609.
** Corresponding author. Tel.: +49 341 9724634; fax: +49 341 9724609.
E-mail addresses: Peter.Illes@medizin.uni-leipzig.de (P. Illes), Patrizia.Rubini@medizin.uni-leipzig.de (P. Rubini).

0028-3908/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.05.017

mammalian brain in specific neurogenic niches, the subgranular zone of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle, contributing mature neurons to the dentate gyrus and olfactory bulb, respectively (Götz and Huttner, 2005; Zhao et al., 2008).

Extracellular nucleotides can influence the proliferation and fate decision of neural progenitor cells (NPCs) both during embryonic development and in the adult brain (Mishra et al., 2006; Rubini et al., 2009). Previous work has demonstrated that $P2Y_1$ receptors are present at NPCs of the SVZ *in vitro* and *in situ* (Mishra et al., 2006; Grimm et al., 2009; Suyama et al., 2012), causing rapid $[Ca^{2+}]_i$ transients and augmenting growth factor-mediated proliferation.

Neurogenesis requires the balance between the proliferation of newly formed progenitor cells and subsequent death of surplus cells. This is of particular relevance in pathological conditions such as traumatic injury, hypoxia/ischemia, and epilepsy that cause excessive proliferation of NPCs (Suh et al., 2009). The functional properties of the P2X7 receptor make it a candidate for counter regulation of excessive neuro- and/or gliogenesis. This receptor mediates various cell damaging effects of ATP, such as actin reorganization/membrane blebbing, interleukin-1 processing/release, channel dilation with or without the involvement of pannexin-1, and caspase activation with subsequent apoptosis (Sperlágh et al., 2006). P2X7 receptors are activated by high concentrations of ATP which are released from dying or metabolically stressed cells (Burnstock et al., 2011).

Although P2X7 receptors were shown to induce apoptosis/necrosis in embryonic NPCs (Delarasse et al., 2009, 2011) and P2X7 mRNA was present in undifferentiated neural cell-lines (Yuahasi et al., 2012) convincing electrophysiological evidence for the membrane location of such ligand-gated cationic channels in neural or non-neural stem cells is missing. We therefore searched for functional P2X7 receptors at both undifferentiated cultured mouse NPCs prepared from the adult SVZ and at NPCs in the SVZ of acute brain slices. We show that in low divalent cation-containing extracellular medium, which favours the generation of epileptiform spontaneous neuronal discharges in hippocampal brain slices (Heinemann et al., 1992), P2X7 receptors become unmasked at NPCs previously exhibiting only a low sensitivity to ATP and its structural analogue dibenzoyl-ATP (Bz-ATP). The activation of these apoptotic/necrotic P2X7 receptors may thus limit excessive neuro- and gliogenesis induced by seizure disorders or other types of neuronal damage.

2. Methods

2.1. Experimental animals

Unless otherwise stated, experiments were performed with primary cultures of adult NPCs obtained from C57Bl/6N wild type (wt) mice. In addition, $P2X7^{-/-}$ (Charles River; Solle et al., 2001), $P2Y_1^{-/-}$ (gift of Dr. Beverly H. Koller, Chapel Hill, NC, USA) (Fabre et al., 1999), and transgenic mice [Tg(nestin/EGFP)] overexpressing enhanced green fluorescent protein (EGFP) under the control of the rat nestin promoter (gift of Dr. Helmut Kettenmann, Berlin, Germany) were used. For the experiments with $P2Y_1^{-/-}$, the corresponding wt background mouse was of the 129Sv strain (Harlan).

2.2. Preparation and culture of neural progenitor cells (NPCs)

Primary cultures of adult NPCs from the SVZ were prepared from 8 to 14 weeks old mice (Grimm et al., 2009). After 7 days of culturing as neurospheres cells were dispersed and seeded onto coated culture dishes (patch-clamp, RT-PCR) or coated glass coverslips (Ca^{2+} imaging, immunocytochemistry, YO-PRO-1 uptake) with proliferation medium containing 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml fibroblast growth factor-2 (FGF-2). A combination of poly-L-ornithin (PLO; 0.5 mg/ml) and fibronectin (5 μg/ml) was used to coat culture dishes or coverslips (both Sigma-Aldrich). For testing the possible dependence of P2X7 receptor function on the type of coating, laminin (20 μg/ml) or collagen (100 μg/ml) were used alternatively (both Sigma-Aldrich). In some of the experiments, adherent NPCs were cultured in the presence of growth factors for only 1 day and thereafter the growth factors were omitted for further 3 days.

Multipotency of NPCs was tested *via* replacing growth factors with 2% foetal calf serum (FCS; Biochrom) in the proliferation medium. After 1–4 days of adherent cultivation in proliferation medium, it was changed to this FCS containing medium, and cells were additionally grown for 6 days at 37 °C and in 5% CO_2.

2.3. RT-PCR and immunocytochemistry

mRNA was isolated from 1 day old adherent NPCs using TRIzol reagent as described in the manual (Invitrogen). cDNA was synthesized using SuperScript® II Reverse Transcriptase and oligo (dT) primer (Invitrogen). A PCR with 35 cycles was performed using GoTaq Flexi DNA polymerase (Promega) and specific primers for P2X1-7 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The forward and reverse primer sequences were the following; P2X1, CCAGGACTTCCGAAGCCTTGC, AGAACTGTGGCCACTCCAAAGATG; P2X2, ATCGGGGTGGGCTCCTTTCTG, TCCCA TATGCTGGTCAAGAGTGTC; P2X3, TCTTGCACGAGAAGGCCTACCAA, GATCTCACA GGTCCGACGGACA; P2X4, GAGAATGACGCTGGTGTGCCAA, CCCTGTAGTACTTGGCAAA CCTGA; P2X5, GAGTGCTGTGGTCACCAAAGTCA, CTGGGCACCAAGCAAAGATCTCA; P2X6, TGTTACCGAGAGAGAACCAAGGAT, GCTTTGGCAAGCTTTACTTCAGCA; P2X7, TTGCTTTGGTGAGCGATAAGCTGT, GCCAGTCTGGATTCCTTTGCTCT; and GAPDH, CAAGGTCATCCATGACAACTTTG, GGGCCATCCACAGTCTTCTG.

Immunocytochemical staining of NPCs was performed by procedures similar to those previously described for astrocytes (Fischer et al., 2009).

Antibodies were used as follows: rabbit anti-P2X7 receptor (1:600, polyclonal; Alomone), rabbit anti-musashi1 (Msi1, 1:200, monoclonal; Abcam), mouse anti-nestin (1:100, monoclonal), mouse anti-CNPase (1:250, monoclonal) (both Chemicon), mouse anti-βIII Tubulin (1:1000, monoclonal; Promega), rabbit anti-active caspase 3 (1:50; MBL International) and mouse anti-GFAP (1:1000, monoclonal; Sigma-Aldrich). The appropriate Cy3- and Cy2-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. Nuclei were stained with Hoechst 33342 (Molecular Probes).

Because of the reported problems with the selectivity of P2X7 receptor antibodies especially for immunohistochemistry in the CNS (Sim et al., 2004; Brass et al., 2012), we purchased two antibodies from different commercial sources. They were tested by Western-blotting for the recombinant human P2X7 receptor transfected in HEK293 cells (not shown). With the antibody of Abcam (ab77413; directed against an epitope at the extracellular loop of P2X7) a specific band at about 75 kDa was observed, but in addition several (at least four) further, non-specific bands appeared, which were also observed in non-transfected HEK293 cells. By contrast, the antibody of Alomone (APR-004; directed against an epitope at the intracellular C terminus of P2X7) exhibited only a single specific band at about 70 kDa in transfected HEK293 cells. Therefore, we used the Alomone antibody in all immunocytochemistry experiments.

Stained cells were examined with a confocal laser scanning microscope (see Fischer et al., 2009). For quantitative evaluation of undifferentiated NPCs, a series of 10 images from 3 independent experiments across the axis of the coverslip were taken with a 40× objective. These pictures were evaluated with ImageJ software; the percentage of Msi1- or nestin-positive cells was calculated in relation to the total number of cell nuclei stained with Hoechst. For documentation of the expression of active caspase 3-immunoreactivity, NPCs from wt and $P2X7^{-/-}$ mice were seeded onto PLO/fibronectin-coated coverslips in Petri dishes and incubated for 2 h at 37 °C in a Bz-ATP (300 μM)-containing or Bz-ATP-free culture medium.

2.4. Whole-cell patch clamp recordings and drug application protocols; cultured NPCs

Whole-cell current-clamp and voltage-clamp recordings were made at room temperature (20–24 °C) on 1–4 days old adherent NPCs, using an Axopatch 200B patch clamp amplifier (Molecular Devices). The pipette solution contained (in mM): K-gluconate 140, NaCl 10, $CaCl_2$ 0.2, $MgCl_2$ 2.3, HEPES 10, EGTA 10, Mg-ATP 4, Li-GTP 0.3 (pH 7.2, with KOH). In a few experiments, EGTA was replaced by BAPTA (5 mM) and K-gluconate was increased correspondingly. Two different bath solutions were used, one of them contained normal and the other one low divalent cation (low X^{2+}) concentrations. For seal formation and some initial experiments, we used a normal artificial cerebrospinal fluid (aCSF; in mM): NaCl 140, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 1.2, HEPES 25, glucose 10.5 (pH 7.3, with NaOH). For low X^{2+} solution $MgCl_2$ was omitted, and the concentration of $CaCl_2$ was decreased to 0.1 mM. Pipettes had resistances of 3–8 MΩ. A relatively high holding potential of −80 mV was selected (by taking into account a calculated liquid junction potential of −14 mV), because adult NPCs were reported to have astrocytic characteristics (Yasuda et al., 2008).

The resting membrane potential (U_m) was measured within 30 s after establishing whole-cell access. Membrane capacitance (C_m) and resistance (R_m) as well as the access resistance (R_a) were monitored and recorded before and during individual experiments. Cells with R_a values higher than 25 MΩ were discarded. The current- and voltage-step protocols are described in chapter 3.2. pClamp 10.2 software (Molecular Devices) was used to store the recorded data for offline analysis/filtering and to trigger the fast drug application system.