Neuropharmacology 61 (2011) 1210–1221

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

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

ERK1/2 activation is involved in the neuroprotective action of $P2Y_{13}$ and P2X7 receptors against glutamate excitotoxicity in cerebellar granule neurons

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ARTICLE INFO

Article history:
Received 22 December 2010
Received in revised form
5 July 2011
Accepted 8 July 2011

Keywords:
Granule neurons
$P2Y_{13}$ receptor
P2X7 receptor
ERK
PI3kinase
Neuroprotection

ABSTRACT

Cerebellar granule neurons express several types of nucleotide receptors, with the metabotropic $P2Y_{13}$ and the ionotropic P2X7 being the most relevant in this model. In the present study we investigated the role of $P2Y_{13}$ and P2X7 nucleotide receptors in ERK1/2 signalling. The nucleotidic agonists 2MeSADP (2-methylthioadenosine-5′-diphosphate) for $P2Y_{13}$ and BzATP (2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate) for P2X7 receptors were coupled to ERK1/2 activation in granule neurons, being able to increase around two-fold the levels of ERK1/2 phosphorylation. These effects were sensitive to the inhibitory action of the antagonists MRS-2211 and A-438079, specific for $P2Y_{13}$ and P2X7 receptors, respectively. Although both receptor subtypes shared the same pattern of transient ERK1/2 phosphorylation, they differed in the intracellular cascades they triggered, being PI3K-dependent for $P2Y_{13}$ and calcium/calmodulin kinase II (CaMKII)-dependent for P2X7. These two different ERK-mediated pathways were involved in the neuroprotective effects displayed by both $P2Y_{13}$ and P2X7 receptors against apoptosis induced by an excitotoxic concentration of glutamate, in a similar manner to the neurotrophin, BDNF. In addition, $P2Y_{13}$ and P2X7 receptor agonists were also able to phosphorylate and activate the ERK-dependent target CREB, which could be involved in their neuroprotective effect. These results indicate that nucleotide receptors share with trophic factors the same survival routes in neurons, such as the ERK signalling route, and therefore, can contribute to the maintenance of granule neurons in conditions in which survival is being compromised.

1. Introduction

Nucleotides are emerging as relevant extracellular signals displaying a broad spectrum of actions that regulate physiological and pathophysiological events. They play important roles in the level of the cardiovascular system, bone physiology, pain transmission and inflammation, and special senses (Erlinge and Burnstock, 2008; Housley et al., 2009; Parandeh et al., 2008; Trang et al., 2006; Tsuda et al., 2010; Wirkner et al., 2007). They act through specific nucleotide receptors that belong to the two families of ionotropic P2X and metabotropic P2Y receptors, which are both widely expressed in different tissues (North, 2002; Roberts et al., 2006; von Kugelgen, 2006). Nucleotide actions at the level of the nervous system are not well characterized, although important roles have been attributed to nucleotide receptors expressed in cells of both glial and neuronal origin (Franke and Illes, 2006; Illes and Ribeiro, 2004; Inoue et al., 2007; Lecca et al., 2008; Neary and Kang, 2005). Among them, cerebellar granule neurons provide a good model for studying nucleotide receptor signalling, as they co-express a great diversity of nucleotide receptors of both P2Y and

Abbreviations: A-438079, 3-(5-(2,3-dicholorophenyl)-1*H*-tetrazol-1-yl) methyl pyridine; BDNF, brain derived neurotrophic factor; BBG, brilliant blue G; BzATP, 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate; CaMKII, calcium-calmodulin kinase II; CREB, cAMP-responsive element binding protein; ERK1/2, extracellular signal-regulated kinase; GF-I, GF-109203X or Gö-6850, 2-(1-(3-dimethylaminopropyl)indol-3-yl)-3-(indol-3-yl) maleimide; GSK3, glycogen-synthase kinase-3; JNK, jun kinase; KN-62, 1-(N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl)-4phenylpiperazine; MAPK, mitogen-activated protein kinase; MEK1, kinase MAP kinase kinase-1; 2MeSADP, 2-methylthioadenosine-5′-diphosphate; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PPADS, pyridoxalphosphate-6-azophenyl-2′-4′-disulfonic acid; MRS-2179, 2′-deoxy-N6-methyladenosine 3′,5′-bisfosfato; MRS-2211, 6-(2′-chloro-5′-nitro-azophenyl)-pyridoxal-alpha5-phosphate; NMDA, N-methyl-D-aspartate; PI3K, phosphatidylinositol-3-kinase; PTX, *Pertussis* toxin; U-0126, 1,4-diamine-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.

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doi:10.1016/j.neuropharm.2011.07.010

P2X types (Hervas et al., 2003). Signalling studies performed in these cells gave evidence of nucleotides functioning as survival promoting factors, as they share the same intracellular signalling pathways as neurotrophins and other survival signals in these cells. In line with this, nucleotides were recently described to be coupled to the phosphorylation and inhibition of the enzyme glycogen synthase kinase-3 (GSK3), which is a key element in the intracellular survival pathways of granule neurons. In these cells, two different subtypes of ADP-P2Y nucleotide receptors co-exist, $P2Y_1$ and $P2Y_{13}$, but only $P2Y_{13}$ is responsible for GSK3 mediated actions through the activation of the classical PI3K/Akt survival pathway. This effect is followed by the translocation of the GSK3 substrate, β-catenin, to the nucleus, where this transcriptional modulator is involved in the regulation of cell survival (Ortega et al., 2008). The effect of $P2Y_{13}$ activation on this GSK3/β-catenin pathway was not reproduced by the activation of other metabotropic nucleotidic receptors, which are less represented in granule neurons, such as $P2Y_4$ and $P2Y_6$ receptors (Hervas et al., 2003). With respect to P2X ionotropic receptors, several agonists selective for P2X1, P2X3 (α,β-methylen-ATP) and P2X7 receptors were analyzed, and it was the P2X7 agonist, BzATP, which had the most significant effect on GSK3 phosphorylation. Activation of P2X7 receptor triggered a different intracellular pathway dependent on extracellular calcium and PKC to induce GSK3 phosphorylation. In addition, the independence from PI3K/Akt axis made it possible for P2X7 receptors to rescue granule neurons from cell death induced by the pharmacological inactivation of this route (Ortega et al., 2009). Moreover, P2X7 cooperated with both NMDA and BDNF receptors in this survival action (Ortega et al., 2010).

Continuing with this line of work, in the present study we investigated whether other signalling cascades were also involved in the neuroprotective actions of $P2Y_{13}$ and P2X7 receptors in granule neurons. The extracellular regulated kinases, (ERKs), which are members of the family of mitogen-activated protein kinases (MAPKs), have been described to be involved in neuronal survival in different neuronal models (Hetman and Gozdz, 2004). In this sense, in granule neurons ERK1/2 proteins play an important role in the neuroprotective effect of factors, such as IGF-I and GDF-15, against apoptosis induced by low potassium concentration, or in the protection elicited by the neurotrophin BDNF against the cytotoxic drug camptothecin (Bonni et al., 1999; Subramaniam et al., 2003; Yamada et al., 2001). These factors are usually coupled to the ERK1/2 activation in a transient way, through a dual phosphorylation event at Thr and Tyr residues by the upstream kinase MAP kinase kinase-1 (MEK1) (Pearson et al., 2001). In the present work, ERK1/2 signalling was analyzed for nucleotide receptors in granule neurons and found that both $P2Y_{13}$ and P2X7 receptors were coupled to ERK phosphorylation and activation, displaying different intracellular cascades that involve PI3K/Akt for $P2Y_{13}$ and Ca^{2+}/CaMKII for P2X7 receptors. In addition, although they activated different intracellular routes, they had in common the coupling to the same ERK1/2-derived target, transcription factor CREB, whose activation could be involved in their neuroprotective action against apoptosis induced by excitotoxic concentrations of glutamate.

2. Materials and methods

2.1. Cell culture and treatment conditions

All experiments carried out at the Universidad Complutense de Madrid followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Cerebellar cultures were performed according to the procedure described by Pons et al. (2001) (Pons et al., 2001). Cerebella from Wistar rat pups (P7) were aseptically removed, and submitted to digestion with papain 100 U/ml (Worthington, Lake Wood, NJ) (previously activated in EBSS buffer containing of 5 mM L-Cys, 2 mM EDTA and 0.067 mM β-mercaptoethanol), in the presence of 100 U/ml of DNAse (Worthington, Lake Wood, NJ), 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The obtained cells were resuspended in neurobasal medium supplemented with B-27 (GIBCO, BRL, Paisley, Renfrewshire, UK) and containing 21 mM KCl, 2 mM glutamine, and antibiotics, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma Aldrich, St Louis, USA), and plated on glass coverslips or plastic Petri dishes (60 and 35 mm) (Falcon Becton Dickinson Labware, Franklin Lakes, USA) precoated with 0.1 mg/ml poly-L-lysine (Biochrom, AG, Berlin) at a density of 200,000 cells/cm^2. They were maintained in a humidified incubator at 37 °C in 5% CO_2. AraC (10 μM) was added to avoid the proliferation of glial cells.

Cerebellar granule neurons were cultured 9–12 DIV (days *in vitro*), prior to stimulation with nucleotide agonists or other agents, cells were routinely washed for about 2 h at 37 °C in Locke solution, (in mM: NaCl, 140; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 5.5; and HEPES (acid), 10; pH 7.4) in order to lower the basal levels of ERK phosphorylation, normally being elevated as a consequence of the culture in high potassium medium. After this washing period, this medium was replaced by Locke solution for additional 5 min, and then nucleotides were added at the required concentrations and incubation times. For experiments analyzing the effect of P2X7 receptor stimulation, medium in the absence of Mg^{2+} was used, as P2X7 receptors are known to be sensitive to the inhibition of divalent cations (North, 2002; Virginio et al., 1997).

2.2. Western blot experiments

Cells were stimulated in the presence of the nucleotide agonists and BDNF with different treatments for the required times. Stimulation was stopped by the addition of lysis buffer (20 mM MOPS, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium ortovanadate, 5 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, pH = 7.2, 1 mM PMSF and protease inhibitor cocktail (Complete, Roche)). Protein determination of the cell extracts was performed and then mixed with sample buffer 4× (50% glycerol, 125 mM Tris; pH 6.8, 4% SDS, 1% bromophenol blue, 5% β-mercaptoetanol, 4.5% H_2O). The samples were heated to 99 °C and aliquots (25 μg) were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis (25 mM Tris, 200 mM glycine, 0.1% SDS, pH = 8.3) using 12% acrylamide gels. Immunotransference was performed on PVDF membranes (Amersham Biosciences Europe GmbH, Barcelona, Spain) (25 mM Tris, 192 mM glycine, 20% methanol). The TBS buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.5) containing 1% (v/v) tween-20 and 5% BSA was used for blocking and for subsequent incubation with antibodies. Incubation with antibodies was performed at the following dilutions: 1:1000 for phospho ERK1/2, total ERK, β-III tubulin and phospho-CREB and 1:500 for caspase-3. Primary antibodies were detected with horseradish peroxidase-conjugated antibodies, 1:4000 for anti-mouse (Santa Cruz) and anti-rabbit (Dako), and visualized by the ECL method (kit Super Signal substrate Western Blotting, from Amersham Biosciences Europe GmbH, Barcelona, Spain). The chemoluminescence images were quantified by densitometry employing the Fluo-S Imager of Bio-Rad (Munich, Germany).

2.3. Cell viability assays

Survival studies required long-term treatment, and in this case, the nucleotide agonists, antagonists and the toxic stimuli were applied in complete culture medium. Cells were treated for 2 h with the nucleotide agonists (in the absence or presence of nucleotide receptor antagonist or transducing protein inhibitors) before the addition of glutamate (100 μM). Cell viability was then evaluated 24 h later. No differences were obtained when the experiments were carried out in the continuous presence of adenosine deaminase (ADA, 1 U/ml).

Neuronal survival was quantified by two methods, LIVE/DEAD viability/cytotoxicity kit from Molecular Probes and MTT assay from Sigma Aldrich.

LIVE/DEAD viability/cytotoxicity kit: After the corresponding treatments cells were washed twice with Locke solution and left in complete darkness for 30 min in Locke solution containing 2 μM calcein and 8 μM ethidium homodimer-1 (supplied by the commercial kit). Then, immunofluorescence images were taken with a NIKON TE-200 microscope and a Kappa DX2 camera controlled by kappa Image Base Control software. The analysis of the photographs was performed using Paint Shop Pro 8.0 (Jasc Software). Calcein stains live cells whereas Ethidium Homodimer-1 stains dead cells. Survival percentage was obtained considering the total number of cells in each image.

MTT assay, in which mitochondrial function is assessed, was also employed. After the corresponding treatments, the tetrasodium salt MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was added to the cultures to a final concentration of 0.5 mg/ml, and maintained for 2 h at 37 °C. Then, an equal volume of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added, following a brief incubation of 30 min at room temperature with orbital shaking. The samples were collected and measured spectrophotometrically at 570 nm. Values were normalized with respect to those obtained from untreated cells, considered as 100% survival.

2.4. Statistical analysis

Data are represented as means ± SD of at least 3 independent experiments obtained from different cultures. Comparison between different treatments and controls was