

P2X₇ Nucleotide Receptor is Coupled to GSK-3 Inhibition and Neuroprotection in Cerebellar Granule Neurons

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Abstract In this study we report the coupling of nucleotide receptors to GSK-3 signalling, a relevant survival pathway in cerebellar granule neurons. P2X₇ agonist BzATP induced a 3–4-fold increase in GSK-3 phosphorylation, which is reported to be associated with the catalytic activity inhibition. This effect was dependent on extracellular calcium and PKC, and independent of PI3-K (phosphatidylinositol-3-kinase)/Akt, the main survival route of neurotrophins. BzATP also prevented the apoptosis of granule neurons induced by the pharmacological inhibition of the PI3-K signalling. Both effects, BzATP-mediated GSK-3 phosphorylation and neuroprotection, were abolished by P2X₇ receptor antagonists, BBG, PPADS and A-438079. We found that BzATP prevented the progressive GSK-3 dephosphorylation and caspase-3 activation occurring under conditions of sustained PI3-K inhibition. These results reveal that P2X₇ receptor activation could provide a relevant survival route alternative to classical neurotrophic factors.

Keywords Neuroprotection · Nucleotide receptors · Granule neurons · GSK-3 · P2X₇ receptors

Introduction

Cerebellar granule neurons provide a valuable cell model widely used for the study of the signalling cascades and mechanisms underlying neuronal death and survival (Contestabile 2002). They can be maintained in culture in the presence of depolarising potassium concentrations (25 mM), and under these conditions, they develop the morphological, electrophysiological and biochemical characteristics of mature neurons (Gallo et al. 1985; Burgoyne et al. 1993). The removal of high potassium, which is mimicking neuronal activity, commits granule neurons to apoptotic cell death, and the signalling events in the apoptotic cascade are well characterised. Several survival agents are able to rescue granule neurons from low potassium-induced apoptosis, these being growth factors such as IGF-I and BDNF, and other trophic agents, like NMDA and cyclic AMP (D'Mello et al. 1993; D'Mello et al. 1997; Zhang et al. 1998). All these factors are usually coupled to the PI3-K (phosphatidylinositol-3-kinase)/Akt axis, this pathway being an important survival signalling route in neurons (Dudek et al. 1997; Pap and Cooper 1998; Zhang et al. 1998; D'Mello et al. 1997; Hetman et al. 1999). Interestingly, in the granule cell model, all these survival promoting factors have in common the inhibition of GSK-3 (glycogen synthase kinase-3) activity, the mandatory downstream event in the PI3-K/Akt signalling, GSK-3 being a convergent point in the survival cascade (Miller et al. 1997; Li et al. 2000; Lafón-Cazal et al. 2002; Yamagashi et al. 2003; Chin et al. 2005). The relevance of GSK-3 inhibition in neural survival has also been demonstrated in other neuronal models, such as PC12 cells and cortical neurons submitted to different apoptotic stimuli, in fact the sole overexpression of GSK-3 leads to cell death (Pap and Cooper 1998; Hetman et al. 2000). These data

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support the role of GSK-3 at the CNS level as a key modulator of neuronal survival.

The multifaceted roles and the multiple mechanisms of regulation of GSK-3 have been compiled in recent reviews (Frame and Cohen 2001; Jope and Johnson 2004). One of the best known mechanisms of GSK-3 activity regulation involves its association with several partners being part of multimeric protein complexes. In this respect, GSK-3 is a key component of the Wnt axis, usually named the canonical pathway. In this route the disassembly of GSK-3 from the axin/APC/ β -catenin complex terminates the inhibitory activity that GSK-3 exerts towards its partners. Among them, it is well characterised that when β -catenin is released from the complex, it accumulates in the cytosol and can be translocated to the nucleus, where it functions as a transcriptional regulator involved in functions related to development and proliferation (Ding et al. 2000; Frame and Cohen 2001). GSK-3 can also associate with other substrates, such as tau and presenilin-1, and regulate cytoskeletal re-arrangement necessary for synaptic remodelling (Hall et al. 2002; Sayas et al. 2002). The second major mechanism of GSK-3 activity regulation implies a phosphorylation mechanism in Ser21/9 residues (for α and β isoforms, respectively), which leads to the inhibition of its catalytic activity. This is the mechanism of action of growth and trophic factors, whose main representative is the IGF family (Ding et al. 2000). Although Akt is the main upstream effector of GSK-3, several other kinases have been reported to phosphorylate GSK-3 both “in vivo” and “in vitro”, as is the case of PKA, PKC and p70-S6K (Frame and Cohen 2001). Indeed, in cerebellar granule neurons, PKA is mediating the survival effect promoted by cAMP (D’Mello et al. 1993; Li et al. 2000). Therefore, it appears that GSK-3 phosphorylation and inhibition can be a general mechanism employed by neurotrophic factors to maintain cell survival. In this respect, it would be interesting to analyse whether, besides trophic factors, other extracellular signals could be coupled to GSK-3 signalling and function as potential survival signals displaying protective actions in neurons.

Among these signals nucleotides are good candidates, as both ionotropic (P2X) and metabotropic (P2Y) nucleotide receptors have been described in different neuronal types (North 2002; Abbracchio et al. 2006; Burnstock 2006). These two families are represented in cerebellar granule neurons, which exhibited specific functions and locations (Amadio et al. 2002; Hervás et al. 2003, 2005; Sánchez-Nogueiro et al. 2005). We recently reported that the P2Y₁₃-Gi-coupled metabotropic receptor was coupled to GSK-3 phosphorylation, by a PI3-K/Akt dependent pathway in this cellular model. This effect led to the accumulation of the GSK-3 substrate, β -catenin, and its translocation to the nucleus, where it functions as a

transcriptional regulator (Ortega et al. 2008). In the present study, we extended these signalling studies to ionotropic receptors, and especially to P2X₇ receptors, which have been described to be coupled to phosphorylation and activation of CaMKII and synapsin I (León et al. 2006; León et al. 2008). We found that the P2X₇ agonist BzATP (2’(3’)-O-(4-benzoyl-benzoyl)-adenosine 5’-triphosphate) was coupled to GSK-3 phosphorylation and inhibition in a PKC-dependent way. This is an alternative signalling mechanism different to the classical PI3-K/Akt route activated by trophic factors. In addition, BzATP exerts protective actions towards apoptosis induced by pharmacological inhibition of PI3-K. This action was exclusive for BzATP and seemed to be mediated by a receptor with the pharmacological profile of P2X₇. These data together give evidence of P2X₇ receptors being coupled to survival responses in granule neurons through its inhibitory action on GSK-3.

Materials and Methods

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). 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 CaCl₂ and 1 mM MgCl₂. 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 μ g/ml amphotericin B (Sigma Aldrich, St Louis, USA), and plated in glass coverslips or plastic Petri dishes (100 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/cm². They were maintained in a humidified incubator at 37°C in 5% CO₂. AraC (10 μ M) was added to avoid the proliferation of glial cells.

Cultured cerebellar granule neurons were used at 9–11 DIV (days in vitro). In the experiments regarding the characterisation of P2X₇-responses on GSK-3 phosphorylation, prior to the stimulation of cells with nucleotide agonists or other agents, cells were routinely washed for about 2 h at 37°C in a low potassium medium (Locke solution, in mM: NaCl, 140; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄,

1.2; MgSO₄, 1.2; glucose, 5.5; and HEPES (acid), 10; pH 7.4) in order to lower the basal levels of GSK-3 (and Akt) 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 in the absence of Mg²⁺ for an additional 5 min, and then nucleotides were added at the required concentrations and incubation times. This medium was used in order to enhance P2X₇-mediated responses, as P2X₇ receptors are known to be sensitive to the inhibition of divalent cations (Virginio et al. 1997).

Nucleotide receptor agonists, antagonists, IGF-I and transducing inhibitors were purchased from Sigma Aldrich (St Louis, USA) (BzATP, ATP, α , β -meATP (adenosine-5'-(α,β -methylene)diphosphate), BBG (Brilliant Blue G)), Tocris Bioscience (Essex, UK) (PPADS (pyridoxalphosphate-6-azophenyl-2'-4'-disulfonic acid), TNP-ATP (2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate), A-438079 (3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine), KN-62), and Calbiochem Co. (San Diego, USA) (IGF-I, LY-294002, SB-216763, GF-109203X (also known as GF-I), U-0126, PP-2). All other reagents not specified were routinely supplied by Sigma, Merck (Darmstadt, Germany) or Roche Diagnostics SL (Barcelona, Spain).

Western Blot Experiments

Cells were stimulated in the presence of the nucleotide agonists and IGF-I 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 orthovanadate, 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 done and then mixed with sample buffer 4 \times (50% Glycerol, 125 mM Tris pH = 6.8, 4% SDS, 1% bromophenol blue, 5% β -mercaptoethanol, 4.5% H₂O). The samples were heated at 99°C and aliquots (25 μ g protein for GSK-3, Akt, caspase-3 and β -III tubulin determinations, 10 μ g protein for Tau) were subjected to sodium dodecyl sulphate (SDS) gel electrophoresis (25 mM Tris, 200 mM glycine, 0.1% SDS, pH = 8.3) using 12% acrylamide gels. Immunotransference was performed in 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% de BSA was employed as blocking medium and in subsequent incubations with the antibodies. Incubation with the antibodies was performed at the following dilutions: 1:750 for phospho α/β GSK-3 (Ser 21/9), 1:1000 for phospho-Akt (Ser-473), total Akt, caspase-3, β -III tubulin (all of them from Cell Signalling Technology, Beverly, MA, USA) and total GSK-3 (Biosource, Nivelles,

Belgium), and 1:500 for PHF-1 (Ser396/404) antibody (a gift from Dr. Peter Davies, Albert Einstein College of Medicine of Yeshiva University, New York). Primary antibodies were detected with horseradish peroxidase-conjugated antibodies, 1:4000 for anti-mouse (Santa Cruz Biotechnology Inc, Santa Cruz, California) and 1:4000 for anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and visualised by the ECL method (kit Super Signal substrate Western Blotting, from Amersham Biosciences Europe GmbH, Barcelona, Spain). The quimioluminescence images were quantified by densitometry employing the Fluo-S Imager of Bio-Rad (Munich, Germany).

Cell Viability Assays and TUNEL

Survival studies required long-term treatment with the PI3-K inhibitor LY-294002, and in this case, the nucleotide agonists were applied in the complete culture medium 10 min before the addition of LY-294002. Cell viability was then evaluated 24 h later. These experiments were routinely carried out in the continuous presence of adenosine deaminase (ADA, 1 U/ml).

Neuronal survival was quantified by the MTT assay, in which mitochondrial function is assessed. After the corresponding treatments, the tetrasodium salt MTT (3-(4,5-dimethylthiazol-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 solubilisation 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 normalised in respect to that obtained from untreated cells, considered as 100% survival.

The TUNEL-labelling (TdT-mediated dUTP nick end labelling) was used to quantify the number of apoptotic nuclei, as this reaction preferentially labels DNA strand breaks generated during apoptosis. The TUNEL assay was performed according to manufacturer's instructions (In Situ Cell Death Detection kit, TMR red, Roche Diagnostics SL, Barcelona, Spain). For each condition, around 1,200 cells were analysed from at least two different wells. After the TUNEL reaction, cells were counterstained with DAPI (Sigma). The percentage of apoptotic nuclei was calculated in respect to total nuclei numbers, quantified by DAPI staining. Cell viability evaluation using both MTT and TUNEL methods rendered similar results.

Statistical Analysis

Data are represented as means \pm s.d. of at least 3 independent experiments obtained from different cultures.

Comparison between different treatments and controls was carried out using the Dunnett test, and comparison between different samples was done by applying the Tukey test. Data were statistically significant at ***/### $P < 0.001$, **/## $P < 0.01$ and */# $P < 0.05$.

Results

BzATP Induces GSK-3 Phosphorylation in Granule Neurons

BzATP is usually employed to analyse P2X₇ mediated responses, although is described to activate other ionotropic P2X nucleotide receptors with the same potency (Bianchi et al. 1998). For this reason stimulations with BzATP were carried out in the absence of Mg²⁺, as P2X₇ responses can be specifically potentiated when extracellular magnesium is reduced or eliminated, this being one of the hallmarks of P2X₇ receptor functioning (Virginio et al. 1997; North 2002).

As shown in Fig. 1a, BzATP at 100 μM concentration was able to increase the phosphorylation of GSK-3 in Ser21/9 residues (for α and β isoforms, respectively), which is associated with the inhibition of its catalytic activity. This effect peaked at 5–10 min stimulation (3–4-fold increase over basal level), and was maintained for

longer stimulation periods of 30 and 60 min with a slow decline. Dose-response curve for BzATP on GSK-3 phosphorylation is shown in Fig. 1b. The EC₅₀ value obtained was 20.77 ± 4.28 μM, which is in the range of that described for the rat P2X₇ receptor (Young et al. 2007). Although the maximal effect of BzATP is reached with 300 μM concentration, 100 μM was used in subsequent studies on GSK-3 phosphorylation.

The Effect of BzATP on GSK-3 Phosphorylation Presents the Pharmacological Profile of a P2X₇ Receptor in Granule Neurons

Deepening our studies on BzATP-mediated responses, we tried to identify in more detail the nature of the receptor activated by BzATP in granule neurons. First, the ionotropic nature of the BzATP response was confirmed in experiments in which extracellular calcium was quelated with EGTA/Tris, as in these conditions the observed effects on GSK-3 phosphorylation were completely abolished (Fig. 2a).

In subsequent experiments several P2X receptor antagonists were used, such as Brilliant Blue G (BBG) and PPADS, which have been reported to abolish P2X₇-mediated responses (Jiang et al. 2000; Hibell et al. 2001), and turned out to inhibit the BzATP-induced increase in GSK-3 phosphorylation in granule neurons. However, this was not

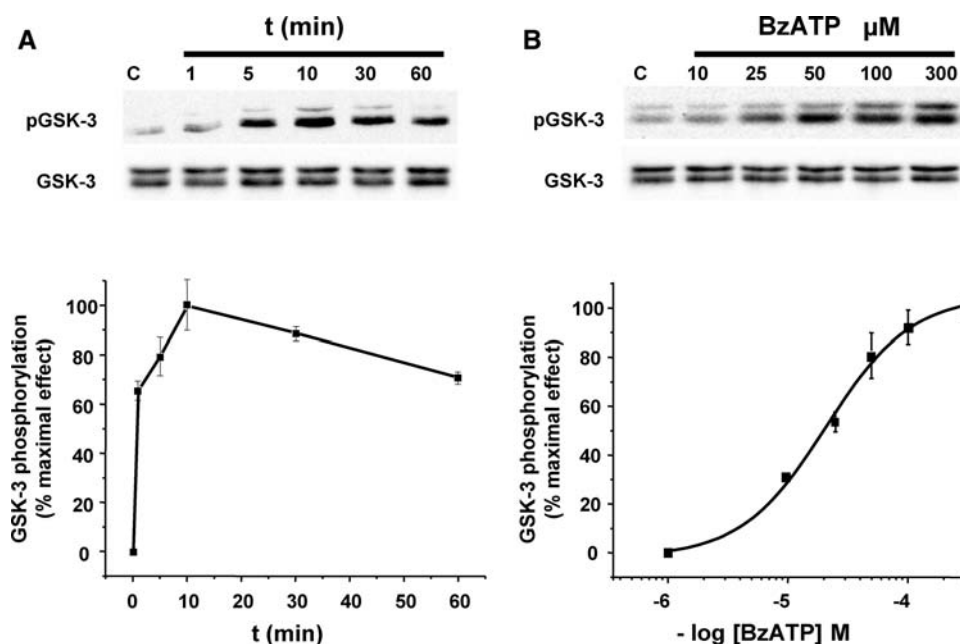


Fig. 1 Time course and dose-dependence of BzATP-induced GSK-3 phosphorylation in granule neurons. Cultured granule neurons were stimulated with 100 μM BzATP at different incubation times (a), or with different BzATP concentrations for 10 min (b). Then cells were harvested and phosphorylation of GSK-3 was analysed by immunoblotting, as described in Sect. Materials and Methods. Data were

obtained by normalisation of densitometric values of phospho-GSK-3 in respect to total GSK-3. Results were normalised in respect to the maximal response elicited by BzATP. In b, the logistic curve was fitted to nonlinear regression (Boltzman analysis). The blots correspond to representative experiments and values are the means ± s.d. of three experiments performed from different cultures

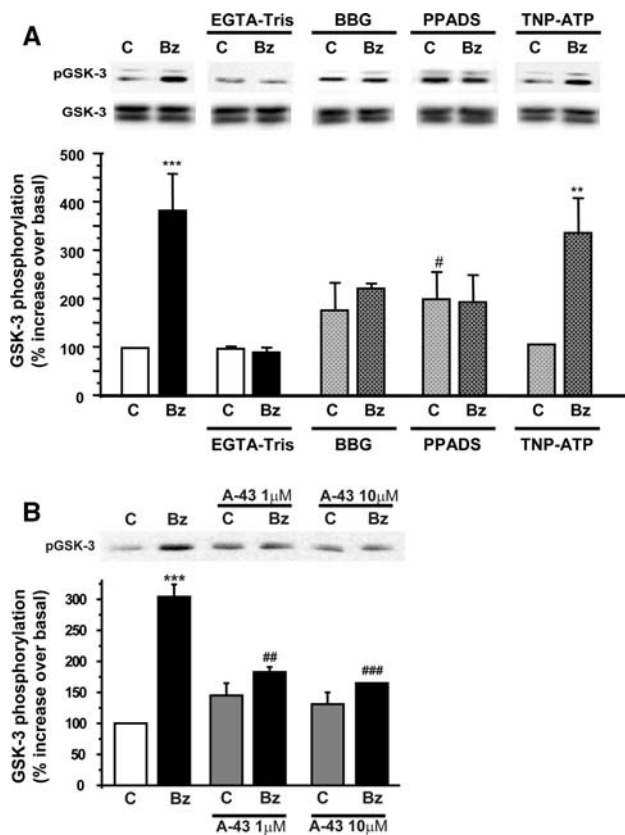


Fig. 2 Characteristics of the response elicited by BzATP on GSK-3 phosphorylation: Effect of extracellular calcium and P2X antagonists. Granule neurons were stimulated with 100 μ M BzATP concentration for 10 min, under different treatments. Cells were pre-incubated for 5 min in medium containing EGTA/Tris to quelate extracellular calcium, and then BzATP was added in the same medium. When the nucleotide receptor antagonists were tested, cells were pre-incubated for 5 min with 10 μ M BBG, 30 μ M PPADS, 2 μ M TNP-ATP (a), and 1–10 μ M A-438079 (b), then, BzATP was added for an additional 10 min, in the continuous presence of these antagonists. Then cells were harvested and phosphorylation of GSK-3 was analysed by immunoblotting, as described in Sect. [Materials and Methods](#). Histograms represent the percentage increase in respect to non-stimulated cells (100% control value), and were obtained by normalisation of densitometric values of phospho-GSK-3 in respect to total GSK-3. Values are the means \pm s.d. of at least three experiments performed from different cultures. The blots correspond to representative experiments. Data were analysed by Dunnet and Tukey tests and were statistically significant at *** P < 0.001 when the effect of BzATP was compared with its respective control, and ## P < 0.01 and # P < 0.05 when the effect of the treatment with the antagonists was compared with the control of non-stimulated cells

reproduced with the specific P2X₁ and P2X₃ antagonist, TNP-ATP (Virginio et al. 1998), discarding the possibility that BzATP could be acting on these receptors (Fig. 2a). In our hands, the specific P2X₁ and P2X₃ agonist $\alpha\beta$ meATP was also able to induce detectable levels of GSK-3 phosphorylation (data not shown). Basal levels of GSK-3 phosphorylation were slightly higher with the use of both BBG and PPADS, resulting in no net effect of BzATP. Nevertheless, this led us to employ more specific antagonists

that could pass up this effect. A novel series of P2X₇ antagonists has been recently synthesised, and among them, A-438079 has been proved to be potent and highly selective and is readily commercially available (Donnelly-Roberts and Jarvis 2007). When we used A-438079 at concentrations reported to only affect P2X₇, we observed that BzATP-mediated effect on GSK-3 phosphorylation was eliminated, without having unspecific effects on its own (Fig. 2b). These data together give evidence of a P2X₇ receptor involved in the actions of BzATP in granule neurons. As expected, the physiological agonist ATP at concentrations in the mM range increased GSK-3 phosphorylation as well (data not shown). As ATP could be acting on several other nucleotide receptors, we used BzATP to analyse P2X₇-mediated responses in following studies.

BzATP-Induced Increase on GSK-3 Phosphorylation is Dependent on PKC and Independent of PI3-K/Akt Signalling Pathway

Next set of experiments were conducted in order to analyse the signalling events triggered by BzATP in granule neurons. Considering that the signalling displayed by BzATP on GSK-3 phosphorylation was strongly dependent on the extracellular calcium we then tested calcium targeted proteins, such as calcium calmodulin kinase II (CaMKII) and PKC. We used KN-62 as a CaMKII inhibitor, which, although it has been described to act as an antagonist for human and mouse P2X₇ receptors, it did not exhibit this ability at the rat orthologue (Humphreys et al. 1998). We found that the effect of BzATP on GSK-3 phosphorylation was not affected in the presence of KN-62, but was completely eliminated with the PKC inhibitor GF-109203X (GF-I), indicating that PKC, but not CaMKII, was a key step in BzATP-induced GSK-3 phosphorylation (Fig. 3a).

The contribution of other signalling mechanisms to BzATP effect was also evaluated. The inhibitor of src-tyrosine kinases, PP-2, reduced basal levels of GSK-3 phosphorylation but did not alter BzATP-mediated effect. However, the treatment with the ERK-activating kinase-1 (MEK-1) inhibitor, U-0126, produced opposite effects and caused increments on GSK-3 phosphorylation basal levels by itself, and in respect to them, BzATP was not able to induce a further increase. Therefore, a cross-talk between MAPKs and GSK-3 cannot be excluded.

As commented before, the PI3-K/Akt axis is the main upstream event in GSK-3 phosphorylation, described so far for growth factors. In this respect, the PI3-K inhibitor LY-294002 was used at concentrations that specifically inhibit PI3-K in granule neurons (D'Mello et al. 1997; Miller et al. 1997), and in these conditions, it was not able to abolish the effect of BzATP (Fig. 3a). These results together imply that BzATP is reaching GSK-3 through an alternative

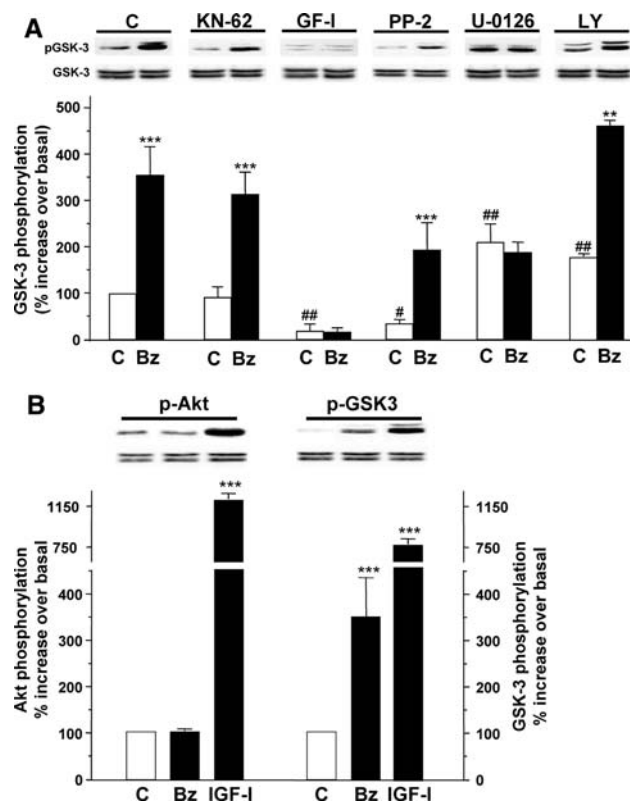


Fig. 3 Effect of various signalling pathway inhibitors in BzATP-induced GSK-3 phosphorylation in granule neurons. **a** Granule cells in culture were submitted to different treatments: 20 min incubation with signalling inhibitors, 10 μ M KN-62, 0.5 μ M GF-I, 1 μ M PP-2, 10 μ M U-0126, and 50 μ M LY-294002. Then cells were stimulated for 10 min with 100 μ M BzATP in the continuous presence of these inhibitors. **b** Cells were stimulated for 10 min in the presence or absence of 100 μ M BzATP and 50 ng/ml IGF-I. In **a** and **b**, the cell extracts were analysed by immunoblotting as described before in Sect. **Materials and Methods**, and the corresponding bands quantified by densitometry. Histograms represent the percentage increase in respect to non-stimulated cells (100% control value), and were obtained by normalisation of densitometric values of phosphoproteins in respect to total forms of GSK-3 (**a** and **b**) and Akt (**b**). Values are the means \pm s.d. of at least three experiments performed from different cultures. The blots correspond to representative experiments. Data were analysed by Dunnet and Tukey tests, and were statistically significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ when the BzATP effect was compared to its respective control (empty bars, in the absence of BzATP) in each individual treatment, and $###P < 0.01$, $#P < 0.05$, when controls of different inhibitor treatments were compared to control in the absence of any treatment (in the absence of inhibitors)

mechanism involving PKC, which is different to the well known and commonly used PI3-K/Akt-dependent pathway. In accordance to this, the ability of BzATP to phosphorylate and activate Akt was evaluated. As expected and shown in Fig. 3b, BzATP was not coupled to Akt phosphorylation, in comparison to that obtained with IGF-I, which induced a strong phosphorylation of both Akt and GSK-3, corroborating that PI3-K/Akt/GSK-3 is the predominant route used by trophic factors in granule neurons.

BzATP Protects Granule Neurons from Apoptosis Induced by PI3-K Inhibition

From the results above, it is clear that BzATP triggers alternative signalling mechanisms, mostly dependent on PKCs, which result in GSK-3 inhibition. The ability of BzATP to bypass the PI3-K pathway, made us investigate if this pathway could represent any advantage for granule neurons, in conditions in which trophic factor signalling and support could be compromised. In this respect, it is well characterised that pharmacological inhibition of PI3-K, which resembles trophic withdrawal conditions, leads to cell death of granule neurons through an apoptotic mechanism (D'Mello et al. 1997; Miller et al. 1997). We employed the specific PI3-K inhibitor LY-294002 to induce apoptosis in granule neurons and examine if BzATP could have any protective effect.

As shown in Fig. 4, in our culture conditions, treatment with LY-294002 for 24 h caused cell death in a dose-dependent way. In fact, the EC_{50} value for PI3-K inhibition is in the low micromolar range, and LY-294002 has no effect on other kinases until 50 μ M (Vlahos et al. 1994). The treatment with BzATP before the addition of the PI3-K inhibitor partially prevented cell death and increased the survival in a percentage around 30% at all doses of LY-294002 (Fig. 4). As BzATP was being added to cells in complete culture media, 300 μ M concentrations were required to obtain more reproducible results. In addition,

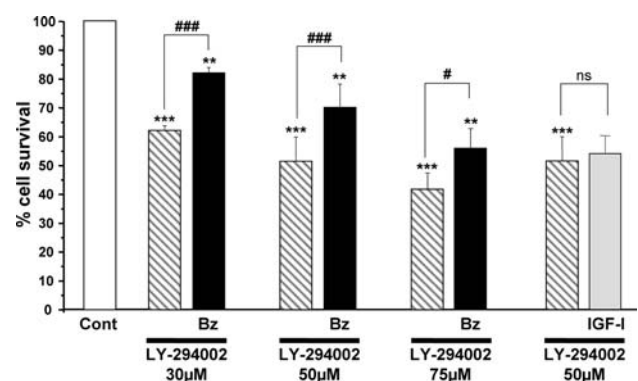


Fig. 4 Effect of treatment with the PI3-K inhibitor LY-294002 on granule cell viability. BzATP-mediated protection. Granule neurons maintained in complete culture medium were treated for 10 min in the presence of 300 μ M BzATP or 50 ng/ml IGF-I, and then LY-294002 was added at several concentrations. These experiments were routinely carried out under the continuous presence of adenosine deaminase (ADA 1U/ml). Cell viability was evaluated after 24 h by the MTT assay as described in Sect. **Materials and Methods**. Data are the means \pm s.d. of at least three experiments performed in duplicate from different cultures. Data were analysed by Dunnet and Tukey tests and were statistically significant at $***P < 0.001$ and $**P < 0.01$ when non-treated cells (empty bars) were taken as reference, and at $###P < 0.001$ and $#P < 0.05$ when each LY-294002 treatment was compared in the presence or absence of BzATP

the BzATP-induced protection did not need long pre-incubation periods, and 10 min stimulation was set before the addition of the PI3-K inhibitor. The specificity of LY-2949002 on PI3-K activity was further confirmed by the lack of rescue effect observed with IGF-I, a growth factor that predominantly signals through PI3-K in granule neurons (Fig. 4) (D’Mello et al. 1997; Yamagashi et al. 2003; Subramaniam et al. 2005).

The apoptotic nature of cell death of granule neurons submitted to LY-294002 treatment was corroborated by TUNEL-labelling experiments, in which “in situ” labelling of DNA fragmentation was assessed. As observed in Fig. 5, the percentage of TUNEL-positive cells that accounted for apoptotic nuclei was 2-fold in cells exposed to 50 μ M LY-294002 for 24 h in respect to untreated cells. The number of apoptotic cells was then significantly decreased in around a 30% percentage when cells were pre-treated with BzATP. In addition, this protective effect of BzATP was

prevented by the pre-incubation with the P2X₇ antagonists BBG and PPADS. These percentages of cell survival were in the same range as those obtained with the MTT assay (Fig. 6a) therefore, confirming that this assay accurately measures neuronal survival. In addition, the most specific P2X₇ antagonist, A-438079, eliminated the protective effect of BzATP as well. The treatment with these nucleotide receptor antagonists neither had a toxic effect on granule neurons nor promoted LY-294002 toxicity. Additionally, the rescue displayed by BzATP (31.25% \pm 9.47%) was reproduced with ATP at 3 mM concentration (Fig. 6a). These results together give evidence of BzATP protective effect being mediated by the activation of a P2X₇ receptor in granule neurons. It is necessary to point out that adenosine deaminase was routinely present in these experiments, in order to eliminate the possible contribution of the adenosine formed by extracellular hydrolysis of nucleotides, and therefore,

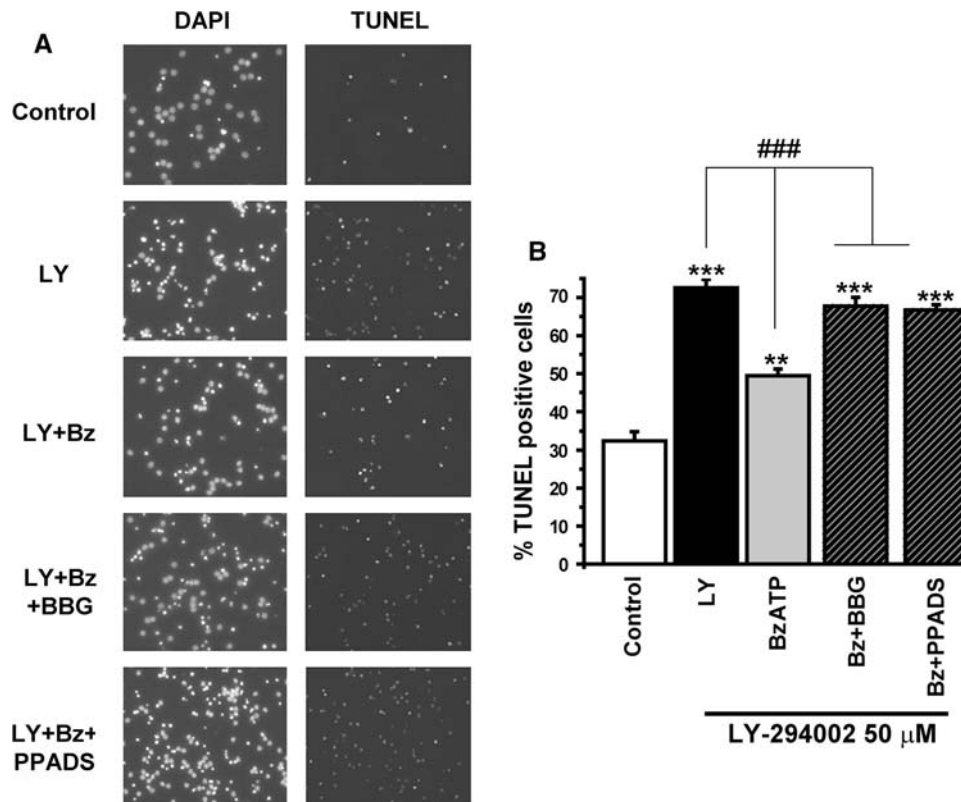


Fig. 5 Quantification of granule neuron apoptosis after PI3-K inhibition. Rescue by BzATP. Granule neurons maintained in complete culture medium were pre-incubated in the presence or absence of the antagonists 10 μ M BBG and 30 μ M PPADS for 5 min, and then 300 μ M BzATP was added for 10 min before the addition of 50 μ M LY-294002. Quantification of apoptotic nuclei by TUNEL reaction was carried out 24 h later, as described in Sect. [Materials and Methods](#). **a** Immunofluorescence images of DAPI staining (*left*) and TUNEL labelling (*right*) were obtained from the same field of granule neurons. DAPI staining was employed to quantify total nuclei in each field, and also, to monitor chromatin condensation and fragmentation

that is associated with morphological changes of nuclei during apoptotic death, which corresponded perfectly to the apoptotic nuclei labelled by TUNEL. **b** Histograms represent the percentage of TUNEL positive cells calculated in respect to total cell numbers (DAPI staining). Data are the means \pm s.d. of at least three experiments performed in duplicate from different cultures. Data were analysed by Dunnet and Tukey tests and were statistically significant at *** P < 0.001 and ** P < 0.01 when non-treated cells (empty bars) were taken as reference, and at ### P < 0.001 when the protective effect of BzATP was the reference

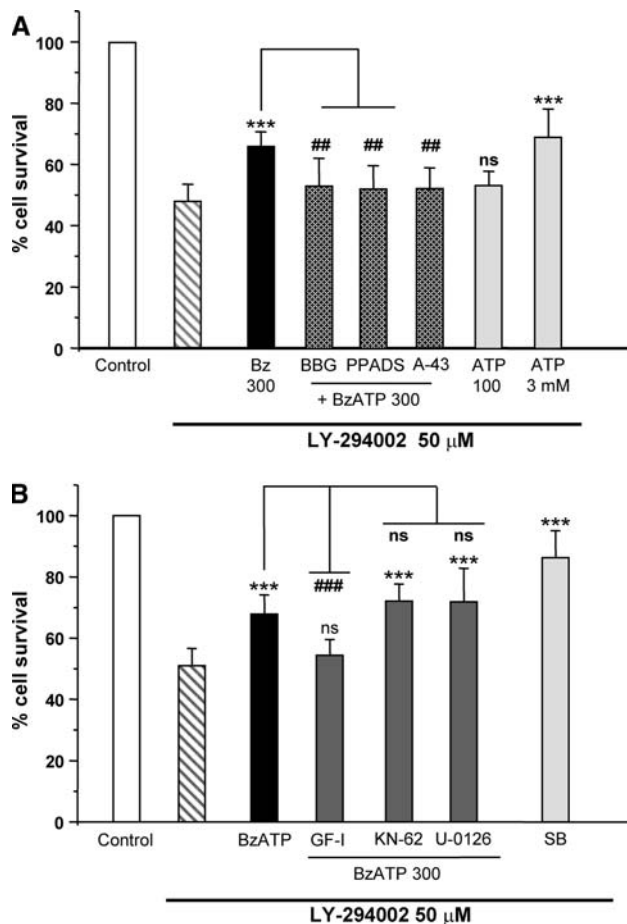


Fig. 6 Effect of different treatments on the protective effect of BzATP against PI3-K inhibition by LY-294002. In **a** Granule neurons in complete culture medium were treated with 300 μM BzATP, 100 μM and 3 mM ATP for 10 min before the addition of 50 μM LY-294002. The nucleotide receptor antagonists 10 μM BBG, 30 μM PPADS and 1 μM A-438079 were added 5 min before the incubation with BzATP. In **b** Cells were pre-treated for 20 min with the following transducing inhibitors: 0.5 μM GF-I, 1 μM KN-62 (for longer incubation times) and 10 μM U-0126, and then 300 μM BzATP was added for 10 min before the addition of 50 μM LY-294002. When the GSK-3 specific inhibitor SB-216763 (3 μM) was used, culture media were changed and were replaced by media containing 1% B-27, then the inhibitor was added at 3 μM concentration for 1 h before the addition of LY-294002. In **a** and **b** Cell viability was evaluated 24 h after the addition of LY-294002 by the MTT assay as described in Sect. **Materials and Methods**. Data are the means ± s.d. of at least three experiments performed in duplicate from different cultures. Data were analysed by Dunnet and Tukey tests and were statistically significant at *** $P < 0.001$ and ** $P < 0.01$ when LY-294002 treatment was taken as reference, and at ### $P < 0.001$, ## $P < 0.01$ and # $P < 0.05$ when comparisons were made in respect to LY-294002 + BzATP treatment

definitively excluding the participation of adenosine receptors in the BzATP-mediated effect.

In parallel with that observed for BzATP-mediated GSK-3 phosphorylation, the survival effect induced by BzATP was only abolished with the pre-treatment with the

PKC inhibitor GF-I. Other transducing inhibitors, such as KN-62 and U-0126, for CaMKII and ERK-1,2 proteins, respectively, did not affect BzATP protective action, and also they did not have any significant effect by themselves. Moreover, cell death was almost totally rescued with the GSK-3 specific inhibitor, SB-216763 (Cross et al. 2001) (Fig. 6b). These data together suggest that BzATP-mediated neuroprotection against PI3-K inhibition could be accomplished by its action on GSK-3 activity.

The BzATP-Mediated Protection is Associated with its Effect on GSK-3 Inhibition

The results above clearly establish a fine parallelism between the BzATP-mediated effect on both GSK-3 activity and rescue from PI3-K inhibition-induced apoptosis. As mentioned before, increased levels of GSK-3 phosphorylation on Ser 21/9 residues are indicative of the inhibition of its catalytic activity, and GSK-3 activity in neurons need to be finely balanced to assure neuronal survival. In this respect, granule cell treatment with the PI3-K inhibitor LY-294002 induced a significant decrease in GSK-3 phosphorylation levels, which were early detectable in the first hours of treatment (Fig. 7a). In parallel with the LY-294002-induced GSK-3 dephosphorylation there was a progressive increase in GSK-3 catalytic activity, which was evaluated measuring the phosphorylation levels of Tau with the PHF-1 antibody (Fig. 7b). This microtubule-associated protein is one of the better known GSK-3 substrates in neurons, and the Ser393/404 residues are specific targets for GSK-3 (Benneceib et al. 2000). When cells were pre-treated with BzATP, GSK-3 phosphorylation levels were partially recovered, and Tau phosphorylation was diminished, these results indicating that BzATP treatment is helping to restore phosphorylated GSK-3 and to avoid excessive activation of GSK-3 catalytic activity (Fig. 7a and b).

Finally, if the survival promoting effect of BzATP was associated with the prevention of apoptotic effectors such as caspase-3 was examined. The apoptotic nature of cell death induced by PI3-K inhibition in granule neurons involves activation of the intrinsic death pathway and cleavage of caspase-3 (Linseman et al. 2004). As reported before and in our experimental conditions, LY-294002 treatment caused a time dependent increase in caspase-3 cleavage, being the 17-kDa active caspase-3 fragment readily detectable at 3 h and markedly increased after 6 and 8 h. As expected, this increase was significantly prevented in cells treated with BzATP confirming the survival promoting effect of this nucleotide. The GSK-3 inhibitor SB-216763 was taken as positive control and reduced almost completely caspase-3 activation (Fig. 7c).

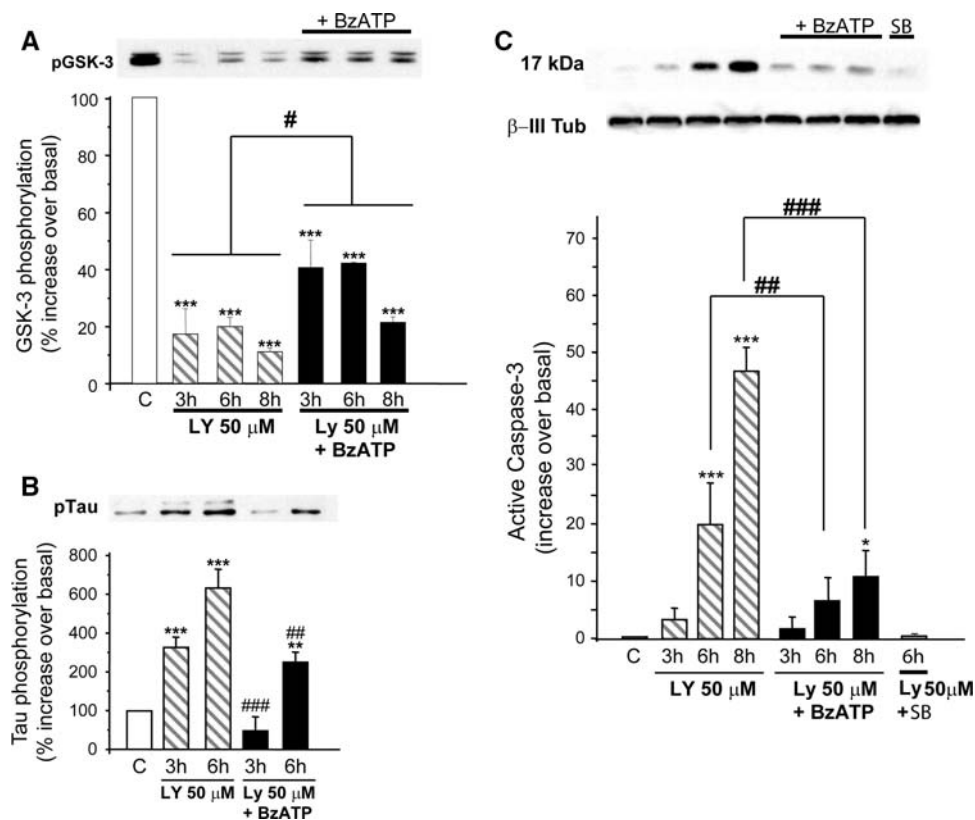


Fig. 7 Analysis of the effect of LY-294002 treatment on phosphorylation levels of GSK-3 and Tau, and caspase-3 activation. Granule neurons in complete culture medium were treated in the presence or absence of 300 μ M BzATP or 3 μ M SB-216763 for 10 min previous to the addition of 50 LY-294002. Cell extracts were obtained at different times of LY-294002 treatment and analysed by immunoblotting as described before for GSK-3 phosphorylation (a), Tau phosphorylation at the PHF-1 epitope (Ser396/404) (b), and for the presence of the 17-kDa active caspase-3 fragment (c). Histograms represent the percentage increase with respect to non-stimulated cells (100% control value for a and b; 1% control value for c, and were

obtained by normalisation of densitometric values of phosphoproteins GSK-3 and Tau, and caspase-3, with respect to that obtained for β -tubuline, employed as a control charge and shown in c. Values are the means \pm s.d. of at least three experiments performed from different cultures. The blots correspond to representative experiments. Data were analysed by Dunnett and Tukey tests and were statistically significant at *** P < 0.001 and * P < 0.05 when compared to the control of untreated cells (empty bars), and ### P < 0.001 ## P < 0.01 and # P < 0.05, when each incubation time with LY-294002 was compared in the presence and absence of Bz-ATP

Discussion

The present study describes the coupling of the nucleotide receptor activated by BzATP in cerebellar granule neurons to a novel signalling mechanism involving GSK-3 phosphorylation and inhibition by a PKC-dependent pathway. This signalling mechanism is responsible for the BzATP survival promoting actions rescuing granule neurons from the apoptosis induced by the inhibition of the PI3-K/Akt axis. Since this route is the most important survival pathway predominantly used by neurotrophic factors, our results support a role for nucleotides as potential neuronal survival promoting signals, acting either alone or in combination with growth factors.

The nature of the receptor activated by BzATP responsible for the neuroprotective actions observed with this nucleotide agonist fits well to the pharmacological profile of a P2X₇ receptor. First, the EC₅₀ value is in the

range (low micromolar) of that described for rat P2X₇ receptors (Young et al. 2007). Second, the protective action of BzATP is only reproduced by millimolar ATP concentrations, and not by 100 μ M that fully activates other P2X receptors present in granule neurons. This is in agreement to the 10-fold lower agonistic affinity described for ATP on P2X₇ receptors in comparison to BzATP (Young et al. 2007). And third, the described BzATP effects are sensitive to the antagonists known to inhibit P2X₇ receptors. We have used the non-selective antagonist PPADS, together with the most selective antagonists currently available, BBG and A-438079 (Jiang et al. 2000; Donnelly-Roberts and Jarvis 2007). Nevertheless, in the light of recent reports providing convincing evidences of new functional heteromers (Nicke et al. 2005; Guo et al. 2007; Lalo et al. 2008) the existence of a heteromeric receptor formed by P2X₇ and any other subunit present in granule neurons cannot be excluded.

Although the presence of P2X₇ receptors in cerebellar granule neurons has been previously reported (Amadio et al. 2002; Hervás et al. 2003), the present study finds a new signalling pathway for the P2X₇ receptor in granule neurons that involves GSK-3. The coupling of nucleotide receptors to GSK-3 signalling was first reported in cortical astrocytes (Neary and Kang 2006), which seemed to involve primarily a PKC-dependent mechanism and to be related to a protective role, in a similar way to that found for BzATP in granule neurons. Other examples in the literature link PKC in the regulation of GSK-3. Indeed, several PKC isoforms have been shown to phosphorylate and inactivate GSK-3 both “in vivo” and “in vitro” (Goode et al. 1992; Fang et al. 2002). In hippocampal neurons, direct PKC activators, such as phorbol esters, were reported to induce GSK-3 phosphorylation in Ser-9 and stabilisation of β -catenin, implying that several members of the Wnt signalling pathway are regulated by PKCs (Garrido et al. 2002). In addition, different extracellular signals have been reported to use the PKC/GSK-3 signalling, such as LPA in Swiss 3T3 cells (Fang et al. 2002), and muscarinic and PDGF receptors, which cooperate to regulate airway myocyte proliferation (Gosens et al. 2007). Interestingly, the classical pathway to reach GSK-3 inhibition by phosphorylation was described for insulin and other growth factors, which involved the PI3-K/Akt axis, as we reported for the metabotropic P2Y₁₃ receptor in granule neurons (Ding et al. 2000; Ortega et al. 2008).

One of the main interesting findings in the present work is the survival promoting effect found for BzATP in granule neurons, which appears to be directly related to its action on GSK-3 inhibition. In fact, GSK-3 is outlined as one of the main effectors of apoptosis in neuronal models (Pap and Cooper 1998; Hetman et al. 2000). In a recent report it was demonstrated that a component of the intrinsic apoptotic cascade, the protein Bax, was identified as a direct target of GSK-3 action. This resulted in the phosphorylation and activation of Bax that led to its mitochondrial re-localisation (Linseman et al. 2004). This implies that signals that can maintain a decreased GSK-3 activity can function as important pro-survival signals. In the case of BzATP, it keeps GSK-3 inhibited in conditions in which the main survival route to GSK-3, the PI3-K/Akt axis, is compromised or inhibited. A similar role to that found for BzATP, was described for NMDA in cortical neurons, since it was able to rescue them from the apoptosis induced by LY-294002 treatment. This protective effect is also dependent on NMDA-mediated inhibition of GSK-3 activity (Habas et al. 2006).

With respect to the role of nucleotides in cell death and survival, both harmful and protective actions have been described at the CNS level, well reviewed in recent reports (Volonté et al. 2003; Sperlagh et al. 2006). Likewise,

nucleotides released after brain insults have been proposed as danger signals that display dual functions, first alerting tissue to trigger protective responses against damage, and then, upon sustained release and activation, can function as amplifiers to augment inflammation and toxicity. Focusing on P2X₇ receptors, sustained activation seems to be coupled to cell death, as was first reported in cells of blood lineage (Surprenant et al. 1996; Di Virgilio 1999) and also in some neuronal and glial cells, such as cortical, dopaminergic neurons and cortical astrocytes (Fumagalli et al. 2004; Kong et al. 2005; Jun et al. 2007). However, P2X₇ is not associated to cell death in other cells, such as cerebellar astrocytes (Delicado et al. 2005), suggesting that the heterogeneity observed in P2X₇-mediated actions can be cell and tissue specific and can depend on differences at P2X₇ receptor expression levels. In this respect, there is plenty of data regarding their presence at presynaptic terminals, which has assigned them a function in the regulation of neurotransmitter release (Armstrong et al. 2002; Sperlagh et al. 2002; Miras-Portugal et al. 2003; Atkinson et al. 2004; Ireland et al. 2004). In addition, P2X₇ receptor expression in astrocytes seems to play a relevant function in neuron-glia communication (Delicado et al. 2005; Duan et al. 2003; Suadcani et al. 2006). All these data give evidence in favour of the versatility and plasticity of the P2X₇ receptor, and its ability to adopt different dispositions and conformations to elaborate particular actions when is expressed natively in different cell types and tissues.

In conclusion, the results presented here in granule neurons support the hypothesis that nucleotides can function in the same way as neurotrophic factors. To support cell survival nucleotides have to reach GSK-3 signalling by another intracellular cascade involving PKC, as an important survival promoting mechanism. These results add new functions to that previously reported for P2X₇ receptors at the CNS level.

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References

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson

- KA, Weisman GA (2006) International union of pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 58:281–341
- Amadio S, D'Ambrosi ND, Cavaliere F, Murra B, Sancesario G, Bernardi G, Burnstock G, Volonté C (2002) P2 receptor modulation and cytotoxic function in cultured CNS neurons. *Neuropharmacol* 42:489–501
- Armstrong JN, Brust TB, Lewis RG, MacVicar BA (2002) Activation of presynaptic P2X7-like receptors depresses mossy fiber-CA3 synaptic transmission through p38 mitogen-activated protein kinase. *J Neurosci* 22:5938–5945
- Atkinson L, Batten TF, Moores TS, Varoqui H, Erickson JD, Deuchars J (2004) Differential co-localisation of the P2X7 receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. *Neuroscience* 123:761–768
- Bennecib M, Gong C-X, Grundke-Iqbal I, Iqbal K (2000) Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain. *FEBS Lett* 485:87–93
- Bianchi BR, Lynch KJ, Touma E, Niforatos W, Burgard EC, Alexander KM, Park HS, Yu H, Metzger R, Kowaluk E, Jarvis MF, van Biesen T (1998) Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J Pharmacol* 376:127–138
- Burgoyne RD, Graham ME, Cambray-Deakin M (1993) Neurotrophic effects of NMDA receptor activation on developing cerebellar granule cells. *J Neurocytol* 22:689–695
- Burnstock G (2006) Historical review: ATP as a neurotransmitter. *Trends Pharmacol Sci* 27:166–176
- Chin PC, Majdzadeh NM, D'Mello SR (2005) Inhibition of GSK-3 β is a common event in neuroprotection by different survival factors. *Mol Brain Res* 137:193–201
- Contestabile A (2002) Cerebellar granule cells as a model to study mechanisms of neuronal apoptosis or survival in vivo and in vitro. *Cerebellum* 1:41–55
- Cross DAE, Culbert AA, Chalmers KA, Facci L, Skaper SD, Reith AD (2001) Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurons from death. *J Neurochem* 77:94–102
- D'Mello SR, Galli C, Ciotti T, Calissano P (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor-I and cAMP. *Proc Natl Acad Sci USA* 90:10989–10993
- D'Mello SR, Borodetz V, Soltoff SP (1997) Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI3-kinase in IGF-I signalling. *J Neurosci* 17:1548–1560
- Delicado EG, Carrasquero LMG, Pérez-Sen RP, Miras-Portugal MT (2005) Identification of functional P2X7 receptor in rat cerebellar astrocytes. In: International proceedings of VII European meeting on glial cell function in health and disease, Amsterdam, The Netherlands, Medimond
- Di Virgilio F (1999) The P2Z/P2X7 receptor of microglial cells: a novel immunomodulatory receptor. *Prog Brain Res* 120:355–368
- Ding VW, Chen R-H, McCormick F (2000) Differential regulation of glycogen synthase kinase 3 β by insulin and Wnt signalling. *J Biol Chem* 275:32475–32481
- Donnelly-Roberts DL, Jarvis MF (2007) Discovery of P2X7 receptor-selective antagonists offer new insights into P2X7 receptor function and indicates a role in chronic pain states. *Br J Pharmacol* 151:571–579
- Duan S, Anderson CM, Keung EC, Chen Y, Swanson RA (2003) P2X7 receptor-mediated release of excitatory amino acids from astrocytes. *J Neurosci* 23:1320–1328
- Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275:628–630
- Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, Mills GB (2002) Convergence of multiple signalling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol Cell Biol* 22:2099–2110
- Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 359:1–16
- Fumagalli M, Trincavelli L, Lecca D, Martini C, Ciana P, Abbracchio MP (2004) Cloning, pharmacological characterization and distribution of the rat G-protein-coupled P2Y₁₃ receptor. *Biochem Pharmacol* 68:113–124
- Gallo V, Kingsbury A, Balazs R, Jorgensen OS (1985) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J Neurosci* 7:2203–2213
- Garrido JL, Godoy J, Alvarez A, Bronfman M, Inestrosa NC (2002) Protein kinase C inhibits amyloid β -peptide neurotoxicity by acting on members of the Wnt pathway. *FASEB J* 16:1982–1984
- Goode N, Hughes K, Woodgett JR, Parker PJ (1992) Differential regulation of glycogen synthase kinase-3 β by protein kinase C isoforms. *J Biol Chem* 267:16878–16882
- Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H, Halayko AJ (2007) Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 293:L1348–L1358
- Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD (2007) Evidence for functional P2X₄/P2X₇ heteromeric responses. *Mol Pharmacol* 72:1447–1456
- Habas A, Kharebava G, Szatmari E, Hetman M (2006) NMDA neuroprotection against a phosphatidylinositol-3 kinase inhibitor, LY294002 by NR2B-mediated suppression of glycogen synthase kinase-3 β -induced apoptosis. *J Neurochem* 96:335–348
- Hall AC, Brennan A, Gould RG, Cleverley K, Lucas FR, Gordon-Weeks PR, Salinas PC (2002) Valproate regulates GSK-3-mediated axonal remodelling and synapsin I clustering in developing neurons. *Mol Cell Neurosci* 20:257–270
- Hervás C, Pérez-Sen R, Miras-Portugal MT (2003) Coexpression of functional P2X and P2Y nucleotide receptors in single cerebellar granule cells. *J Neurosci Res* 73:384–399
- Hervás C, Pérez-Sen R, Miras-Portugal MT (2005) Presence of diverse functional P2X receptors in rat cerebellar synaptic terminals. *Biochem Pharmacol* 70:770–785
- Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 274:22569–22580
- Hetman M, Cavanaugh JE, Kimelman D, Xia Z (2000) Role of glycogen synthase kinase-3 β in neuronal apoptosis induced by trophic withdrawal. *J Neurosci* 20:2567–2574
- Hibell AD, Thompsom KM, Xing M, Humphrey PP, Michel AD (2001) Complexities of measuring antagonist potency at P2X7 receptor orthologs. *J Pharmacol Exp Ther* 296:947–957
- Humphreys BD, Virginio C, Surprenant A, Rice J, Dubyak GR (1998) Isoquinolines as antagonists of the P2X₇ nucleotide receptor: high selectivity for the human versus rat receptor homologues. *Mol Pharmacol* 54:22–32
- Ireland MF, Noakes PG, Bellingham MC (2004) P2X₇-like receptor subunits enhance excitatory synaptic transmission at central synapses by presynaptic mechanisms. *Neuroscience* 128:269–280

- Jiang LH, Mackenzie AB, North RA, Surprenant AM (2000) Brilliant blue G selectively blocks ATP-gated rat P2X₇ receptors. *Mol Pharmacol* 58:82–88
- Jope RS, Johnson GVW (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29:95–102
- Jun D-J, Kim J, Jung SY, Song R, Noh J-H, Park Y-S, Ryu S-H, Kim J-H, Kong Y-Y, Chung J-M, Kim K-T (2007) Extracellular ATP mediates necrotic cell swelling in SN4741 dopaminergic neurons through P2X₇ receptors. *J Biol Chem* 282:37350–37358
- Kong Q, Wang M, Liao Z, Camden JM, Yu S, Simonyi A, Sun GY, Gonzalez FA, Erb L, Seye CI, Weisman GA (2005) P2X₇ nucleotide receptors mediate caspase-8/9/3-dependent apoptosis in rat primary cortical neurons. *Purinergic Signal* 1:337–347
- Lafón-Cazal M, Pérez V, Bockaert J, Marín P (2002) Akt mediates the anti-apoptotic effect of NMDA but not that induced by potassium depolarization in cultured cerebellar granule cells. *Eur J Neurosci* 16:575–583
- Lalo U, Pankratov Y, Wichert SP, Rossner MJ, North RA, Kirchhoff F, Verkhratsky A (2008) P2X₁ and P2X₅ subunits form the functional P2X receptor in mouse cortical astrocytes. *J Neurosci* 28:5473–5480
- León D, Hervás C, Miras-Portugal MT (2006) Activation of P2Y₁ and P2X₇ receptors induce calcium/calmodulin-dependent protein kinase II phosphorylation in cerebellar granule neurons. *Eur J Neurosci* 23:2999–3013
- León D, Sánchez-Nogueiro J, Marín-García P, Miras-Portugal MT (2008) Glutamate release and synapsin-I phosphorylation induced by P2X₇ receptors activation in cerebellar granule neurons. *Neurochem Int* 52:1148–1159
- Li M, Wang X, Maintzer MK, Laessig T, Birnbaum MJ, Heidenreich KA (2000) Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3 beta. *Mol Cell Biol* 20:9356–9363
- Linseman DA, Butts BD, Precht TA, Phelps RA, Le SS, Laessig TA, Bouchard RJ, Florez-McClure ML, Heidenreich KA (2004) Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. *J Neurosci* 24:9993–10002
- Miller TM, Tansey MG, Johnson EM, Creedon DJ (1997) Inhibition of phosphatidylinositol 3-Kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule neurons. *J Biol Chem* 272:9847–9853
- Miras-Portugal MT, Días-Hernández M, Giráldez L, Hervás C, Gómez-Villafuertes R, Sen RP, Gualix J, Pintor JJ (2003) P2X₇ receptors in rat brain: presence in synaptic terminals and granule cells. *Neurochem Res* 28:1597–1605
- Neary JT, Kang Y (2006) P2 purinergic receptors signal to glycogen synthase kinase-3beta in astrocytes. *J Neurosci Res* 84:515–524
- Nicke A, Kerschensteiner D, Soto F (2005) Biochemical and functional evidence for heteromeric assembly of P2X₁ and P2X₄ subunits. *J Neurochem* 92:925–933
- North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067
- Ortega F, Pérez-Sen RP, Miras-Portugal MT (2008) Gi-coupled P2Y-ADP receptor mediates GSK-3 phosphorylation and beta-catenin nuclear translocation in granule neurons. *J Neurochem* 104:62–73
- Pap M, Cooper GM (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. *J Biol Chem* 273:19929–19932
- Pons S, Trejo JL, Martínez-Morales JR, Martí E (2001) Vitronectin regulates Sonic hedgehog activity during cerebellum development through CREB phosphorylation. *Development* 128:1481–1492
- Sánchez-Nogueiro J, Marín-García P, Miras-Portugal MT (2005) Characterization of a functional P2X(7)-like receptor in cerebellar granule neurons from P2X(7) knockout mice. *FEBS Lett* 579:3783–3788
- Sayas CL, Ávila J, Wandosell F (2002) Regulation of neuronal cytoskeleton by lysophosphatidic acid: role of GSK-3. *Biochim Biophys Acta* 1582:144–153
- Sperlagh B, Kofalvi A, Deuchars J, Atkinson L, Milligan CJ, Buckley NJ, Vizi ES (2002) Involvement of P2X₇ receptors in the regulation of neurotransmitter release in the rat hippocampus. *J Neurochem* 81:1196–1211
- Sperlagh B, Vizi ES, Wirkner K, Illes P (2006) P2X₇ receptors in the nervous system. *Prog Neurobiol* 78:327–346
- Suadicaní SO, Brosnan CF, Scemes E (2006) P2X₇ receptors mediate ATP release and amplification of astrocytic intercellular Ca²⁺ signalling. *J Neurosci* 26:1378–1385
- Subramaniam S, Shahani N, Strelau J, Laliberté C, Brandt R, Kaplan D, Unsicker K (2005) Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. *J Neurosci* 25:2838–2852
- Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X₇). *Science* 272:735–738
- Virginio C, Church D, North RA, Surprenant A (1997) Effects of divalent cations, protons and calmidazolium at the rat P2X₇ receptor. *Neuropharmacol* 36:1285–1294
- Virginio C, Robertson G, Surprenant A, North RA (1998) Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X₁, P2X₃ and heteromeric P2X_{2/3} receptors. *Mol Pharmacol* 53:969–973
- Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269:5241–5248
- Volonté C, Amadio S, Cavaliere F, D'Ambrosi N, Vacca F, Bernardi G (2003) Extracellular ATP and neurodegeneration. *Current Drug Targets CNS Neurol Disord* 2:61–72
- Yamagashi S, Matsumoto T, Yokomaku D, Hatanaka H, Shimoke K, Yamada M, Ikeuchi T (2003) Comparison of inhibitory effects of brain-derived neurotrophic factor and insulin-like growth factor on low potassium-induced apoptosis and activation of p38 MAPK and c-Jun in cultured cerebellar granule neurons. *Mol Brain Res* 119:184–191
- Young MT, Pellegrin P, Surprenant AM (2007) Aminoacid residues in the P2X₇ receptor that mediate differential sensitivity to ATP and BzATP. *Mol Pharmacol* 71:92–100
- Zhang FX, Rubin R, Rooney TA (1998) N-methyl-D-aspartate inhibits apoptosis through activation of phosphatidylinositol 3-kinase in cerebellar granule neurons. *J Biol Chem* 273:26596–26602