P2X7 receptor-induced death of motor neurons by a peroxynitrite/FAS-dependent pathway

Mandi Gandelman[1,2], **Mark Levy**[2], **Patricia Cassina**[3], **Luis Barbeito**[1,4], and **Joseph S Beckman**[3,*]

[1]Neurodegeneration Laboratory, Institut Pasteur, Montevideo, Uruguay

[2]Linus Pauling Institute, Oregon State University

[3]Departamento de Histología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

[4]Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

Abstract

The P2X7 receptor/channel responds to extracellular ATP and is associated with neuronal death and neuroinflammation in spinal cord injury and amyotrophic lateral sclerosis (ALS). Whether activation of P2X7 directly causes motor neuron death is unknown. We found that cultured motor neurons isolated from embryonic rat spinal cord express P2X7 and underwent caspase-dependent apoptosis when exposed to exceptionally low concentrations of the P2X7 agonist 3′-O-(4-benzoyl)-ATP (BzATP). The P2X7 inhibitors BBG, oATP and KN-62 prevented BzATP-induced motor neuron death. The endogenous P2X7 agonist ATP induced motor neuron death at low concentrations (1-100 µM). High concentrations of ATP (1 mM) paradoxically became protective due to degradation in the culture media to produce adenosine and activate adenosine receptors. P2X7-induced motor neuron death was dependent on neuronal nitric oxide synthase-mediated production of peroxynitrite, p38 activation and autocrine FAS signaling. Taken together, our results indicate that motor neurons are highly sensitive to P2X7 activation, which triggers apoptosis by activation of the well-established peroxynitrite/FAS death pathway in motor neurons.

Introduction

Neurologic disorders like trauma, ischemia and inflammation cause a marked increase in extracellular adenosine-5′-triphosphate (ATP) levels (Wang *et al.* 2004, Phillis *et al.* 1993, Melani *et al.* 2005, Piccini *et al.* 2008). The P2X7 receptor is a non-desensitizing ATP-gated cation channel that can induce pro-inflammatory responses in glia and immune cells that can indirectly initiate neuronal death (Apolloni *et al.* 2009, Burnstock 2008). P2X7 can also directly induce neuronal death (Burnstock 2008). Inhibition of P2X7 is neuroprotective in animal models of traumatic spinal cord injury, experimental autoimmune encephalomyelitis

*Corresponding author joe.beckman@oregonstate.edu.
MG: mandi.gandelman@gmail.com ML: mark.levy@us.usana.com PC: pcassina@fmed.edu.uy LB: barbeito@pasteur.edu.uy

and Alzheimer's and Huntington's disease (Ryu & McLarnon 2008, Matute *et al.* 2007, Diaz-Hernandez *et al.* 2009, Jun *et al.* 2007).

Motor neurons are the most vulnerable neurons after traumatic spinal cord injury and in motor neuron diseases like amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy. A role for P2X7 in inducing motor neuron death during traumatic spinal cord injury has been uncovered in a rat model, where ATP was dramatically increased in the peripheral regions of the injury (Wang et al. 2004). Activation of P2X7 in spinal cord motor neurons caused an irreversible increase in intracellular calcium (Wang et al. 2004), while systemic inhibition of P2X7 protected motor neurons, promoted functional recovery, decreased microglia and astrocyte activation and limited neutrophil infiltration into the spinal cord (Peng *et al.* 2009).

In ALS patients as well as in the ALS animal models expressing mutant Cu,Zn superoxide dismutase (SOD1^{G93A}), growing evidence indicates that P2X7 could cause motor neuron death acting through astrocytes or microglia. Spinal cord microglia display increased immunoreactivity for P2X7 during the disease (Yiangou *et al.* 2006, Casanovas *et al.* 2008). Furthermore, cultured SOD1^{G93A} microglia display an increased sensitivity to ATP and P2X7 activation drives their pro-inflammatory activation (D'Ambrosi *et al.* 2009).

We have previously shown that P2X7 activation in astrocytes triggers a phenotype change and causes astrocytes to induce death of motor neurons in a co-culture model (Gandelman *et al.* 2010). In astrocytes cultured from SOD1^{G93A} rats, P2X7 is basally activated in an autocrine manner that maintains their neurotoxic phenotype towards motor neurons (Gandelman et al. 2010). These results indicate that part of the dysfunction of SOD^{G93A} astrocytes involves the release of enough ATP to activate P2X7 in vivo. Here, we investigated whether ATP might also activate P2X7 in motor neurons and result in motor neuron death.

Methods

Chemicals and reagents

Cell culture, PCR reagents and secondary antibody were from *Life Technologies*. FAS:FC and cleaved caspase 3 antibodies were from *Cell Signaling Technologies* (Danvers, MA). An antibody directed against the intracellular C-terminal domain of P2X7 was from *Alomone Labs* (Jerusalem, Israel). All other reagents were purchased from *Sigma-Aldrich* (St. Louis, MO).

Animals

Timed pregnant Sprague-Dawley rats were purchased from Harlan Laboratories (Livermore, CA). Procedures using laboratory animals were in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Care Organization of the School of Medicine, Universidad de la República (Montevideo, Uruguay) and by the Oregon State University IACUC.

Primary motor neuron cultures

Motor neurons were prepared from embryonic day 15 rat spinal cords as previously described (Henderson *et al.* 1995, Gandelman et al. 2010). Briefly, the ventral horns of spinal cords were dissected and incubated in 0.05% trypsin for 15 minutes at 37°C, followed by mechanical dissociation. Motor neurons were then purified by centrifugation on an Optiprep cushion, followed by isolation of p75^{NTR} expressing motor neurons by immunoaffinity selection with the IgG-192 monoclonal antibody. Approximately 1500

motor neurons were plated in each well of a 24-well plate coated with poly-L-ornithine and laminin. Motor neurons were cultured in Neurobasal media supplemented with 2% horse serum, 2% B-27 supplement, 25 μM L-glutamate, 25 μM 2-mercaptoethanol and 500 μM L-glutamine (Henderson et al. 1995). Survival was maintained by the addition of GDNF (1ng/ml). Unless otherwise stated, motor neurons were treated 2 hours after seeding, after they had attached and started differentiating. Motor neuron survival was assessed after culturing for 48 hours by counting all cells displaying intact neurites longer than 4 cell diameters in 2 diameters of the well. Motor neurons were plated to yield 130 motor neurons per well under optimal growth conditions with each condition replicated in triplicate and then repeated with at least three separate motor neuron preparations. This method of measuring survival has been extensively used and validated for measuring motor neuron survival (Henderson et al. 1995, Estevez *et al.* 1998, Estevez *et al.* 1999).

RT-PCR

Motor neurons were plated in 35mm dishes and after 18 hours RNA was extracted using Trizol according to the manufacturer's instructions. RT-PCR was performed using SuperScript® III One-Step RT-PCR System by adding specific primers (P2X7 forward AAGGGAAAGAAGCCCCACGG, P2X7 reverse CCGCTTTTCCATGCCATTTT, Actin forward GAGCAATGATCTTGATCTTCATGGTG, Actin reverse CCTTCCTTCCTGGGTATGGAATCC).

Immunofluorescence

Motor neurons were fixed with ice-cold 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 15 minutes. Cultures were permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked for 1 hour with 10% goat serum, 2% bovine serum albumin, and 0.1% Triton X-100 in PBS. Anti-cleaved caspase 3 or P2X7 monoclonal antibody diluted in blocking solution (1:100) was incubated overnight at 4°C. After washing, cultures were incubated for 1 hour at room temperature with Alexa Fluor 488 or 568-conjugated goat anti-mouse antibody (1:500). Nuclei were stained with DAPI (1 μg/mL).

Statistics

Each experiment was repeated at least three times with separate motor neuron preparations and data are reported as mean $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance, followed by a Student–Newman–Keuls test. Differences were declared statistically significant if $p < 0.05$. Statistics were performed using GraphPad Prism 4.

Results

Activation of P2X7 in motor neurons leads to cell death

P2X7 was expressed in spinal motor neurons in culture (Fig 1A). The presence of the mRNA for P2X7 receptor was evidenced by RT-PCR. In addition, the soma and neurites of all motor neurons showed intense immunoreactivity for the receptor (Fig. 1A and B). To investigate the effect of P2X7 activation on motor neurons, increasing concentrations of the P2X7 agonist BzATP were added to the cultures two hours after isolation and survival was assessed after 48 hours. Concentrations of BzATP from 0.1 to 100 μM decreased motor neuron survival similarly, ranging from 74$\pm$3% to 69$\pm$5% (Fig. 2A). Only 0.01 μM BzATP had no observable effect on motor neuron survival. We saw no difference in death induced by BzATP whether motor neurons were exposed to BzATP immediately after plating or if treatment was delayed overnight to allow motor neurons to attach and extend neurites (not shown). Thus, BzATP decreased survival independently of motor neuron attachment and subsequent differentiation.

Inhibiting the P2X7 receptor with BBG, oATP and KN-62 (1 μM) prevented motor neuron death caused by BzATP (at concentrations of 0.1, 10 and 100 μM), which strongly suggests the specific involvement of P2X7 in the induction of motor neuron death by BzATP (Fig. 2A). To investigate the effects of P2X7 activation in motor neurons in the rest of this study, we used treatments with 10 μM BzATP, which causes death of 32±2% motor neurons and is generally recognized to activate P2X7 according to the typical pharmacological profile of this receptor (Donnelly-Roberts *et al.* 2009).

P2X7 activation triggers apoptosis by activation of the Fas/p38/NO pathway

An increase in Fas-L production followed by autocrine activation of the Fas death receptor results in caspase-dependent apoptosis in motor neurons deprived of trophic factors or expressing ALS-associated mutant SOD1. In this death pathway, Fas signals to p38, leading to NO production that causes motor neuron apoptosis (Raoul *et al.* 2006, Raoul *et al.* 2002, Raoul *et al.* 1999). To test whether this death pathway was activated following P2X7 stimulation, we blocked activation of Fas and found that motor neuron survival after BzATP exposure was restored (92±7% survival). In addition, the p38 inhibitor SB203580 (1 μM) was able to inhibit motor neuron death (96±4%, Fig. 3A). Motor neuron death was also prevented by the general NOS inhibitor L-NAME (100 μM) or the specific nNOS inhibitor TRIM (10μM, Handy et al, 1995). The effect of both inhibitors was reversed by the simultaneous addition of DETA-NONOate (10 μM, Fig. 3B). This concentration of DETA-NONOate will maintain a flow of 40 nM nitric oxide over a 24 h period and does not induce motor neuron death by itself (Estevez et al. 1998).

Because P2X7 is known for elevating intracellular calcium levels and NO production by nNOS is activated by calcium, we investigated whether chelation could prevent motor neuron death. Pre-incubation of the cultures with BAPTA-AM (1 μM) prevented motor neuron death induced by BzATP (Fig. 3B) and this protective effect was reversed by addition of DETA NONOate (Fig. 3B). These results indicate a key role for calcium regulation of nNOS in motor neuron death initiated by BzATP. The regulation of intracellular calcium likely also involves additional modulation by endoplasmic reticulum and mitochondrial transporters in addition to P2X7.

NO itself is not toxic to motor neurons, but its diffusion-limited reaction with superoxide to form peroxynitrite is ultimately responsible for motor neuron death (Estevez et al. 1998). Because P2X7 activation is known to initiate production of superoxide in numerous immune and non-immune cell types (Hewinson & Mackenzie 2007), we tested whether superoxide and peroxynitrite played a role in BzATP-induced motor neuron death. The superoxide scavenger MnTBAP (10 μM) and the peroxynitrite scavenger FeTMPyP (10 μM) completely rescued motor neurons from death after P2X7 activation (Fig. 3C). In addition, urate (200 μM), which scavenges peroxynitrite-derived radicals and thereby inhibits tyrosine nitration of proteins (Santos *et al.* 1999, Robinson *et al.* 2004), was also able to prevent motor neuron death caused by P2X7 activation (Fig. 3C).

P2X7 activation triggered caspase-dependent death in motor neurons, as it was prevented by the general caspase inhibitor zVAD-fmk (10 μM) and the caspase 3 inhibitor DEVD-fmk (10 μM, Fig. 4A). In addition, extensive immunoreactivity for activated caspase 3 is evidenced in motor neurons 24 hours after treatment with BzATP, in coincidence with fragmented nucleus, known hallmark of apoptosis (Fig. 4B). This agrees with previous reports providing evidence that Fas activates caspase-dependent apoptosis in motor neurons (Raoul, Henderson et al. 1999).

ATP degradation to adenosine prevents P2X7-mediated motor neuron death

To more closely resemble conditions during injury or disease in the nervous system, we exposed motor neurons to ATP, the endogenous P2X7 agonist. Addition of 100 μM ATP induced death of 27±7% of motor neurons while 1mM ATP did not cause any significant death (8±4%, Fig. 5A). Because ATP quickly degrades to adenosine, which can increase motor neuron survival by trans-activating Trk receptors (Wiese, Jablonka et al. 2007), we hypothesized that degradation of 1mM ATP could produce enough adenosine to prevent P2X7-mediated death. Indeed, we found that with caffeine (0.5 μM; an inhibitor of P1 receptors), addition of 1mM ATP caused death of 26±7% motor neurons. In addition, high concentrations of adenosine (200 μM) prevented motor neuron death initiated by BzATP (Fig. 5B), while lower doses (1 μM) had no effect, explaining why this antagonizing effect was not seen in motor neurons exposed to lower concentrations of ATP. In agreement with these results, exogenous addition of high doses of adenosine (100 and 200 μM) caused a small but significant increase in basal survival (115±4 and 116±6% respectively) while 1 μM adenosine had no effect (Fig. 5C).

Discussion

Accumulating evidence indicates that P2X7 mediates multiple detrimental effects in many nervous system diseases (Koles *et al.* 2005, Sperlagh *et al.* 2006). One of the earliest events after spinal cord trauma is ATP release in areas surrounding experimental spinal cord injury that is also associated with a P2X7 dependent irreversible increase in intracellular calcium (Wang et al, 2004). Surprisingly, the role of P2X7 has received limited investigation in ALS. Increased immunoreactivity for P2X7 in microglia has been observed in ALS tissues (Yiangou et al. 2006). We have recently shown that P2X7 activation caused astrocytes to become neurotoxic to motor neurons (Gandelman et al. 2010). Furthermore, P2X7 was basally activated in SOD1[G93A] expressing astrocytes and was needed to maintain their neurotoxic phenotype. Here, we found motor neurons themselves can be a direct target of elevated extracellular ATP through activation of P2X7.

P2X7 activation in motor neurons triggered a peroxynitrite-fueled apoptotic cascade, previously described as a distinctive death pathway extensively characterized in the death of motor neurons from trophic factor deprivation, activation of the p75 receptor and expression of mutant SODs (Raoul et al. 2006, Raoul et al. 1999, Raoul et al. 2002, Pehar *et al.* 2005, Pehar *et al.* 2007, Pehar *et al.* 2006). The downstream signaling triggered by P2X7 involves activation of p38 MAPK that in turn activates an autocrine loop involving neuronal NOS activation and Fas signaling. It involves cycles of amplification of increasing nitric oxide synthesis by neuronal NOS, phosphorylation of p38 through DAXX/ASK1, and further FAS-L release. Calcium entry due to P2X7 activation could increase nitric oxide synthesis by stimulating neuronal NOS activity. Supporting this concept, we found that the protection afforded by intracellular calcium chelation from P2X7 activation was reversed by a low steady state concentration of 40 nM nitric oxide.

We have previously shown that nitric oxide is not directly toxic to motor neurons via this pathway, but rather reacts with superoxide to form the oxidant peroxynitrite (Sahawneh *et al.* 2010). Here, motor neuron death triggered by P2X7 was prevented by two different peroxynitrite scavengers as well as by urate, a radical scavenger that strongly inhibits tyrosine nitration. Thus, the transformation of nitric oxide to peroxynitrite can serve as a switch controlling the survival versus death of stressed motor neurons. Peroxynitrite scavengers have also been shown to be protective in mutant SOD1 transgenic mouse models of ALS (Soon *et al.* 2011, Wu *et al.* 2003). Additionally, nitration of a single tyrosine near the ATP binding pocket of HSP90 has recently been shown to be sufficient to activate motor

neuron death through a mechanism that involves P2X7 and the release of FAS (Franco *et al.* 2013).

Fas is strongly implicated in controlling peroxynitrite-induced apoptosis of motor neurons in ALS (Sahawneh et al. 2010), and involved in spinal ischemia, axotomy of the facial nerve, sciatic nerve avulsion and spinal cord injury (Matsushita et al., 2000, Sakurai et al., 1998, Ugolini et al., 2003, Martin et al, 2005, Casha et al, 2005, Demjen et al, 2004). Silencing Fas in the spinal cord of animal models of ALS models improves motor function and extends survival (Locatelli et al, 2007). In spinal cord injury, Fas inhibition specifically spares neurons and axons in the areas surrounding the injury (Casha et al, 2005, Demjen et al, 2004). Here, we found evidence that P2X7 activates Fas-induced death of cultured motor neurons via peroxynitrite, but whether P2X7 triggers this death pathway in vivo remains to be determined. Activation of P2X7 has recently been shown to be involved in FAS-induced death of Jurket cells (Aguirre *et al.* 2013).

The sensitivity of motor neurons to ATP in vitro was modulated by cleavage to adenosine, which can be catalyzed by extracellular nucleotidases such as CD39 as well as apyrase in culture serum. Adenosine has well established roles as anti-inflammatory and neuroprotective agent (Jacobson & Gao 2006). In our assays, direct addition of high doses of adenosine prevented motor neuron death triggered by BzATP while lower doses had no protective effect. This likely explains how high concentrations of ATP (1 mM) paradoxically protected motor neurons while lower concentrations activated apoptosis via P2X7. Adenosine signaling through the A_{2A} receptor leads to transactivation of the neurotrophin TrkB receptor and prevents motor neuron death in vivo after facial nerve axotomy and in vitro caused by trophic factor deprivation (Wiese *et al.* 2007). In addition, A_{2A} activation reduces neuronal apoptosis and neurological deficit after spinal cord trauma (Genovese *et al.* 2010). Both P2X7 antagonists and A_{2A} agonists protect motor neurons from spinal cord trauma (Genovese et al. 2010, Wang et al. 2004, Peng et al. 2009). Thus, ATP-degrading enzymes in the microenvironment surrounding motor neurons might modulate disease progression by limiting the activation of P2X7 while stimulating adenosine receptors. Taken together our results reveal a new regulatory mechanism of motor neuron survival by being a target for elevated extracellular ATP released during the disease. Further knowledge of the interactions between these two pathways could help optimize the design of new highly targeted therapies to prevent motor neuron death in ALS and spinal cord injury.

Acknowledgments

Abbreviations

ALS	Amyotrophic lateral sclerosis
ASK1	Apoptosis signal-regulating kinase 1
ATP	adenosine-5′-triphosphate
A2A	Adenosine receptor
A2a BBG	Brilliant Blue G
BzATP	2′(3′)-*O*-(4-Benzoylbenzoyl) -ATP

DAXX	Death-associated protein 6
DEVD-fmk	Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone
DETA-NONOate	(Z)-1-[N- (2-aminoethyl)- N- (2-ammonioethyl)amino]diazen-1-ium-1, 2 diolate
FeTMPyP	5,10,15,20-Tetrakis(N-methyl-4′-pyridyl)porphinato Iron (III)
Chloride GDNF	Glial cell-derived neurotrophic factor
KN-62	4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester
L-NAME	NG-Nitro-L-arginine methyl ester
MnTBAP	Mn(III)tetrakis(4-benzoic acid)porphyrin chloride
nNOS	neuronal nitric oxide syntahse
oATP	oxidized ATP
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SOD1	Cu/Zn superoxide dismutase-1 SOD1G93A Cu/Zn superoxide dismutase-1 Glycine 93 to Alanine mutation
TRIM	-(2-Trifluoromethylphenyl) imidazole
Trk	tropomyosin-receptor-kinase zVAD-fmk - carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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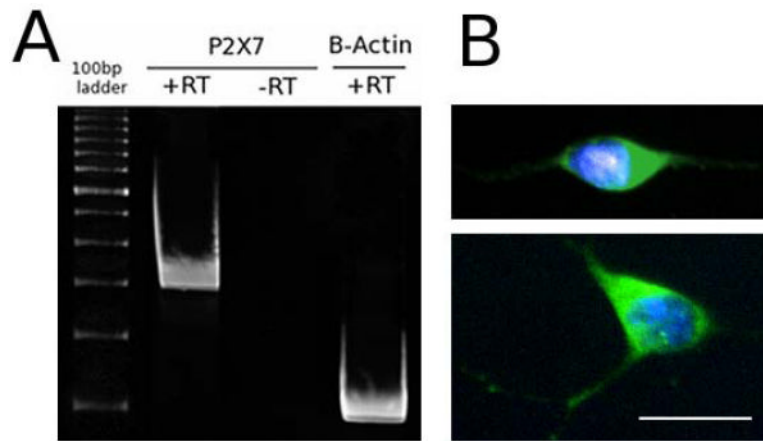
Fig. 1. Motor neurons in primary culture express P2X7
(A) Total RNA was extracted from cultured motor neurons 18 hours after isolation and RT-PCR was performed to reveal the presence of P2X7 mRNA. (B) Motor neurons cultured for 18 hours display immunoreactivity for P2X7 (green) in the soma and neurites. Nuclei were stained with DAPI (blue). The scale bar represents 20 μm.
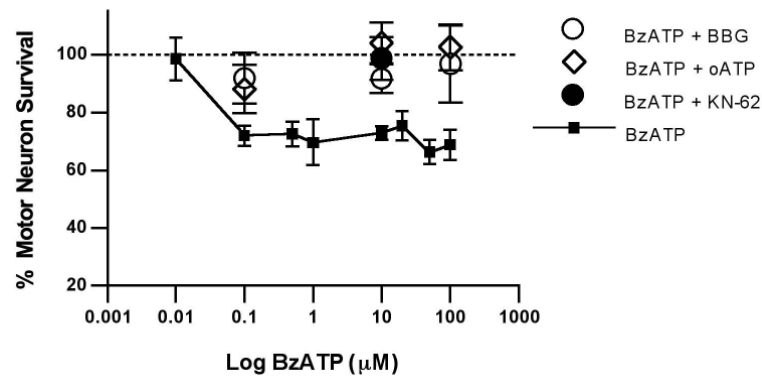
Fig. 2. Cultured motor neurons are highly sensitive to BzATP
Motor neuron survival in response to 0.1 to 100µM BzATP (squares). The P2X7 inhibitors
BBG (1 µM, triangles), oATP (1 µM, inverted triangles) and KN-62 (1 µM, diamonds)
prevented motor neuron death induced by 0.1, 10 and 100 µM BzATP. Dotted line indicates
survival in absence of BzATP (100%). All BzATP treatments at or above 0.1 µM resulted in
significantly decreased survival compared to control cultures ($p < 0.05$). Survival after all of
the antagonist treatments was not statistically different from the control cultures.
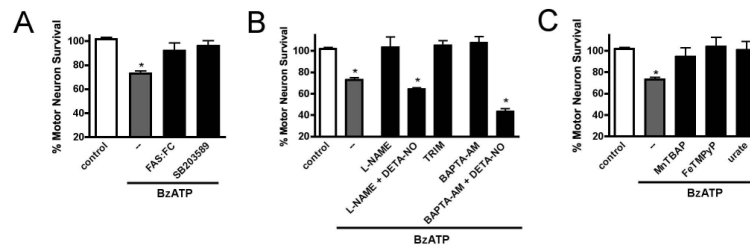
Fig. 3. BzATP triggered autocrine FAS/p38/nNOS signaling
(A) Motor neuron death triggered by BzATP (10 μM) was prevented by SB203580 (10 μM) and FAS:FC. (B) L-NAME (100 μM), TRIM (1 μM) and BAPTA-AM (1 μM) prevented motor neuron death initiated by BzATP, while addition of DETA-NO (10 μM) reinstated death. (C) Motor neuron death caused by BzATP was prevented by addition of MnTBAP (10 μM), FeTMPyP (10 μM) or urate (200 μM). * p<0.05, significantly different from control.
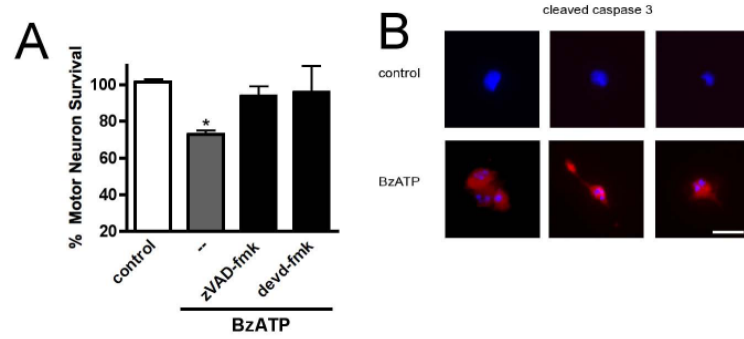
Fig. 4. BzATP triggered caspase-dependent apoptosis
(A) Caspase inhibitors zVAD-fmk and devd-fmk (10 μM) prevented motor neuron death triggered by BzATP (10 μM). (B) Immunofluorescence of cultured motor neurons for cleaved caspase 3 (red), apparent only in motor neurons treated with BzATP. Nuclear morphology was evident with DAPI staining (blue). * p<0.05, significantly different from control. The scale bar represents 20 μm.
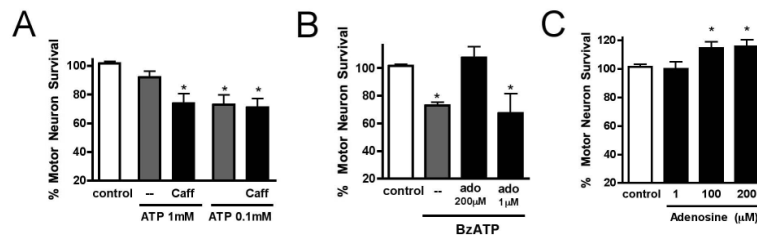
Fig. 5. High concentrations of adenosine produced by ATP degradation can prevent BzATP-induced motor neuron death

(A) Motor neuron survival in response to the indicated concentrations of ATP (1mM and 100 μM) in presence or absence of caffeine (0.5 μM). (B) Adenosine at a concentration of 200 μM but not at 1 μM prevented motor neuron survival induced by BzATP (10 μM) (C) Basal motor neuron survival increased in response to 1, 100 and 200 μM adenosine. * p<0.05, significantly different from control.