

Effectors of large-conductance calcium-activated potassium channel modulate glutamate excitotoxicity in organotypic hippocampal slice cultures

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Mitochondria have been suggested as a potential target for cytoprotective strategies. It has been shown that increased K⁺ uptake mediated by mitochondrial ATP-regulated potassium channels (mitoK_{ATP} channel) or large-conductance Ca²⁺-activated potassium channels (mitoBK_{Ca} channel) may provide protection in different models of cell death. Since recent findings demonstrated the presence of BK_{Ca} channels in neuronal mitochondria, the goal of the present study was to test the potential neuroprotective effects of BK_{Ca} channel modulators. Using organotypic hippocampal slice cultures exposed to glutamate, we demonstrated that preincubation of the slices with the BK_{Ca} channel opener NS1619 resulted in decreased neuronal cell death measured as reduced uptake of propidium iodide. This neuroprotective effect was reversed by preincubation with the BK_{Ca} channel inhibitors paxilline and Iberiotoxin (IbTx). Moreover, mitochondrial respiration measurements revealed that NS1619 induced an IbTx-sensitive increase in state 2 respiration of isolated brain mitochondria. In addition, electrophysiological patch-clamp studies confirmed the presence of BK_{Ca} channels in mitoplasts isolated from embryonic hippocampal cells. Taken together, our results confirm presence of BK_{Ca} channel in rat hippocampal neurons mitochondria and suggest putative role for mitoBK_{Ca} in neuroprotection.

Key words: potassium channel, glutamate, hippocampal slice cultures, mitochondria, respiration, patch-clamp

INTRODUCTION

Mitochondria, the key regulators of cellular metabolism, are involved in ATP synthesis and the accumulation of cytoplasmic calcium ions, as well as in reactive oxygen species (ROS) synthesis (Feissner et al. 2009). It is also known that mitochondria play a central role in the initiation of the intrinsic apoptotic signaling cascade. This dual function makes mitochondria particularly interesting in the context of cytoprotective strategies (Halestrap et al. 2007, Busija et al. 2008, Murphy and Steenbergen 2007). Potential targets for neuroprotective intervention include the permeability transition pore (PTP) and uncoupling proteins. In addition, recent studies have implicated mitochondrial potassium channels in the mechanism of preconditioning, a promising approach for cytoprotection (Facundo et al. 2006, Szewczyk et al. 2009, 2010, Szabo and Zoratti 2014).

A growing body of evidence has demonstrated that ischemic preconditioning, a phenomenon in which brief episodes of ischemia increase tissue tolerance to subsequent lethal insult, could be mimicked by administration of

potassium channel openers (KCOs) (O'Rourke 2007). In contrast to the initial understanding of plasma membrane potassium channels as major mediators of preconditioning, it is now thought that the primary cytoprotective effects are derived from potassium channels in the inner mitochondrial membrane (Szewczyk and Marban 1999, O'Rourke 2007). Increased potassium transport into brain mitochondria catalyzed by mitochondrial ATP-regulated potassium channels (mitoK_{ATP} channels) has been shown to affect mitochondrial membrane potential and the rate of respiration, leading to subsequent beneficial effects on neuronal survival (Rodriguez-Pallares et al. 2009, Watanabe et al. 2008, Gaspar et al. 2008a, Raval et al. 2007). In addition, recent research targeted mitochondrial large-conductance Ca²⁺-regulated potassium channels (mitoBK_{Ca} channel) for cardioprotective strategies (Xu et al. 2002).

Large-conductance Ca²⁺-activated potassium channels (BK_{Ca}) were originally identified in the plasma membranes of smooth muscle cells, neurons and chromaffin cells as well as in the kidney and inner ear (Grimm and Sansom 2007, Eichhorn and Dobrev 2007). Due to their sensitivity to changes in both membrane potential and calcium ions, BK_{Ca} channels were

found to be crucial for processes such as neurotransmitter release, cell excitability and smooth muscle tone (Latorre and Brauchi 2006, Facler and Adelman 2008).

Further studies revealed that BK_{Ca} channels are also present in mitochondria (Siemen et al. 1999, Xu et al. 2002). The activity of the putative mitochondrial BK_{Ca} channel (mitoBK_{Ca}), thought to be stimulated by Ca²⁺ and blocked by charybdotoxin, was initially detected in mitoplasts from a glioma cell line (Siemen et al. 1999). Later, similar single-channel recordings were performed for the inner membranes of heart, skeletal muscle, brain and endothelium mitochondria (Xu et al. 2002, Skalska et al. 2008, 2009, Douglas et al. 2006, Fahnrik-Babaei et al. 2011, Bednarczyk et al. 2013a). The BK_{Ca} channel opener NS1619 was found to increase K⁺ influx of intact heart mitochondria in a way that was sensitive to BK_{Ca} channel blockers, charybdotoxin and Iberitoxin (Xu et al. 2002). Furthermore, it has been reported that pharmacological preconditioning using NS1619 led to heart protection against infarction (Xu et al. 2002). The mechanism of this process seems to be partially related to the reduced driving force for mitochondrial Ca²⁺ influx or to superoxide radical synthesis (Sato et al. 2005, Kulawiak et al. 2008). In last time, it was shown that substrates of the respiratory chain can decrease the activity of the mitoBK_{Ca} channel and the effect is abolished by inhibitors of the respiratory chain. The putative interaction of the β 4 subunit of mitoBK_{Ca} with cytochrome c oxidase was demonstrated using blue native electrophoresis. These findings indicate functional and structural coupling of the mitoBK_{Ca} channel with the mitochondrial respiratory chain in human astrocytoma U-87 MG cells (Bednarczyk et al. 2013b).

Evidence was found for the expression of BK_{Ca} α and β 4 subunits in the inner membrane of neuronal mitochondria (Piwonska et al. 2008). The identification of BK_{Ca} channels in brain mitochondria led to speculation about the potential role of this channel in neuroprotection. Thus, the goal of the present study was to investigate the effects of BK_{Ca} channel modulators on neuronal damage in organotypic hippocampal slice cultures exposed to glutamate.

METHODS

Organotypic hippocampal cultures

Organotypic hippocampal slice cultures (OHC) were prepared from 7- to 9-day-old Wistar rats (P7-9) using a modification of the interface method previously described (Stoppini et al. 1991). After decapitation, the brain was removed, the hippocampi were dissected and 350 μ m thick transverse sections were cut on a McIlwain tissue chopper (The Mickle Laboratory Engineering, Guildford, UK). The

slices were carefully separated and transferred to sterile, 0.4 μ m porous Millicell membranes (Millipore, Molsheim, France) placed in 6-well plates containing serum-based medium [74% culture medium (HME03; Cell Concept GmbH, Umkirch, Germany), 0.5% L-glutamine (Biochrom, Berlin, Germany), 0.5% gentamycin (Biochrom), 25% horse serum (Gibco, Eggenstein, Germany)]. For the following 3 days, the cultures were maintained at 37°C in 5% CO₂, with a medium change performed on the first day *in vitro* (DIV). Thereafter, cultures were transferred to serum-free medium [74% culture medium (HME03; Cell Concept GmbH), 0.5% L-glutamine (Biochrom), 0.5% gentamycin (Biochrom), 25% Neurobasal-A (Gibco) with B27 supplement (Gibco)] and were kept in a humidified incubator at 33°C in 5% CO₂ with the medium changed three times per week.

Slice cultures were used for experiments after 12 DIV. Before the experiment, the slices were stained with propidium iodide (PI) (2 μ M; Sigma, Deisenhofen, Germany) to exclude damaged cultures. PI was added to the culture medium for 12 h, and PI-negative OHCs were selected using a fluorescence microscope (Eclipse TE 300, Nikon, Japan).

Glutamate excitotoxicity

Glutamate excitotoxicity was assessed by 45-minute exposure of the slices to 15 mM glutamate (Sigma) at 37°C followed by 23-hour incubation in glutamate-free medium (serum-free medium) at 33°C. Control cultures were maintained for the same time interval at 37°C in serum-free medium and then returned to their original culture conditions at 33°C for the next 23 h.

Pharmacological treatment

Contribution of NMDA receptor activation to glutamate-induced neuronal death was tested using the glutamate receptor antagonist (5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801; 10 μ M; Tocris, USA). MK-801 was applied 2 h before excitotoxicity induction and remained in the medium (see Organotypic hippocampal cultures) during the insult and after glutamate withdrawal.

To test the effects of BK_{Ca} channel modulators on the viability of slices exposed to glutamate, the BK_{Ca} channel activator NS1619 (10 nM–30 μ M; Sigma) as well as BK_{Ca} channel inhibitors paxilline (Pax) (2 μ M; Tocris) and Iberitoxin (IbTx) (100 nM; Alomone Labs, Israel) were used. The compounds were applied 2 h before glutamate exposure, and they remained in the medium (see Organotypic hippocampal cultures) during the insult and after glutamate withdrawal. Because NS1619 and paxilline were pre-dissolved in DMSO (final

concentration 0.01%), an additional group of slices treated with DMSO alone was included.

In the experiments investigating the temporal profile of the effects induced by NS1619 and paxilline, test agents were applied to the cultures for 2 h prior glutamate exposure, for 45-min co-incubation with glutamate or for 23 h just after glutamate withdrawal.

Quantification of neuronal death

Neuronal damage was evaluated by cellular uptake of PI. At 22 h after glutamate application, PI (10 μ M) was added to the medium for 2 h at 33°C. For detection of PI fluorescence, slices were excited with light at 510–560 nm, and the emitted signal was acquired at 610 nm using an inverted fluorescence microscope (Eclipse TE 300; Nikon, Japan). Fluorescent and transmission images were captured using a CCD camera (Visitron Systems, Puchheim, Germany) and were analyzed using LUCIA image analysis software (Nikon, Japan). The area of analysis for each culture was determined based on transmission images. The neuronal damage was quantified as the percentage of CA1-CA3 subfields expressing PI fluorescence above the background level and was calculated in relation to the total slice area. The value obtained for slices exposed to glutamate alone was defined as the standard damage and normalized to 100% damage. All other data are given as the percentage of standard damage. Normalized values of neuronal cell death obtained from at least three independent experiments are given as mean $\pm$ SEM. The Mann-Whitney U test was used to compare neuronal damage of OHCs treated with BK_{Ca} channel modulators to the standard damage (100% damage).

Immunostaining of rat brain sections

For immunohistochemical studies, adult male Wistar rats (150–200 g) were transcardially perfused with 4% paraformaldehyde (PFA). The brains were removed, immersed in the same fixative for 24 h and then washed in phosphate buffered saline (PBS). Then, the tissue was processed for paraffin embedding. Paraffin-embedded coronal 4- μ m thick sections were dewaxed, rehydrated and incubated with 3% hydrogen peroxide for 30 min. For antigen retrieval, the sections were heated in a microwave oven in citrate buffer (pH 6.0) for 2 $\times$ 7 min. Nonspecific antibody binding sites were blocked by preincubating the sections with 5% normal donkey serum (NDS) diluted in PBS for 30 min. For the simultaneous detection of the BK_{Ca} β 4 subunit and the neuronal marker microtubule associated protein 2 (MAP2), sections were incubated overnight at 4°C in 5% NDS containing rabbit polyclonal anti-BK_{Ca} β 4 subunit (KCNMB4) antibody (1:50 dilution, Alomone Labs) and mouse

monoclonal anti-MAP2 antibody (1:100 dilution, Chemicon). The immunoreactivity was visualized using AlexaFluor 488-conjugated donkey anti-mouse antibody and AlexaFluor 555-conjugated donkey anti-rabbit antibody (Molecular Probes). The sections were then mounted in VECTASHIELD HardSet Mounting Medium with DAPI (Vector). Confocal images were acquired using a Leica TCS SP2 microscope with Ar (488 nm) and GeNe (543 nm) lasers applied to the excitation of AlexaFluor 488 and AlexaFluor 555, respectively. To avoid cross-talk between the two fluorophores, special care was taken to adjust detector settings, and the images were sequentially scanned.

Isolation of rat brain mitochondria

Rat brain mitochondria were prepared according to a standard protocol. In brief, each male Wistar rat was anesthetized with chloroform and then sacrificed by cervical dislocation. The brain was rapidly removed, washed and placed in ice-cold buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, BSA (1 mg/ml), 1 mM EGTA, pH 7.4. The tissue was then minced with scissors, placed in 10 ml of isolation medium supplemented with nargarse (0.5 mg/ml) and then homogenized using a motor-driven Teflon-glass Potter homogenizer. The homogenate was diluted two times. After then, the homogenate was centrifuged at 2,000 g for 9 min. The supernatant was decanted and centrifuged at 12,000 g for 11 min. To permeabilize synaptosomes, the pellet was suspended in an isolation buffer supplemented with digitonin (0.2 mg/ml) and homogenized manually. Finally, the suspension was centrifuged at 12,000 g for 11 min. The crude mitochondrial pellet was resuspended in isolation medium at a protein concentration of 20–40 mg/ml. All procedures were carried out at 4°C.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured at 25°C using Oroboros oxygraph (Anton Paar, Austria) in a medium containing 10 mM KH₂PO₄, 60 mM KCl, 60 mM Tris, 110 mM mannitol and 0.5 mM EDTA at pH 7.4. For each measurement, 12 μ l of mitochondrial suspension was added to 2.1 ml of medium supplemented with 5 mM MgCl₂ and the respiratory substrates (5 mM malate and 10 mM glutamate). State 3 respiration was induced by the addition of 2 mM ADP.

Preparation of mitochondria from embryonic rat hippocampal neurons

Pregnant female Wistar rats (18–20 days of pregnancy) were euthanized using CO₂. Embryos were removed from the uterus and decapitated. The brains were dissected out and

placed in cold Hank's balanced salt solution medium (HBSS) supplemented with 1 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptomycin solution (Invitrogen, Paisley, UK). After isolation of hippocampi under a dissecting microscope, the tissue was rinsed with cold HBSS and digested at 37°C in HBSS medium supplemented with 0.2% trypsin (Invitrogen) for 15 min. The trypsin solution was gently pipetted off, the tissue was rinsed with warm HBSS and the cells were dissociated by trituration. After sedimentation of non-dispersed tissue, the supernatant was transferred to an Eppendorf tube and centrifuged at 5,000 g for 10 min. The pellet was resuspended in a preparation buffer containing 250 mM sucrose and 5 mM HEPES at pH 7.2 and homogenized using a glass-glass homogenizer. Then, to remove synaptosomes, the resultant homogenate was centrifuged at 9,200 g for 10 min. The pellet was resuspended in preparation buffer and centrifuged at 790 g for 10 min. Next, the supernatant containing purified mitochondria was centrifuged at 9,200 g for 10 min and the resultant pellet was resuspended in storage buffer containing 150 mM KCl and 10 mM HEPES at pH 7.2 centrifuged again under the same conditions. The final mitochondrial pellet (crude) was resuspended in 500 µl of isotonic solution containing 150 mM KCl, 10 mM HEPES, 100 µM CaCl₂, pH 7.2. All procedures were carried out at 4°C (Bednarczyk et al. 2010).

Patch-clamp experiments

Patch-clamp experiments were carried out essentially as previously described (Cheng et al. 2008, Bednarczyk et al. 2010). In brief, mitoplasts were prepared from mitochondria isolated from embryonic rat hippocampal neurons. To induce mitochondrial swelling and rupture of the outer membrane, samples of the mitochondrial suspension were transferred into a hypotonic solution containing 5 mM HEPES and 100 µM CaCl₂ at pH 7.2 for about 1 min. Then, addition of a hypertonic solution that consisted of 750 mM KCl, 30 mM HEPES and 100 µM CaCl₂ restored isotonicity.

The patch-clamp pipette was filled with an isotonic solution containing 150 mM KCl, 10 mM HEPES, and 100 µM CaCl₂ at pH 7.2. To apply substances to the ion channels present in the patch we used the method of back-filling. The calcium dependence of the channel activity was analyzed using a low calcium solution containing 150 mM KCl, 10 mM HEPES, 1 mM EGTA, and 0.752 mM CaCl₂ at pH 7.2. The sensitivity of the single-channel current to the BK_{Ca} channel inhibitor was tested using 10 µM paxilline. Isotonic solution was used as a control solution.

The single-channel currents were recorded using an Axopatch 200B amplifier (Molecular Devices, USA). The currents were low-pass filtered at 1 kHz and sampled at a frequency of 100 kHz using Clampex 10 software (Molecular Devices, USA). Recording was performed in

mitoplast-attached mode using a pipette of borosilicate glass (Harvard Apparatus, UK) pulled by a Flaming/Brown Micropipette Puller (P-97, Sutter Instruments, USA) with a resistance of 10–20 MΩ measured in the isotonic solution.

Collected data were analyzed using Clampfit 10 software (Molecular Devices, USA). The channel recordings illustrated in the results section are representative of the most frequently observed conductance under the given conditions. The conductance was calculated from the current-voltage relationship. The open probability as well as the mean time of channel closure and opening was determined using the single-channel search mode of the Clampfit 10 software. Data from the experiments are reported as mean value ±SD.

RESULTS

Effects of BK_{Ca} channel modulators on neuronal cell death induced by glutamate in organotypic hippocampal slice cultures

A 45-min exposure of hippocampal slice cultures to 15 mM glutamate resulted in pronounced cell death in the neuronal cell layers as assessed by propidium iodide uptake (Fig. 1A). Basal PI fluorescence was very low (Fig. 1A; control) but increased significantly 24 h after the insult (Fig. 1A; Glu). Application of the NMDA receptor blocker (10 µM MK-801) resulted in decreased PI uptake (Fig. 1A; MK-801) and reduced neuronal cell death by 80.9±2% (n=50) (Fig. 1B; MK-801).

Concentration-dependent effects of the BK_{Ca} channel opener NS1619 on the viability of hippocampal slices exposed to glutamate

To test whether BK_{Ca} channel openers could modulate the effects of glutamate, we applied NS1619 in a concentration range that varied from 10 nM to 30 µM. Using an experimental protocol with 2-h pre-exposure of the slices to NS1619 followed by 45-min incubation with 15 mM glutamate in the presence of NS1619 and its subsequent application after glutamate withdrawal (Fig. 1C), we found a neuroprotective effect for the BK_{Ca} channel opener. This effect was observed for high drug concentrations and was particularly pronounced at 10 µM NS1619 (Fig. 1D). As depicted in Fig. 1D, slice cultures exposed to glutamate in the presence of 10 µM NS1619 showed 49.7±5% (n=20) reduction in neuronal cell death as compared to cultures exposed to glutamate alone. Statistically significant decreases in neuronal damage were also detected at 30 µM and 100 nM NS1619. At these concentrations, neuronal cell death was reduced by 43.7±1% (n=8) and 36.3±4% (n=19), respectively (Fig. 1D). Application of 1 mM and 10 nM NS1619 did not significantly affect culture viability (Fig. 1D).

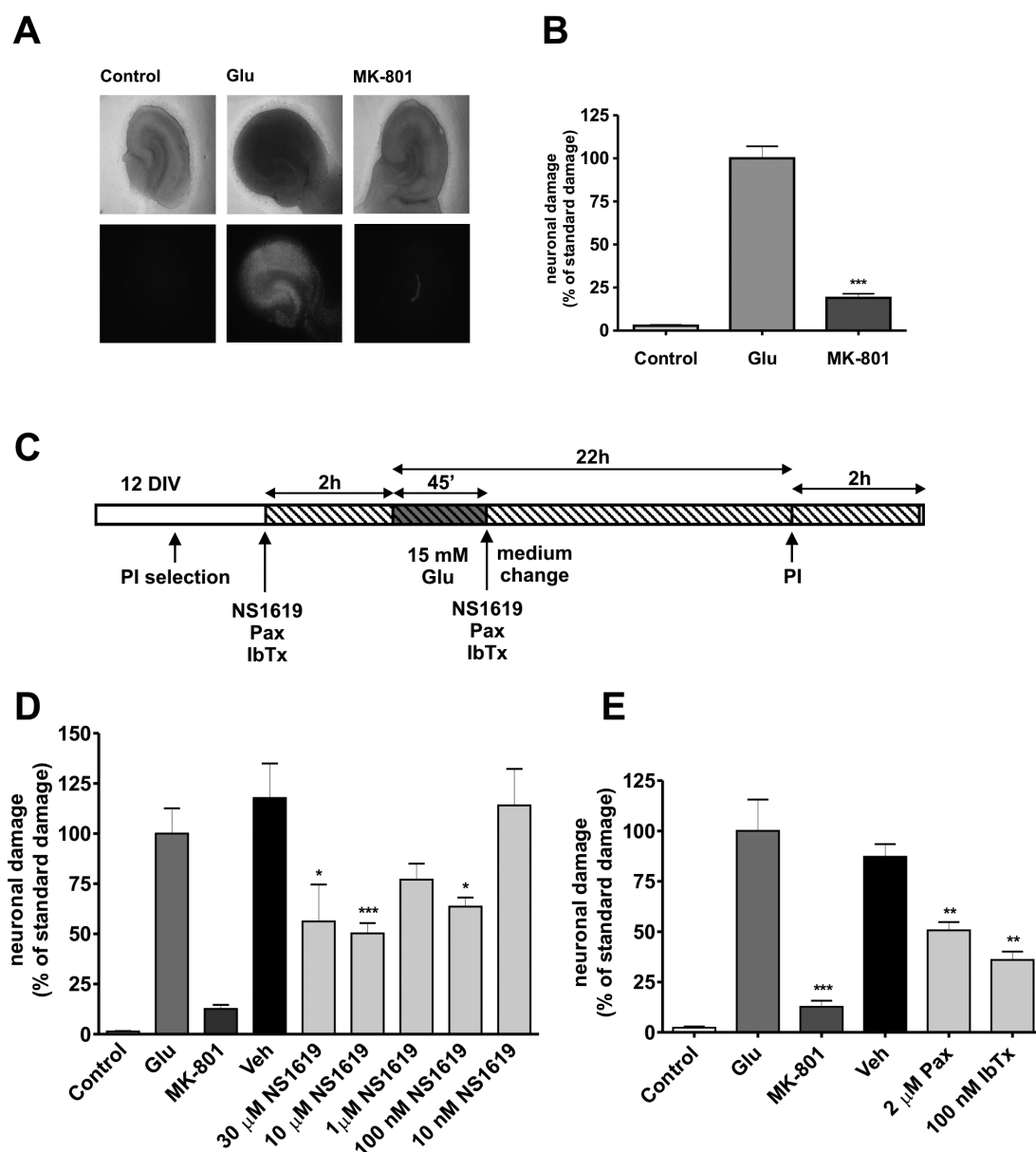


Fig. 1. Effects of BK_{Ca} channel modulators on glutamate excitotoxicity induced in hippocampal organotypic slice cultures. (A) Representative transmission and fluorescent light images of organotypic hippocampal (OHC) slice cultures under control and excitotoxic conditions. OHCs prepared from P7–9 rats were cultivated for 12 DIV and, after selection with PI, were exposed for 45 min to 15 mM Glu. MK-801 was used as a positive control for neuroprotection. OHCs were treated with MK-801 (10 μ M) for 2 h before, during and 24 h after glutamate exposure. (B) Statistical analysis of neuronal damage measured as PI uptake in control cultures (Control), or in cultures exposed to glutamate alone (Glu) or in combination with the NMDA receptor antagonist MK-801 (MK-801). The data are expressed as the mean $\pm$ SEM; *** P < 0.001 vs. glutamate (Mann-Whitney U-test). (C) Experimental protocol of OHC treatment with BK_{Ca} channel modulators under excitotoxic conditions. 12 DIV PI-negative OHCs were treated with the BK_{Ca} channel activator NS1619 (10 nM–30 μ M) or inhibitors paxilline (Pax; 2 μ M) or Iberitoxin (IbTx; 100 nM) for 2 h before, during and 23 h after glutamate exposure. (D) Concentration-dependent effects of BK_{Ca} channel opener NS1619 on the viability of OHCs exposed to glutamate. The most pronounced neuroprotective effect of NS1619 was observed at 10 μ M. The data are expressed as the means $\pm$ SEM of three independent experiments; *** P < 0.001, * P < 0.1 vs. glutamate (Mann-Whitney U-test). Experimental media (Veh) were used as described in Methods sections (Organotypic hippocampal cultures and Pharmacological treatments). (E) Effects of BK_{Ca} channel inhibitors on neuronal cell death induced by glutamate. Quantification of neuronal damage showed neuroprotective effects of BK_{Ca} channel blockers paxilline (Pax; 2 μ M) and Iberitoxin (IbTx; 100 nM). The data are expressed as the means $\pm$ SEM of three independent experiments; *** P < 0.0001, ** P < 0.01 vs. glutamate (Mann-Whitney U-test). Experimental media (Veh) were used as described in Methods sections (Organotypic hippocampal cultures and Pharmacological treatments).

Effects of BK_{Ca} channel inhibitors on neuronal cell death induced by glutamate

Next, we tested the effects of BK_{Ca} channel inhibitors on glutamate-induced neuronal damage. Paxilline (Pax; 2 μ M) and Iberiotoxin (IbTx; 100 nM) were applied to the medium 2 h before, during and 23 h after glutamate exposure (Fig. 1C). As shown in Fig. 1E, BK_{Ca} channel blockers were effective for promoting neuroprotection against glutamate-induced excitotoxicity. The PI uptake measurements showed that addition of 2 μ M Pax reduced neuronal cell death by 49.3 $\pm$ 4% (n=11), whereas 100 nM IbTx decreased the cellular damage by 64 $\pm$ 4% (n=6) (Fig. 1E).

Temporal profile of neuroprotective effects induced by BK_{Ca} channel modulators NS1619 and paxilline

In the next step, to determine the application time window crucial for the effects observed for NS1619 and Pax, cultures were preincubated with test agent for 2 h before glutamate exposure (Fig. 2A), co-treated with the compound together with glutamate for 45 min (Fig. 2B), or incubated with the compound for 23 h after glutamate withdrawal (Fig. 2C). As shown in Fig. 2D, the 10 μ M concentration of NS1619 was neuroprotective when applied for 2 h before the insult. Preincubation with NS1619 resulted in a 72 $\pm$ 4% (n=21) reduction in neuronal cell death (Fig. 2D; NS1619 before). In contrast, NS1619 did not affect neuronal viability when it was present during the incubation with glutamate or when it was applied after the insult. In both conditions, do not observed statistically significant changes in neuronal damage (Fig. 2D; NS1619 during, NS1619 after).

Analysis of PI fluorescence in slices treated with 2 μ M paxilline demonstrated that the most pronounced cytoprotective effect was elicited by co-exposure of the cultures to Pax together with glutamate. The presence of the BK_{Ca} channel blocker during the insult resulted in a 75.3 $\pm$ 4% (n=17) decrease in neuronal death (Fig. 2D; Pax during). Moreover, as shown in Fig. 2D, a less pronounced but still statistically significant decrease in slice damage was also found in cultures pretreated with Pax (45.1 $\pm$ 9%; n=16; Pax before). However, since the slices were not washed after the removal of the compound, it seems plausible that this effect may result from the residual presence of Pax during the incubation with glutamate. The significant neuroprotective effect was no longer observed when the cultures were subjected to Pax in the recovery period after glutamate withdrawal (Fig. 2D; Pax after).

Modulation of NS1619-induced neuroprotection by BK_{Ca} channel inhibitors

Next, to determine the contribution of BK_{Ca} channel activation to neuroprotection induced by NS1619, we

tested whether these effects could be modulated by BK_{Ca} channel inhibitors. Having established the drug application time windows crucial for the neuroprotective effects of the BK_{Ca} channel activator and inhibitors, we concentrated on the NS1619-important pretreatment

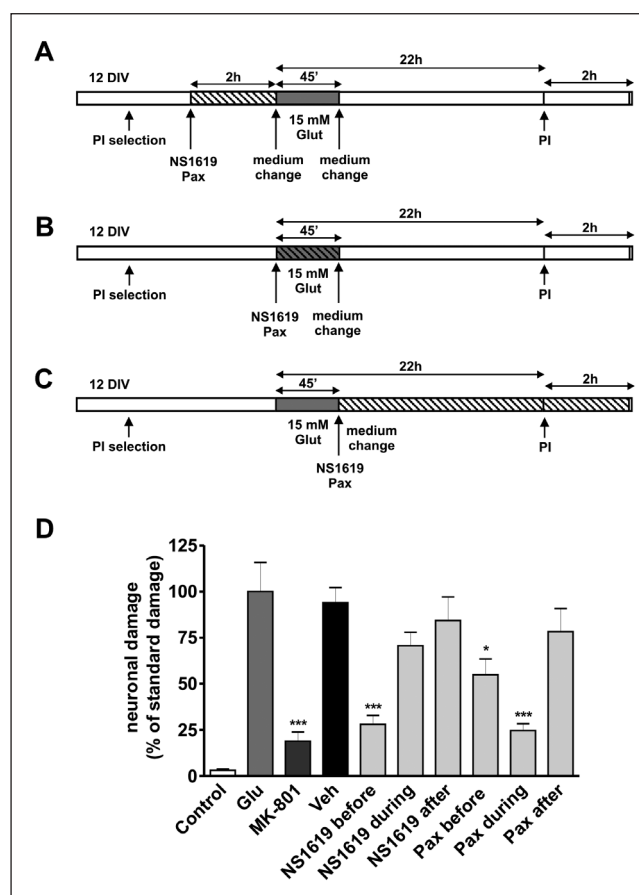


Fig. 2. Temporal profile of neuroprotective effects of BK_{Ca} channel modulators NS1619 and paxilline on neuronal damage induced by glutamate. (A–C) Experimental protocols presenting the time windows of BK_{Ca} channel modulator application under excitotoxic conditions. 12 DIV PI-negative OHCs were treated with NS1619 (10 μ M) or paxilline (2 μ M) for 2 h before excitotoxicity induction (A), during 45-min glutamate exposure (B) or for 23 h after glutamate withdrawal (C). (D) Statistical analysis of time-dependent effects of BK_{Ca} channel modulators on neuronal viability in OHCs exposed to glutamate. Quantification of neuronal damage showed neuroprotective effects of BK_{Ca} channel activator NS1619 when applied for 2 h before glutamate exposure (NS1619 before). The most pronounced cytoprotective effect of the BK_{Ca} channel inhibitor was observed in cultures exposed to paxilline in the presence of glutamate (Pax during). The data are expressed as the means $\pm$ SEM of three independent experiments; ***P<0.001, *P<0.1 vs. glutamate (Mann-Whitney U-test). NS1619 before, Pax before: cultures were exposed to BK_{Ca} channel modulators according to protocol A (2 h before glutamate exposure); NS1619 during, Pax during: cultures were exposed to BK_{Ca} channel modulators according to protocol B (during glutamate exposure); NS1619 after, Pax after: cultures were incubated with BK_{Ca} channel modulators according to protocol C (for 23 h after glutamate withdrawal). Experimental media (Veh) were used as described in Methods sections (Organotypic hippocampal cultures and Pharmacological treatments).

phase for the following experiments. We co-treated the cultures with NS1619 and Pax or IbTx for 2 h before glutamate exposure. Using the protocol presented in Fig. 3A, we were able to exclude the neuroprotective effects elicited by BK_{Ca} channel inhibitors when co-applied with glutamate. We found that pretreatment of the slices with 10 μ M NS1619 resulted in a 72.5 $\pm$ 4% (n=17) reduction in neuronal cell death when compared to the cultures exposed to glutamate alone (Fig. 3B; NS1619). Co-application of 10 μ M NS1619 together with 2 μ M Pax significantly decreased the neuroprotective effect of NS1619 alone, resulting in approximately 52.7 $\pm$ 6% (n=18) neuronal damage (Fig. 3B; NS1619/Pax). As shown in Fig. 3B, the complete reversal of NS1619-induced neuroprotection was assessed using 100 nM IbTx. PI uptake measurements showed that the BK_{Ca} channel activator applied in combination with IbTx non-significantly decreased neuronal damage to 85.2 $\pm$ 6% (n=15) (Fig. 3B; NS1619/IbTx).

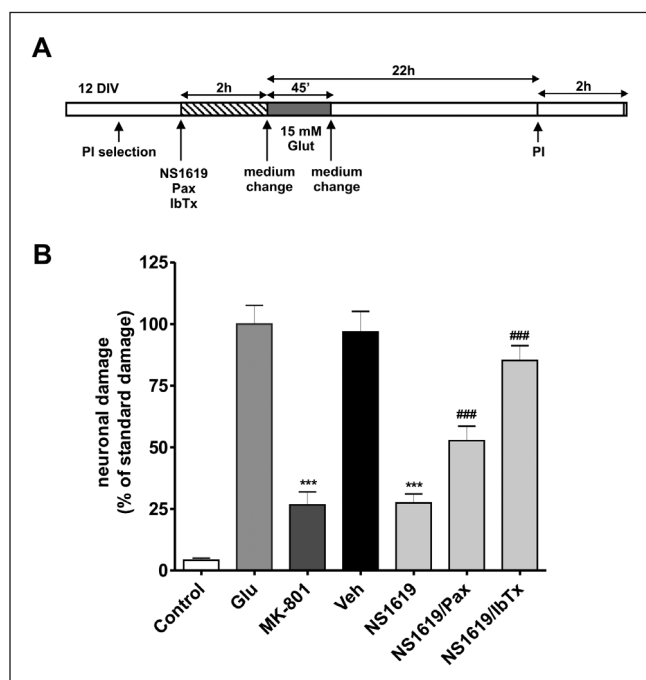


Fig. 3. Modulation of NS1619-induced neuroprotection by BK_{Ca} channel inhibitors. (A) Experimental protocol presenting the time window of BK_{Ca} channel modulator application under excitotoxic conditions. 12 DIV PI-negative OHCs were treated with NS1619 (10 μ M) together with paxilline (Pax; 2 μ M) or Iberitoxin (IbTx; 100 nM) for 2 h before excitotoxicity induction. (B) Statistical analysis of the effects elicited by BK_{Ca} channel inhibitors on NS1619-induced neuroprotection. PI uptake measurements revealed that application of BK channel inhibitors 2 μ M paxilline (NS1619/Pax) or 100 nM Iberitoxin (NS1619/IbTx) partially reversed neuroprotective effects of NS1619 (10 μ M). The data are expressed as the means $\pm$ SEM of three independent experiments; ***P<0.001 vs. glutamate ###P<0.001 vs. NS1619 (Mann-Whitney U-test). Experimental media (Veh) were used as described in Methods sections (Organotypic hippocampal cultures and Pharmacological treatments).

Effects of mitoK_{ATP} channel modulators on neuronal cell death induced by glutamate in organotypic hippocampal slice cultures

To test the hypothesis that mitochondrial potassium influx plays a role in neuroprotection, we tested whether the activation of another mitochondrial potassium channel, the mitoK_{ATP} channel, leads to the similar effects. We pre-treated cultures with 30 μ M BMS191095, which is a specific activator of the mitoK_{ATP} channel (Fig. 4A) (Grover et al. 2001). Analysis of PI fluorescence demonstrated that BMS191095 reduced neuronal damage by 73 $\pm$ 4% (n=17) (Fig. 4B; BMS191095); this was very similar to the effects induced by NS1619. Moreover, 5-HD, an inhibitor of the mitoK_{ATP} channel (Hu et al. 1999), abolished the neuroprotection elicited by BMS191095. As presented in Fig. 4B, co-application of these compounds together resulted in a statistically non-significant 94.1 $\pm$ 6% (n=16) decrease in cellular cell death (Fig.4B; BMS191095/5-HD).

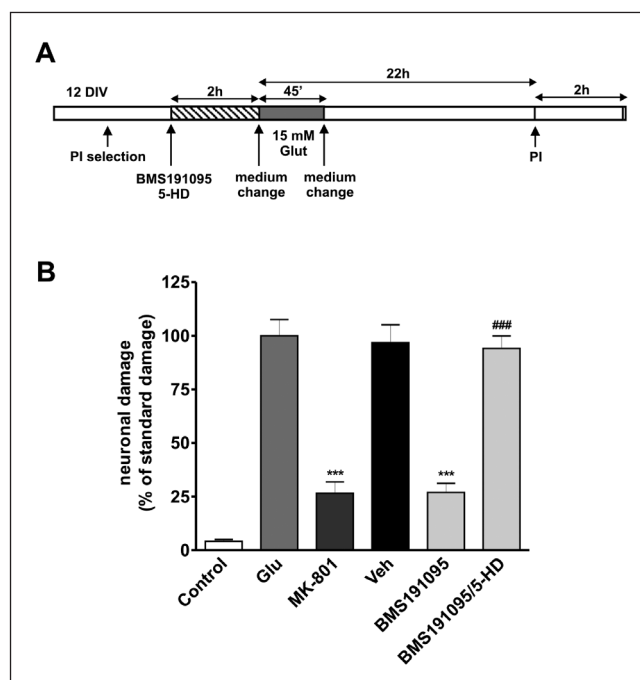


Fig. 4. Effects of mitoK_{ATP} channel modulators on neuronal cell death in OHCs exposed to excitotoxicity. (A) Experimental protocol presenting the time windows of mitoK_{ATP} channel modulator application under excitotoxic conditions. 12 DIV PI-negative OHCs were treated with BMS191095 (30 μ M) and 5-hydroxydecanoic acid (5-HD; 500 μ M) for 2 h before glutamate administration. (B) Statistical analysis of the effects of mitoK_{ATP} channel modulators on neuronal viability in OHCs exposed to glutamate. PI uptake measurements revealed that the mitoK_{ATP} channel activator BMS191095 (30 μ M) protected neurons against glutamate excitotoxicity. This effect was reversed by the mitoK_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD; 500 μ M). The data are expressed as the means $\pm$ SEM of three independent experiments; ***P<0.0001 vs. glutamate ###P<0.0001 vs. BMS191095 (Mann-Whitney U-test). Experimental media (Veh) were used as described in Methods sections (Organotypic hippocampal cultures and Pharmacological treatments).

Immunohistochemical identification of the BK_{Ca} channel β 4 subunit in the rat hippocampus

To determine the distribution of the BK_{Ca} channel in the rat hippocampus, we performed double-labeling of paraffin-embedded rat brain sections with antibodies against the β 4 subunit of BK_{Ca} channel and microtubule-associated protein 2 (MAP-2) as a neuronal marker. As shown in Fig. 5, we observed granular labeling of BK_{Ca} β 4 (Fig. 5A) that closely overlapped with MAP-2 staining (Fig. 5B) in CA3 region of rat hippocampus. Such a pattern was also detected in the main hippocampal cell layers, including the pyramidal cell layer of CA1–CA3 fields as well as the dentate gyrus granule cell layer (data not shown). Analysis of the subcellular localization of BK_{Ca} β 4 in neurons revealed that β 4-immunoreactive clusters were localized to cytochrome c oxidase-positive mitochondria (Piwonska et al. 2008).

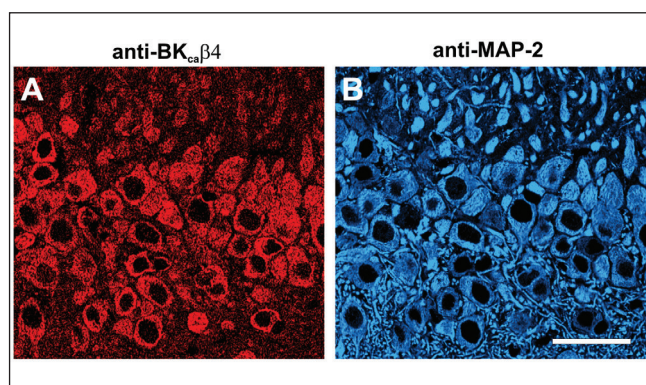


Fig. 5. Immunofluorescent detection of the BK_{Ca} β 4 subunit in the rat hippocampus. (A) Localization of the BK_{Ca} β 4 subunit in the rat hippocampal CA3 region. The majority of BK_{Ca} β 4 subunit immunoreactivity (red) was localized in MAP-2-positive neurons (blue) – see below. (B) Localization of the microtubule-associated protein 2 (MAP-2). Microtubule-associated protein 2 (MAP-2) was used as a neuronal marker. Immunostaining was performed as described in Methods section (see Immunostaining of rat brain sections).

Effects of BK_{Ca} channel modulators on the respiration rate of isolated brain mitochondria

We next analyzed the effects of the BK_{Ca} channel activator on mitochondrial respiration in the presence of glutamate and malate as respiratory substrates. As shown in the representative traces in Fig. 6A, application of 10 μ M NS1619 to a suspension of isolated mitochondria resulted in increased oxygen consumption (oxygen concentration), and an almost 100% increase in the rate of respiration (rate of respiration). The addition of 2 mM ADP induced further stimulation of respiratory chain until the maximal state 3 respiration was reached (Fig. 6A; rate of respiration). Statistical analysis of results obtained in four independent experiments confirmed our observations and revealed that 10 μ M NS1619 increased state 2 respiration by 102.4 $\pm$ 5% over the control (Fig. 6B).

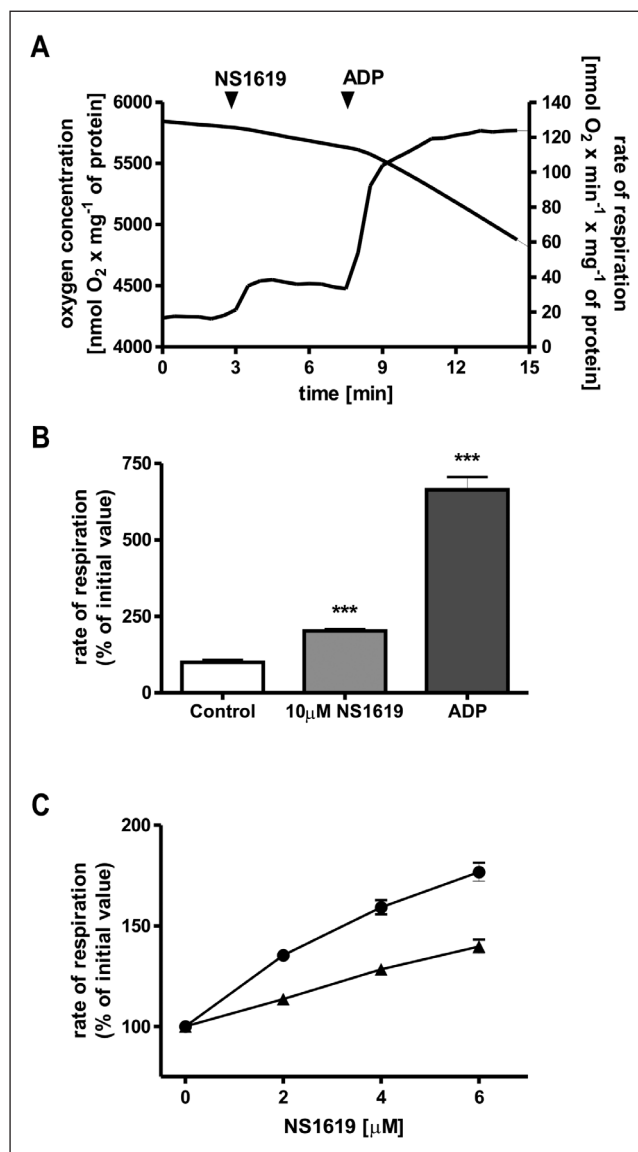


Fig. 6. Effects of BK_{Ca} channel modulators on respiration rate of isolated brain mitochondria. (A) Representative traces presenting the effects of NS1619 on oxygen consumption and respiratory rates of isolated brain mitochondria. The application of NS1619 (10 μ M) to a suspension of rat brain mitochondria increased oxygen consumption and stimulation of respiratory chain. Additions: NS1619 10 μ M; ADP 2 mM. (B) Statistical analysis of the changes in state 2 respiration induced by NS1619 (10 μ M) and ADP (2 mM). The data are expressed as the means $\pm$ SEM of four independent experiments; ***P<0.0001 vs. control; the respiratory rate in the control was set as 100 % and reached a value of approximately 21.4 $\pm$ 2 nmol O₂/min/mg of protein. (C) Effects of Iberiotoxin on NS1619-induced stimulation of mitochondrial respiration. Measurements of respiratory rate were made in potassium medium in the presence of NS1619 alone (2–6 μ M) (circles) or NS1619 (2–6 μ M) in combination with Iberiotoxin (IbTx; 200 nM) (triangles). Statistical analysis revealed that the NS1619-induced increase in respiration rate was partially reversed by IbTx. The data are expressed as the mean $\pm$ SEM of four independent experiments; the respiratory rate in the control was set as 100 % and reached a value of approximately 20.9 $\pm$ 1 nmol O₂/min/mg of protein.

To test whether the stimulation of the respiratory chain by NS1619 was due to mitoBK_{Ca} activation, we analyzed the effects of this compound in the presence of the BK_{Ca} channel inhibitor Iberitoxin. As shown in Fig. 6C, NS1619 accelerated the rate of respiration in a concentration-dependent manner (circles), and these effects were partially reversed by 200 nM IbTx (triangles). The addition of three subsequent aliquots of 2 μ M NS1619 increased state 2 respiration by 35.3 $\pm$ 2% (2 μ M NS1619), 59.2 $\pm$ 3% (4 μ M NS1619) and 76.8 $\pm$ 4% (6 μ M NS1619) over the control (Fig. 6C, circles). The effectiveness of NS1619 was significantly reduced in the presence of the BK_{Ca} channel inhibitor IbTx. As presented in Fig. 6C, co-application of NS1619 with IbTx resulted in the following increases of respiratory rate over the control: 13.6% for 2 μ M NS1619, 28.4 $\pm$ 2% for 4 μ M NS1619 and 39.8 $\pm$ 3% for 6 μ M NS1619 (Fig. 6C, triangles).

Single-channel recordings of the mitoBK_{Ca} channel

To further address the presence of functional mitoBK_{Ca} channels in rat hippocampal mitochondria, we performed patch-clamp experiments on mitoplasts isolated from embryonic hippocampal neurons. Electrophysiological studies revealed the existence of single-channel currents ($n=12$) similar to those of the mitoBK_{Ca} channel previously reported in glioma, cardiac and brain mitochondria. Single-channel recordings obtained at different voltages in isotonic solution are presented in Fig. 7A. The channel conductance, calculated based on current-voltage relationships, was equal to 289 $\pm$ 3 pS. Statistical analysis of the open probability distribution as well as the mean time of channel opening and closure revealed an apparent dependence on holding potential (Fig. 7B). We observed a voltage-dependent increase in the open probability from about 0.09 at -60 mV to 0.95 at +10 mV. Further shifts to positive potentials up to +60 mV did not significantly affect the activity of the channel. Moreover, the mean time of channel opening was low at negative voltages and increased with increasing holding potentials (Fig. 7B; middle panel). As shown in Fig. 7B (right panel), the longest closed states of the channel were recorded at negative potentials. The shift to positive voltages led to decreases in the mean time of closure (Fig. 7B, right panel).

To gain further insight into the properties of the analyzed current, we applied substances known to modulate the activity of the mitoBK_{Ca} channel. Single-channel recordings performed under control and low-calcium conditions revealed the dependence of the channel activity on calcium concentration (Fig. 7C; upper trace). As shown in Fig. 7C (upper trace), after exchanging the control medium for a solution containing

1 μ M Ca²⁺, a significant decrease in channel activity was observed. Moreover, we demonstrated that the single-channel current recorded under control conditions was sensitive to paxilline, a potent inhibitor of the large-conductance calcium-activated potassium channel (Fig. 7C; lower trace). The application of paxilline to the pipette control solution led to irreversible inhibition of the channel activity.

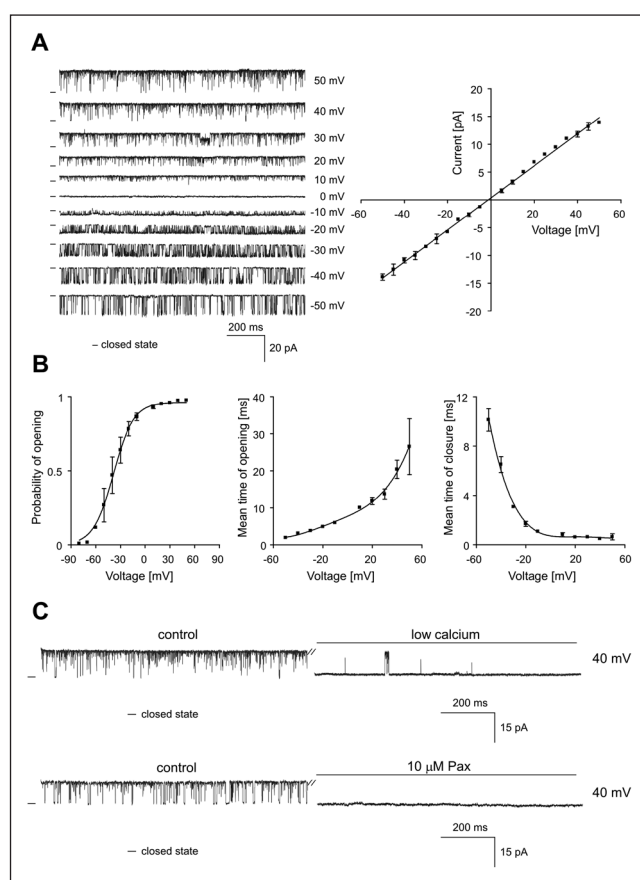


Fig. 7. Electrophysiological identification of the BK_{Ca} channel in mitoplasts isolated from embryonic hippocampal neurons. (A) Single channel recordings at different voltages (left panel) and current-voltage characteristics of single-channel events (right panel) in isotonic solution. The holding potential is indicated to the right. Dash (–) indicates the closed state of the channel. Recordings were low-pass filtered at 1 kHz. (B) Kinetic analysis of mitoBK_{Ca} channel activity recorded at different voltages. Electrophysiological studies revealed voltage dependence of the open probability (left panel), as well as of the mean time of opening (middle panel) and closure (right panel). Data were taken from single channel recordings in isotonic solution. (C) Effect of low calcium and paxilline on the activity of the mitoBK_{Ca} channel. Upper panel shows the dependence of channel activity on calcium concentration. Single-channel currents were recorded in isotonic solution (control) and under low-calcium conditions (low calcium). Lower panel demonstrates the sensitivity of single-channel currents to paxilline. Single-channel recordings of a large-conductance potassium channel in isotonic solution (control) and after addition of 10 μ M paxilline. The holding potential is indicated to the right. Dash (–) indicates the closed state of the channel. Recordings were low-pass filtered at 1 kHz.

Taken together, our data indicate that the observed single channel activity is similar to that of the mitoBK_{Ca} channel previously reported in glioma, cardiac, skeletal muscle and endothelium mitochondria (Siemen et al. 1999, Xu et al. 2002, Skalska et al. 2009, Bednarczyk et al. 2013a).

DISCUSSION

The role of mitochondrial potassium influx in neuroprotection was extensively studied after the beneficial effects of selective mitoK_{ATP} channel activation were demonstrated. In view of recent data showing the presence of BK_{Ca} channel in neuronal mitochondria, we hypothesized that pharmacological preconditioning using BK_{Ca} channel openers could serve as an alternative neuroprotective approach (Piwonska et al. 2008, Skalska et al. 2009). Thus, in the current study we investigated the effects of BK_{Ca} channel modulators on the viability of hippocampal neurons under excitotoxic conditions (Fig. 8). Our findings demonstrated that the BK_{Ca} channel opener NS1619 effectively decreased neuronal cell death induced by glutamate. Moreover, the role of BK_{Ca} channel activation in the mechanism of neuroprotection was confirmed by the inhibitory effect of the BK_{Ca} channel blockers Pax and IbTx. It remains unclear whether the mitochondrial or the plasma membrane BK_{Ca} channel is the major mediator of pharmacological preconditioning. Both blockers affect mitochondrial

channel activity (Bednarczyk et al. 2013a, 2013b), but organotypic hippocampal slice culture (applied in this manuscript) may differentiate access of these modulators to plasma membrane or mitochondrial BK_{Ca} channels.

There are several reports demonstrating that activation of plasma membrane K⁺ channels contribute to preconditioning. Application of the BK_{Ca} channel opener BMS204352 provided significant levels of neuroprotection in stroke and traumatic brain injury models (Gribkoff et al. 2001, Cheney et al. 2001). However, these effects are typically ascribed to the post-injury action of BMS204352. It has been proposed that potentiation of BK_{Ca} channels due to increased K⁺ efflux leads to plasma membrane hyperpolarization, thereby decreasing neuronal excitability. This supports the idea that increased open probability of the plasma membrane BK_{Ca} channel is particularly important during disturbances of energy homeostasis such as hypoxia or ischemia.

In contrast to these results, we found in the current study that neuroprotection induced by NS1619 was observed its presence of this drug in the medium only during preincubation phase. This likely reflects a mitochondrial-dependent preconditioning phenomenon, in which early activation of mitochondrial rather than plasma membrane potassium channels seems to play a crucial role. Consistent with this idea, a previous study performed on knockout mice lacking SUR1-based K_{ATP} channels demonstrated that increased K⁺ efflux across the plasma membrane is not obligatory for neuronal preconditioning induced by transient ischemia or diazoxide (Munoz et al. 2003). On the other hand, the role of mitoK_{ATP} channels was confirmed by data showing inhibition of cytoprotective effects by 5-HD, a selective mitoK_{ATP} channel blocker (Munoz et al. 2003). These findings were additionally strengthened by the observation that targeted expression of Kir6.2 in mitochondria confers protection against hypoxic stress (Ljubkovic et al. 2006). Furthermore, extensive evidence for neuroprotection induced by activation of mitochondrial K⁺ channels was provided by experiments that applied selective mitoK_{ATP} channel modulators. Consistent with these data, we found that preincubation of organotypic hippocampal slices with the mitoK_{ATP} channel opener BMS191095 led to cytoprotective effects similar to those obtained with NS1619 preconditioning. The specificity of BMS191095 action (Grover et al. 2001) was confirmed with 5-HD, a specific inhibitor of the mitoK_{ATP} channel (Hu et al. 1999). Parallel application of 5-HD with BMS191095 lead the total reversal of the neuroprotective effect induced by potassium channel opener of the mitoK_{ATP} channel (Fig. 4B). Since previous results excluded the effects of BMS191095 on neuronal membrane potential (Mayanagi et al. 2007, Gaspar et al. 2008b), it is reasonable to propose that increased mitochondrial K⁺ influx is the key determinant of neuroprotection in our experimental model. Despite the applied model is based on increased Ca²⁺ influx (induced by glutamate) via NMDA receptor.

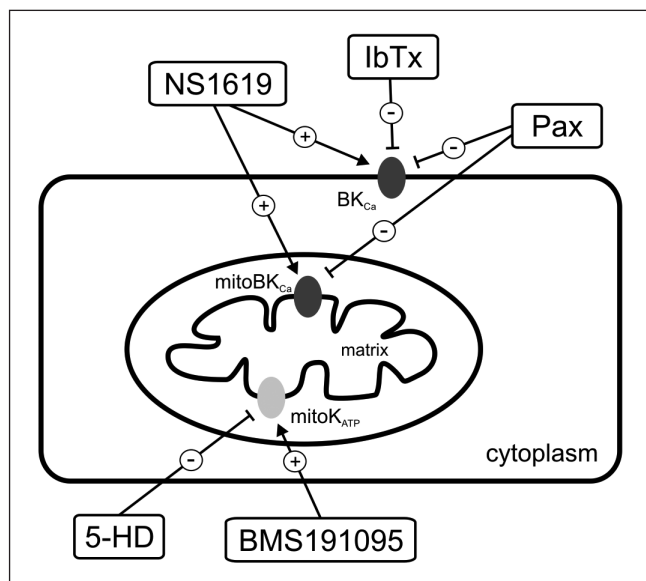


Fig. 8. Targeting of potassium channels effectors (potassium channel opener and blocker) described in this manuscript. It was marked: NS1619 as an activators of mitochondrial large-conductance Ca²⁺-activated potassium channel (mitoBK_{Ca}) and plasma membrane large-conductance Ca²⁺-activated potassium channel (BK_{Ca}); IbTx (iberiotoxin) as an inhibitor of BK_{Ca} channel; Pax (paxilline) as an inhibitor of mitoBK_{Ca} and BK_{Ca} channel. Additionally, 5-HD as an inhibitor and BMS191095 as an activator of mitochondrial ATP regulated potassium channel (mitoK_{ATP}) was shown.

Cytoprotection induced by MK-801, a potent and selective NMDA receptor antagonist, confirms this injury pathway.

This hypothesis is in line with data showing that selective activation of the mitoK_{ATP} channel contributes to increased neuronal viability under excitotoxic conditions (Kis et al. 2004, Gaspar et al. 2008b). Although the detailed mechanism of mitochondrial-targeted preconditioning is still unclear, studies using BMS-191095 have demonstrated that the signaling cascade initiated by mitoK_{ATP} channel opening likely involves mitochondrial depolarization, phosphorylation of protein kinase C (Kis et al. 2004) and activation of the phosphoinositide 3-kinase pathway (Gaspar et al. 2008b). Moreover, it appears that preconditioning neurons with BMS-191095 leads to a subsequent reduction of ROS generation during glutamate exposure (Kis et al. 2004). On the other hand, since the mitochondrial Ca²⁺ overload seems to be an early critical event in the mechanism of excitotoxicity (White and Reynolds 1996, Nieminen et al. 1996, Stout et al. 1998, Pivovarov et al. 2008), the role of mitochondrial K⁺ influx in the attenuation of calcium uptake cannot be excluded (Gaspar et al. 2008b).

However, evidence for a mitoBK_{Ca} channel-independent mechanism of neuronal preconditioning with NS1619 has been provided (Gaspar et al. 2008a). Although NS1619 protected neurons in a concentration-dependent manner, co-application of BK_{Ca} channel blockers did not antagonize this effect. It appears, therefore, that either mitochondrial or plasma membrane BK_{Ca} channel opening was not required to initiate the cytoprotective signaling cascade in this experimental setting. On the other hand, NS1619 was found to depolarize isolated mitochondria and trigger increased ROS production (Gaspar et al. 2008a). The previous data showing nonspecific effects of high concentrations of NS1619 (Debska et al. 2003, Kicinska and Szewczyk 2004, Heinen et al. 2007a, 2007b), may also contribute to cell death/survival. Similar results were previously obtained using potassium channel openers – diazoxide (Nagy et al. 2004). The improvement of neuronal viability was attributed to the inhibitory effects of the mitoK_{ATP} channel opener on the respiratory chain complex II. In support of this hypothesis, neuroprotective effects of 3-NPA, an inhibitor of succinate dehydrogenase, have been reported (Kis et al. 2003).

To support presence of mitoBK_{Ca} channel in brain mitochondria effects of channel modulators on respiration rate was measured. Results, confirms that the channel opener NS1619 increase the influx of potassium ions into mitochondria. Additionally, with the use of the patch-clamp technique we were able to show for the first time single channel activity and biophysical properties of the BK_{Ca} channel in hippocampal mitochondria. Taken together, our results suggest the putative role of the mitoBK_{Ca} channel in neuroprotection.

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