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The Cytoprotective Action of the Potassium Channel Opener BMS-191095 in C2C12 Myoblasts is Related to the Modulation of Calcium Homeostasis

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Key Words
Potassium channel opener BMS-191095 • Mitochondria • Cytoprotection • Calpains • Calcium

Abstract
BMS-191095 is an opener of the mitochondrial ATP-regulated potassium channel, which has been shown to provide cytoprotection in models of ischemia-reperfusion induced injury in various tissues. This study aimed at checking the protective action of BMS-191095 under the conditions of oxidative stress or disruption of intracellular calcium homeostasis. Methods: The cytoprotective potential of BMS-191095 was tested in C2C12 myoblasts injured by treatment with H_2O_2 or calcium ionophore A23187. The influence of the opener on intracellular calcium levels, calpain activity and respiration rates were determined. Results: BMS-191095 protected myoblasts from calcium ionophore A23187-induced injury, but not from H_2O_2-induced injury. A23187-mediated cell damage was also prevented by calpain inhibitor PD 150606. A23187 administration led to a transient increase in cytosolic calcium levels, concomitant activation of calpains and a decrease in state 3 respiration rates, indicating mitochondrial dysfunction. Co-administration of BMS-191095 diminished calpain activation in A23187-treated cells but did not prevent mitochondrial damage. In the presence of BMS-191095, restoration of cytosolic calcium concentrations to basal levels after A23187 treatment was considerably faster which may underly the reduced activation of calpains. Conclusion: The BMS-191095-mediated cytoprotection observed in C2C12 myoblasts results probably from modulation of intracellular calcium transients leading to prevention of calpain activation.

Introduction

The cytoprotective properties of certain potassium channel openers (KCOs), such as diazoxide or pinacidil, have been extensively observed and studied under conditions of ischemia and reperfusion [1]. After the discovery that potassium channels are present also in the inner mitochondrial membrane and finding that they are potential targets for KCOs, the influence of KCOs on mitochondrial function has received considerable attention [2-4]. In the inner mitochondrial membrane several potassium channels have been identified including the ATP-

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regulated potassium channel (mitoK$_{ATP}$) [5], large conductance calcium-activated potassium channel (mitoBK$_{Ca}$) [6] and voltage-dependent potassium channel (mitoKv1.3) [7]. At present, it is broadly accepted that pharmacological preconditioning with KCOs leads to cytoprotection through the activation of potassium channels localised in the mitochondria [8, 9].

Increased permeability of the inner mitochondrial membrane to potassium ions can decrease the mitochondrial membrane potential, increase the respiration rate and induce matrix swelling. Such effects are observed upon KCOs administration [10, 11]. Still, the link between the modulation of mitochondrial function by KCOs and cytoprotection remains unclear. It has been proposed that this phenomenon is a consequence of matrix volume modulation [12], diminished calcium uptake into the mitochondria due to decreased membrane potential [13, 14] or mild stimulation of mitochondrial ROS production leading to the activation of pro-survival signalling pathways [15, 16]. However, the assumption that pharmacological preconditioning with KCOs arises from the opening of mitochondrial potassium channels has been questioned due to the doubtful specificity of available potassium channel modulators [17]. Diazoxide, which has been used in the majority of studies concerning KCOs-mediated cytoprotection, has also channel-independent actions: it has been shown to inhibit succinate dehydrogenase (SDH) [18, 19] and to have protonophoric properties [20]. Thus, it has been suggested that diazoxide-mediated cardioprotection can be attributed to these non-specific activities rather than to effects on mitochondrial potassium fluxes.

BMS-191095 [(3R)-trans-4-(4-chlorophenyl)-N-(1H-imidazol-2-ylmethyl)dimethyl-2H-1-benzopyran-6-carbonitril monohydrochloride] is a mitoK$_{ATP}$ opener that has been reported to be cytoprotective in the conditions of ischemia-reperfusion in cardiac [21], brain [22] and skeletal muscle tissue [23]. The influence of this drug on cardiac mitoK$_{ATP}$ channels was confirmed with functional studies [24], as well as with single channel recordings [25]. Moreover, BMS-191095 is devoid of non-specific activities described for diazoxide such as increased mitochondrial ROS generation attributed to SDH inhibition [26, 27] and vasodilatory activity resulting from activation of plasmalemmal K_{ATP} channels in vascular smooth muscle cells [21]. Therefore, the cytoprotective properties of BMS-191095 strengthen the evidence that the beneficial effects of potassium channel openers on cell survival result from the modulation of potassium flux across the inner mitochondrial membrane. However, BMS-191095 has been until now much less studied than diazoxide and its high selectivity toward mitochondrial potassium channels still needs to be confirmed.

The aim of this study was to compare the cytoprotective potential of BMS-191095 under the conditions of oxidative stress and elevated intracellular calcium levels and to delineate the mechanism underlying the potential cytoprotective action of this potassium channel opener. As a model we used C2C12 myoblasts, in which cell injury was induced by treatment with hydrogen peroxide or with calcium ionophore A23187. The results suggest a link between BMS-191095-mediated cytoprotection and the beneficial effects of the drug on the maintenance of intracellular calcium homeostasis under stress conditions.

Materials and Methods

Reagents

Most of the chemicals used in this study were purchased from Sigma, with the exception of calpain inhibitors PD 150606 and N-acetylleucylleucylnorleucinal - ALLN (Calbiochem), fluo-3-AM (Molecular Probes), foetal bovine serum and L-glutamine (Gibco). BMS-191095 was obtained from Bristol-Myers-Squibb. Stock solutions of BMS-191095 (10 mM), PD150606 (20 mM), ALLN (20 mM), Z-vad-FMK (20 mM), cyclosporine A (1 mM) and A23187 (2 mM) were prepared in DMSO. Sodium 5-hydroxydecanoate was dissolved in water. Directly before the experiments, appropriate dilutions of the chemicals were prepared in the medium used in the particular measurement (HBSS-HEPES or DMEM) and used for cell treatment.

Cell culture conditions

C2C12 murine myoblasts were obtained from ECACC. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% foetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell injury tests and calpain activity assays were performed in culture medium containing 0.1% FBS.

Determination of cell injury levels

Cells grown in 96-well plates (at about 6 x 10^3 cells per well) were treated for 6 h with damaging agents. Then, cell survival was estimated using the lactate dehydrogenase (LDH) release test or the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay.

LDH release was measured using the Cytotoxicity Detection Kit (Roche Molecular Biochemicals). After incubation with tested chemicals, cell medium was collected and cell debris was removed by centrifugation. In parallel, 0.1% Triton X-100 was added to each well to lyse attached cells. LDH activity in cell lysates and in the incubation medium was measured colorimetrically according to the instructions provided by the

producer. The amount of LDH released from the cells was calculated as follows:

% of LDH release = (LDH_{medium} / LDH_{total}) × 100%,

where LDH_{medium} - LDH activity detected in incubation medium, LDH_{total} - sum of the LDH activities detected in incubation medium and in cell lysates.

To test cellular survival using the MTT reduction assay, cells were chemically treated and the cell medium was discarded. To each well, 50 µl of DMEM containing 0.5 mg/ml of MTT was added. After 2 h of incubation, 50 µl of lysis buffer (20% sodium dodecylsulfate, 50% N,N-dimethylformamide, 2% acetic acid and 25 mM HCl) was added to each well and the plates were kept overnight at 37°C. The amount of MTT formazan formed was determined by absorbance measurement at 570 nm. As a reference wavelength, 650 nm was used. The results were normalised against values measured in untreated controls in each of the experiments.

Calpain activity assays

Calpain activity in C2C12 cells was measured with the Calpain-Glo Protease Assay (Promega). The assay detects the summaric activity of calpains, without distinguisning between the particular isoforms. Cells grown in 96-well plates were treated for 2 h with tested substances in the presence of 20 µM of the proluminescent calpain substrate succinyl-LLVY-aminoluciferin. Then cells were lysed by adding Triton X-100 to a final concentration of 1%. To prevent further cleavage of the proluminescent substrate, 50 µM ALLN, an inhibitor of calpain and proteasome, was also added. The lysates were mixed 1:1 with the luciferin detection reagent provided by the manufacturer and after 10 minutes of incubation the amount of the cleaved substrate was assessed by luminescence measurements. The results were normalised against calpain activity detected in untreated controls in corresponding experiment.

The direct effect of BMS-191095 and PD 150606 on the activity of purified µ-calpain from porcine pancreas and on the activity of calpains in C2C12 cells was investigated using the fluorescent probe N-succinyl-LLVY-7-amido-4-methylcoumarin (succ-LLVY-AMC). Calpain-mediated cleavage of the probe was monitored by measuring the increase in fluorescence at λ_{ex} = 380 nm and λ_{em} = 460 nm in presence of 10 µM of succ-LLVY-AMC. The measurement buffer consisted of 120 mM KCl, 5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES and 5 mM EGTA, pH 7.3. For measurement of calpain activity in C2C12 myoblasts, around 0.3 million cells were detached by trypsinisation and suspended in PBS. The suspension was divided into three equal samples that were incubated for 15 minutes with, respectively, 30 µM BMS-191095, 100 µM PD 150606 or in PBS alone (control sample). Afterwards, the cells were permeabilised with digitonin (40 µg/ml) and calpain activity was measured in the constant presence of tested chemicals. The slopes of fluorescence increase were compared to the slope obtained for the control sample and the results were expressed as % of calpain activity detected in control cells.

Single cell measurements of cytosolic calcium levels

Cells grown on coverslips were incubated for 1 h at 37°C in HBSS-HEPES (137 mM NaCl, 0.4 mM Na_2HPO_4, 5.4 mM KCl, 0.4 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 10 mM HEPES, pH 7.4) containing 5.5 mM glucose, 5 µM fluo-3-acetoxymethyl ester and 0.025% pluronic-127. The cells were then washed three times with HBSS-HEPES and the coverslips were placed in an inverted fluorescent microscope (IX 70, Olympus) equipped with a xenon lamp light source with monochromator (Polychrome IV, Till Photonics GmbH) and a CCD camera (Till Imago, Till Photonics GmbH). The experiments were performed in HBSS-HEPES. The images of fluorescence intensities at 488 nm excitation and 510 nm longpass emission were recorded every 2 seconds. Changes in fluo-3 fluorescence in individual cells were analysed with Till Vision software. After background subtraction, the results were normalised against the initial fluorescence levels recorded in the analysed cell.

Respiration measurements

For determination of the respiration rate, C2C12 cells were detached by trypsinisation, suspended in DMEM containing 0.1% FBS, and oxygen consumption in the suspension was monitored with an oxygraph (Oroboros, Oxygraph-2k). For measurement of respiratory control ratios (RCR) the cells were suspended in the measurement buffer (120 mM KCl, 5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES and 5 mM EGTA, pH 7.3) and permeabilised with 40 µg/ml of digitonin. Oxygen consumption was determined in the presence of 10 mM glutamate and 5 mM malate alone (state 2) and after an addition of 1 mM ADP (state 3). RCR was calculated as state 3/state 2 respiration rate.

Measurements of calcium uptake by isolated sarcoplasmic reticulum

Sarcoplasmic reticulum from rat skeletal muscle was isolated as described before [28] with slight modifications. Briefly, back and leg muscles from Wistar rat were homogenized (homogenization buffer: 100 mM NaCl, 5 mM imidazole, 0.1 mM PMSF, pH 7.4) and the debris was removed by two centrifugations at 4,000 x g for 20 min. The supernatant was centrifuged at 110,000 x g for 60 min, and the resulting pellet was further fractionated on a sucrose (w/w) step gradient (45%, 38%, 34%, 32%, 27% sucrose, centrifugation at 110,000 x g for 3 h). The fractions between 32%/34% (Ca^{2+} ATP-ase (SERCA) rich L-SR fraction) and between 38%/45% (H-SR fraction with lower content of SERCA pumps) were collected, diluted with 10 mM HEPES-Tris (pH 7.4), 100 mM NaCl, 0.1 mM PMSF and sedimented for 1 h at 125,000 x g. The pellets were resuspended in storage buffer (10 mM HEPES-Tris, 100 mM NaCl, 250 mM sucrose, 0.1 mM PMSF), aliquoted and stored at -70ºC. The fluorimetric measurements of Ca^{2+} uptake were performed with the fura-2 fluorescent probe at excitation waveleghts 340 nm and 380 nm, and emmission wavelenght 510 nm. Calcium concentration in the measurement medium is reflected by ratio of 340 nm to 380 nm fluorescence. The measurement solution (pH 7.1) consisted of 100 mM KCl, 100 mM sucrose, 20 mM HEPES, 2 mM $MgCl_2$, 2 mM NaN_3, 5 mM potassium oxalate ($K_2C_2O_4$), 2 µM fura-2 and SR (5 µg protein/ml of L-SR or 7.5 µg protein/ml of H-SR). At the beginning of the measurement, an excess of ATP was added, to enable calcium uptake by the SERCA pump.

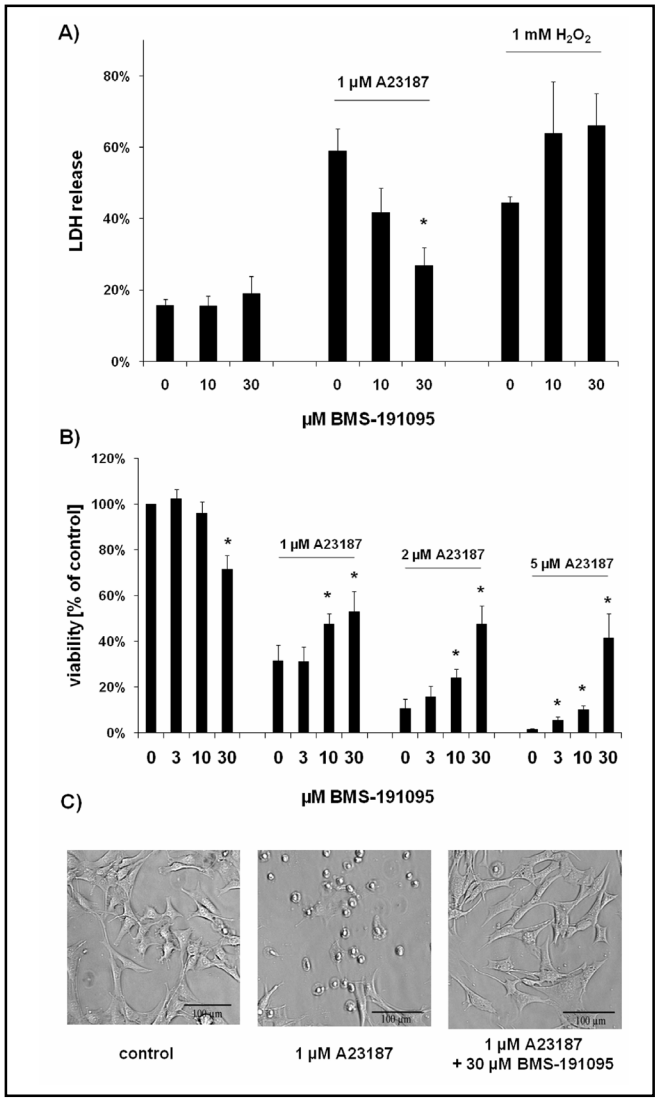

Fig. 1. BMS-191095 protects against A23187-induced cell death in C2C12 cells. Cells were treated for 6 h with the tested substances and cell injury was measured by the LDH release test (A) and MTT test (B). Data are presented as mean values from n = 4-5 independent experiments performed in triplicates ± SEM. * p= 0.05 vs. cells treated with A23187 alone. (C) Morphology of the cells after 6 h of treatment with tested chemicals.

The velocity of Ca^{2+} uptake was observed after addition of 10 µM $CaCl_2$. At the end of each trace 0.1 mM $MnCl_2$ was added, to determine the level of backround fluorescence and background correction was performed.

Statistical analysis

Statistical significance of the results was checked with two-sided Student's t-test (measurements of respiration and cytosolic calcium levels) or paired two-sided Student's t-test (cell injury and calpain activity measurements).

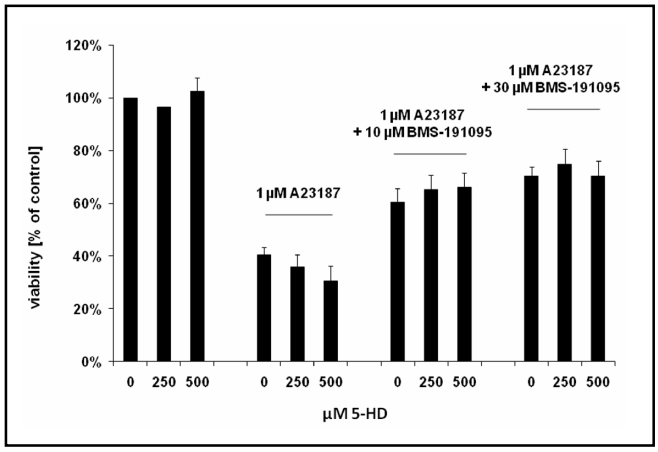

Fig. 2. Cytoprotective effect of BMS-191095 is not abolished by the mitoK$_{ATP}$ inhibitor 5-hyroxydecanoate (5-HD). Cells were treated for 6 h with the tested substances and cell injury was measured by the MTT test. Data are presented as mean values from n = 4 independent experiments performed in triplicates ± SEM.

Results

Cytoprotective properties of BMS-191095 and calpain inhibitor PD 150606 in injured C2C12 cells

We tested the cytoprotective potential of the mitoK$_{ATP}$ channel opener BMS-191095 in C2C12 cells under two different stress conditions: oxidative stress induced by H_2O_2 treatment and disruption of calcium homeostasis caused by calcium ionophore A23187. BMS-191095 was administered 10 minutes before the addition of the damaging agents (H_2O_2 or A23187) and was present throughout the incubation time (6 h). The level of cellular damage was determined with two independent assays: the LDH release test, which measures the loss of cell membrane integrity, and the MTT reduction assay, which estimates the number of viable cells by measuring the activity of mitochondrial dehydrogenases.

Cytoprotection by BMS-191095 was not observed when cellular damage was induced by H_2O_2 (Fig. 1A). In contrast, both tests demonstrated a decrease in A23187-induced injury in the presence of BMS-191095 (Fig. 1). However, the cytoprotective effect of this potassium channel opener was not reversed by 500 µM 5-hydroxydecanoic acid, an inhibitor of mitoK$_{ATP}$ channel (Fig. 2). DMSO alone did not have any protective effects in the applied model. The cytoprotective effect was very strongly pronounced in presence of 30 µM BMS-191095. Therefore such concentration was applied in

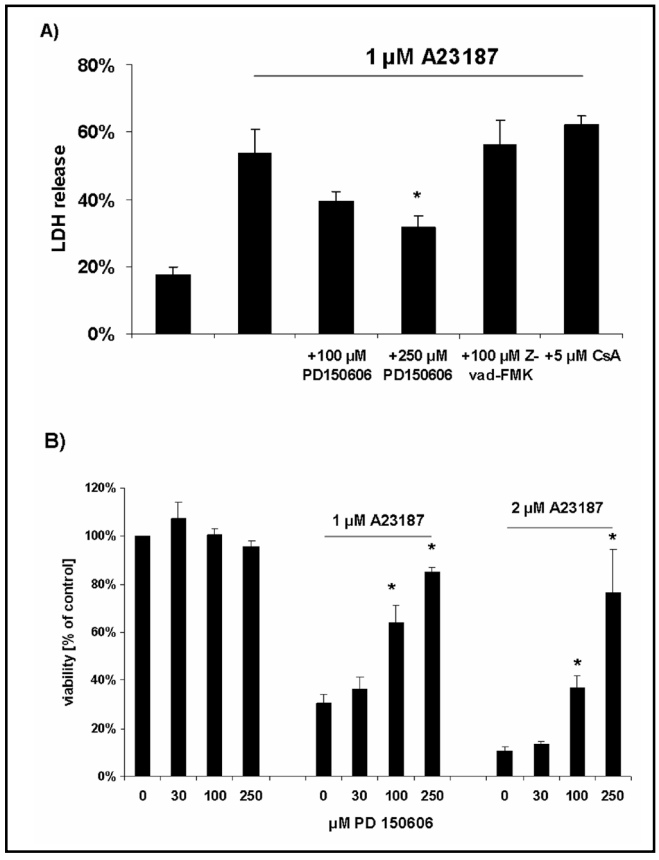

Fig. 3. The calpain inhibitor PD 150606 rescues A23187-induced cell injury in C2C12 cells. Cells were treated for 6 h with A23187 alone or in combination with CsA (5 µM), Z-vad-FMK (100 µM) or PD 150606 (100 µM and 250 µM), and cell viability was measured using the LDH release test (A, n=3). The protective effect observed in presence of calpain inhibitor PD 150606 was confirmed with the MTT test (B, n=4-5). Data are presented as mean values from n = 3-5 independent experiments performed in triplicates ± SEM. * p≤ 0.05 vs. cells treated with A23187 alone.

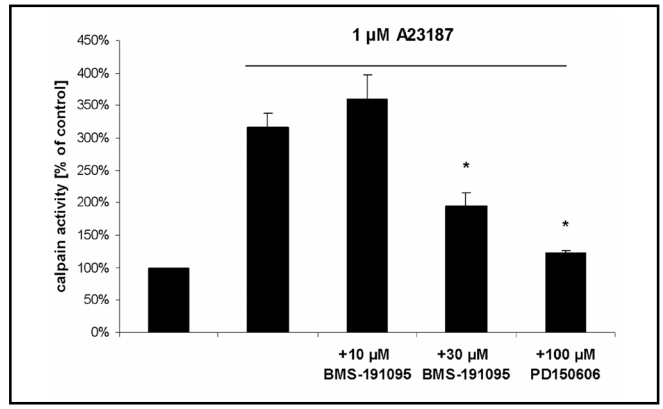

Fig. 4. BMS-191095 and PD 150606 treatments reduce *in vivo* calpain activation in A23187-injured C2C12 myoblasts. Cells were incubated for 2 h with tested substances in presence of succinyl-LLVY-aminoluciferin and the amount of luciferase released was measured. Results are expressed as mean values from n = 4-9 independent experiments performed in triplicates ± SEM. * p≤ 0.01 vs. cells treated with A23187 alone.

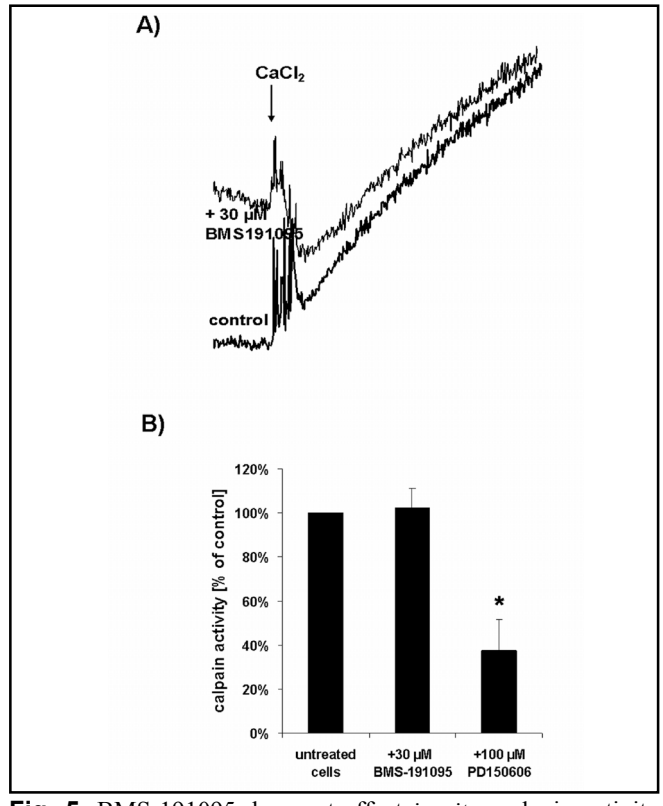

Fig. 5. BMS-191095 does not affect *in vitro* calpain activity measured with a Succ-LLVY-AMC fluorescent probe. (A) Representative traces of measurements of purified µ-calpain (90 nM) activity in the presence of tested chemicals. The enzyme was activated by addition of 10 mM $CaCl_2$ (free calcium concentration: 5 mM). (B) Influence of BMS-191095 and PD 150606 on calpain activity measured in C2C12 cells. Tested substances were present throughout the whole measurement as well as during 15 minutes of preincubation. Results are expressed as mean values ± SEM from n=3 independent experiments. * p≤ 0.01 vs. control cells.

further experiments, despite the fact that 30 µM BMS-191095 alone induced a mild decrease in C2C12 cells viability.

To determine which mechanisms are crucial for cell death observed upon A23187 treatment, we investigated whether A23187-induced cell injury could be limited by the caspase inhibitor Z-vad-FMK, the calpain inhibitor PD 150606 or by cyclosporin A, an inhibitor of mitochondrial permeability transition. As revealed by the LDH-release test, only the calpain inhibitor PD 150606 was protective under the applied conditions (Fig. 3A). The rescue effects of PD 150606 were confirmed by the MTT reduction assay (Fig. 3B).

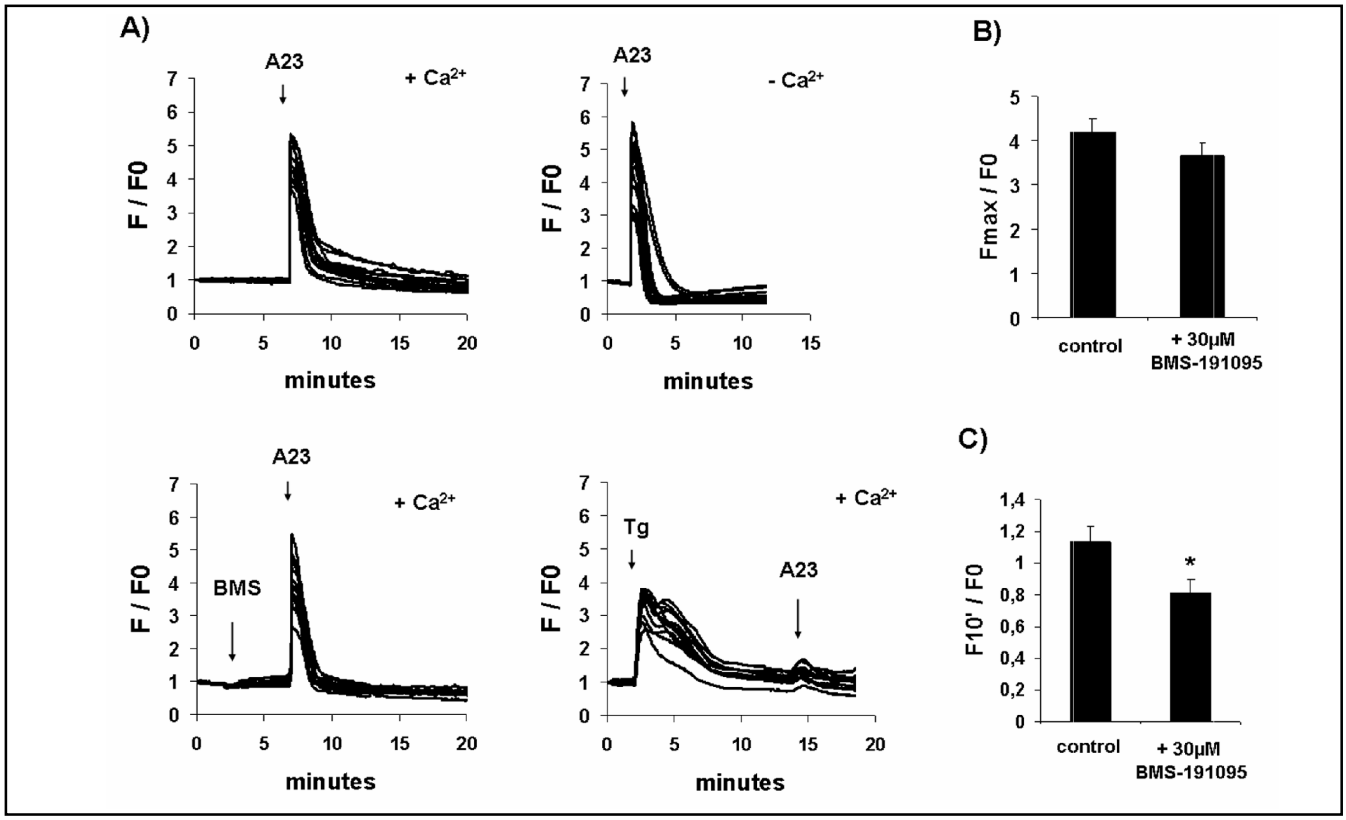

Fig. 6. BMS-191095 reduces A23187-induced intracellular calcium transients. Experiments were performed in calcium-free HBSS ($-Ca^{2+}$) or in HBSS containing 1.8 mM $CaCl_2$ ($+Ca^{2+}$). (A) Representative experiments of monitoring the fluo-3 fluorescence in C2C12 cells. The traces represent fluorescence changes recorded in single cells monitored during one experiment. Arrows indicate the following additions: A23 - 1 μM A23187, BMS - 30 μM BMS-191095, Tg - 1 μM thapsigargin. (B) Maximal increase in intracellular calcium levels observed after addition of 1 μM A23187. (C) Calcium levels registered 10 min. after A23187 addition. The data in figs. B and C are mean values ± SEM derived from n = 8 independent experiments similar those presented in A. In total, 90 individual cells have been analyzed in the control group, and 94 in the BMS-treated group. *$p \leq 0.05$ vs. control experiments.

Influence of BMS-191095 and PD 150606 on calpain activation by A23187

To verify the dependence of cell survival on calcium ionophore-induced calpain activation, we measured calpain activity in C2C12 cells treated with A23187 alone or in combination with BMS-191095 or PD 150606. As shown in Fig. 4, a strong increase in calpain activity was visible after 2 h of A23187 treatment. This increase was markedly limited by the presence of 100 μM PD 150606 or 30 μM BMS-191095, which correlated with the observed protection against cell injury. However, 10 μM BMS-191095, which already demonstrated mild cytoprotective properties, failed to decrease calpain activity. This shows that prevention of calpain activation may be an important, but not the only, mechanism underlying the protective effects of BMS-191095 observed in this model.

The fluorimetric measurements of short-term effects of the tested substances on calpain activity revealed that 15 minutes of preincubation with BMS-191095 did not influence calpain activity in C2C12 cells in contrast to PD 150606, which reduced the activity by approximately 50% (Fig. 5B). Also, no effect of BMS-191095 on the activity of purified μ-calpain was detected (Fig. 5A).

Modulation of A23187-induced calcium transients by BMS-191095

Since activation of calpains by A23187 is mediated by calcium ions, we tested whether the influence of BMS-191095 on the activity of these enzymes is related to the modulation of intracellular calcium levels. Fluo-3 measurements revealed that the addition of 1 μM A23187 caused a transient elevation in cytosolic calcium levels in C2C12 myoblasts, which in individual cells ranged between 3- and 7-fold as compared to initial levels (Fig. 6A, first panel). Such an increase was observed regardless of extracellular Ca^{2+} concentration (measurement media containing 1.8 mM or 0 mM calcium were used,

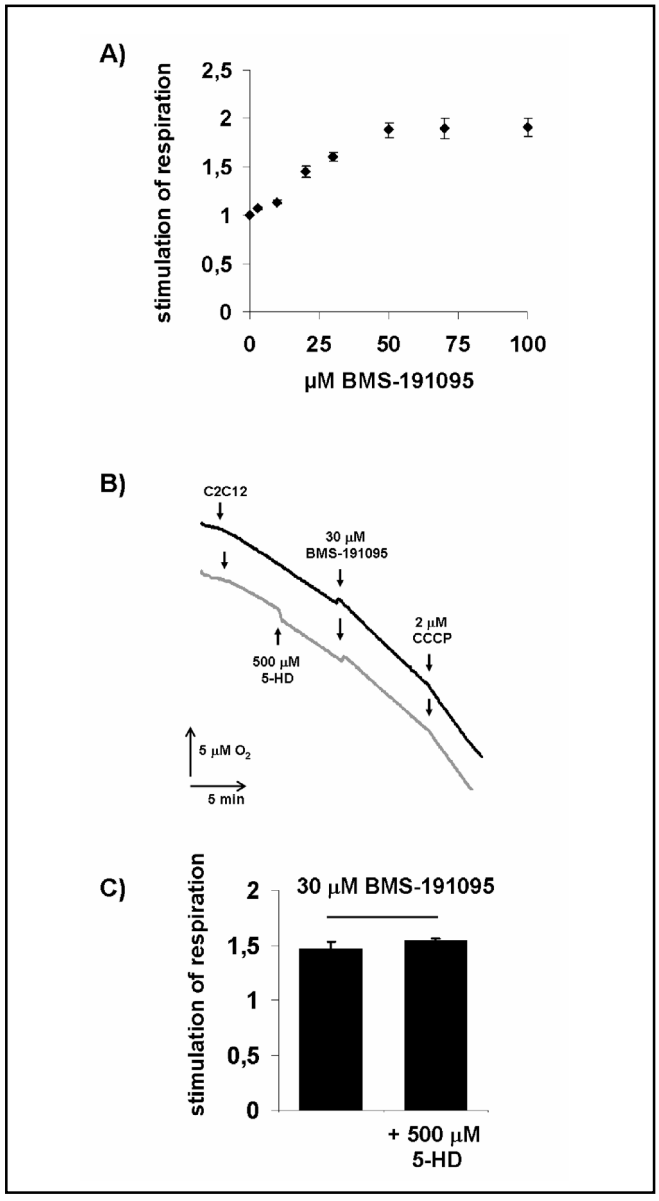

Fig. 7. BMS-191095 stimulates oxygen consumption in C2C12 myoblasts. (A) Stimulation of myoblast respiration observed in presence of different concentrations of BMS-191095. (B) Representative traces of the oxygen consumption measurements. For each measurement around 0.2 million of cells/ml was added. (C) Lack of effect of 5-hydroxydecanoate on stimulatory effect of 30 µM BMS-191095 on C2C12 cells respiration. Results are expressed as mean values ± SEM from n=4 independent experiments similar to the ones presented in B.

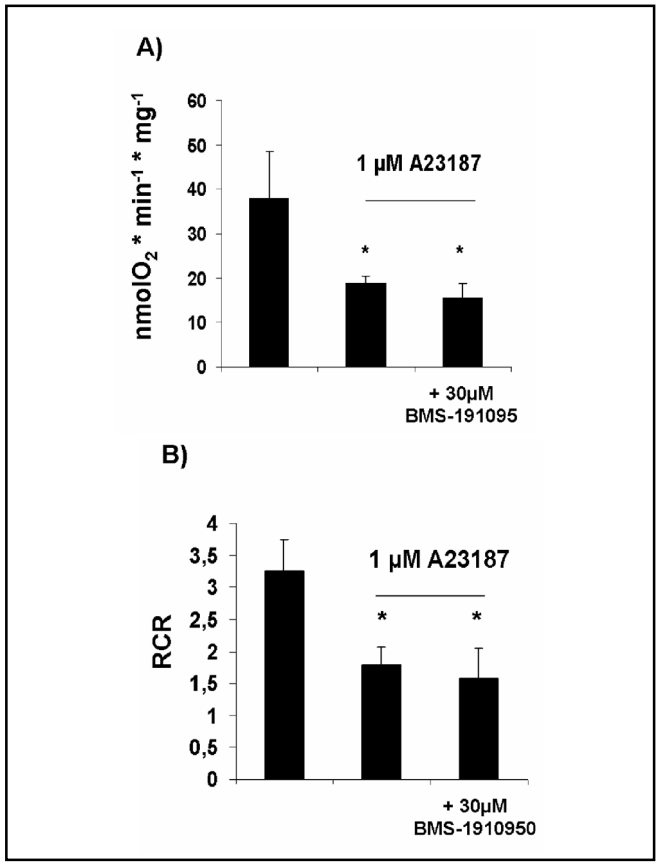

Fig. 8. BMS-191095 does not improve the impaired oxidative phosphorylation of A23187-treated C2C12 cells. (A) State 3 respiration and (B) respiratory control ratios in C2C12 cells after 6 h of treatment with 1 µM A23187 alone or in combination with 30 µM BMS-191095. Results are expressed as mean values ± SEM from n=4 independent experiments. *p ≤ 0.05 vs. untreated cells.

Fig. 5A, third panel). On the other hand, the A23187-induced cytosolic calcium increase was strongly decreased or completely prevented by the depletion of intracellular calcium stores with thapsigargin, an inhibitor of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATP-ase) pumps (Fig. 6A, fourth panel). This shows that in C2C12 cells, A23187 increases cytosolic calcium levels mainly by releasing calcium ions from intracellular stores. Also, if administred after A23187, thapsigargin did not lead to any further increase in cytosolic calcium (data not shown), indicating that calcium removal from the reticulum by A23187 was very efficient.

After an initial increase caused by A23187 administration, cytosolic calcium levels were declining quite quickly. However, in most of analysed cells initial calcium concentrations were not reached even 10 minutes after A23187 addition. In contrast, when the cells were pretreated with 30 µM BMS-191095, the drop in cytosolic calcium levels was markedly faster and more efficient (Fig. 6C), despite the fact that the initial peak did not differ significantly from the one observed in control cells (Fig. 6B and Fig. 6A, second panel). The influence of the potassium channel opener itself on cytosolic Ca^{2+} levels was either very weak or not detectable (data not shown).

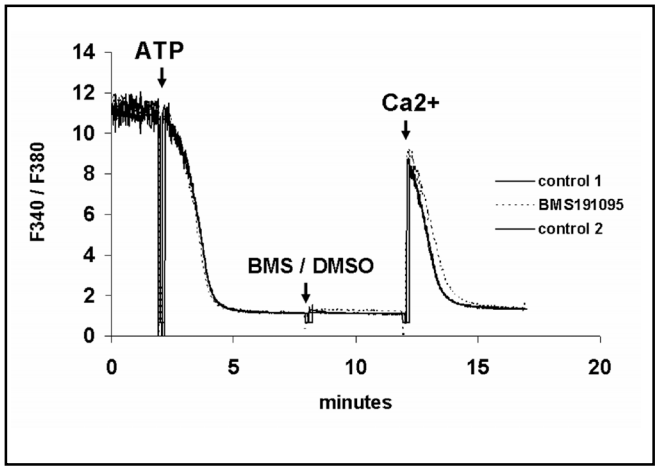

Fig. 9. BMS-191095 does not improve calcium uptake by isolated skeletal muscle sarcoplasmic reticulum. Representative measurement of calcium uptake by isolated H-SR fraction, performed with fura-2 fluorescent probe. The experiment was repeated three times for each of the fractions (L-SR and H-SR), each time with similar outcome. Arrows indicate additions of 0.5 mM ATP, 30 μM BMS-191095 (or, in the control traces, corresponding amount of DMSO), 10 μM $CaCl_2$.

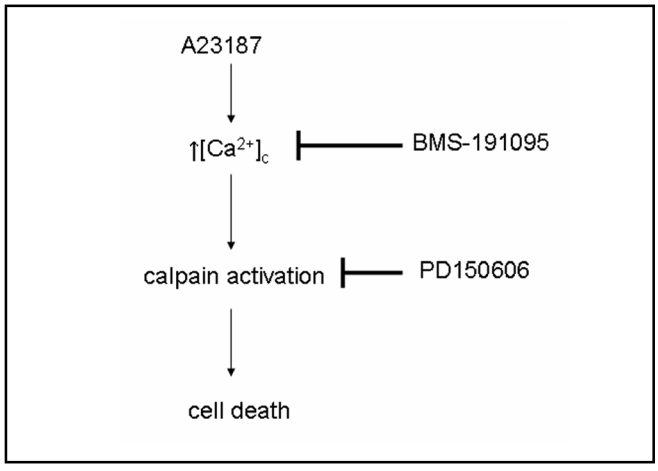

Fig. 10. Cytoprotective action of BMS-191095 and PD 150606 in C2C12 cells treated with calcium ionophore A23187. PD 150606 directly inhibits calpain activity and consequently increases cellular survival, while BMS-191095 prevents calpain activation presumably by attenuating the increase in cytosolic calcium levels.

Influence of BMS-191095 and A23187 on oxygen consumption rates in C2C12 myoblasts

Since mitochondria have been proposed to be the target of BMS-19095 action, we investigated the effect of the drug on respiration rates in C2C12 myoblasts. In presence of the drug, up to a 2-fold increase in oxygen consumption was observed (Fig. 7A). 30 μM BMS-191095 stimulated respiration by 1.6-fold (with ± 0.22 SD). This stimulation was not affected by the presence of 500 μM 5-HD (Fig 7B, C).

To evaluate the extent of mitochondrial damage in A23187-treated C2C12 cells, we measured respiratory control ratios (RCR) after 6 h of treatment with the tested substances. The RCRs were determined in digitonin-permeabilised cells with glutamate + malate as respiratory substrates. Treatment with 1 μM A23187 led to a decrease in RCR (Fig. 8B) due to reduced state 3 respiration rates (Fig. 8A), indicating that A23187 treatment induced mitochondrial damage. Co-administration of 30 μM BMS-191095 did not prevent mitochondrial injury measured as described above.

Influence of BMS-191095 on calcium uptake by isolated sarcoplasmic reticulum

Improved efficacy of calcium removal from the cytosol can result from faster extrusion of Ca^{2+} to the extracellular environment or from stimulation of calcium uptake by endoplasmic reticulum. The measurements performed with the use of isolated sarcoplasmic reticulum revealed, that BMS-191095 not only did not improve but even mildly decreased the speed of calcium uptake by the SR (Fig. 9). Similar results were obtained for both heavy (H-SR) and light (L-SR) fractions of skeletal muscle sarcoplasmic reticulum. Thus, such influence of BMS-191095 on sarcoplasmic reticulum is not likely to contribute to the observed cytoprotective action.

Discussion

Potassium channel openers are known for their beneficial effects on cell survival in different stress conditions [8]. BMS-191095 was demonstrated to decrease cellular damage caused by ischemia-reperfusion [22-24, 29], glutamate excitotoxicity and oxygen-glucose deprivation [30]. In such models, cell injury is a consequence of different pathological processes including elevation of cytosolic calcium levels, increased generation of reactive oxygen species and ATP depletion (for review see: [31]).

In our study, cell death was induced by treatment of C2C12 myoblasts with H_2O_2 or calcium ionophore A23187. Both models of cell injury are often used to mimic the events responsible for cellular damage during ischemia-reperfusion [32-35]. Hydrogen peroxide acts primarily by causing oxidative damage to proteins, lipids and

DNA, while A23187 increases intracellular calcium levels by inducing calcium influx from the extracellular environment and calcium release from intracellular stores [36-38]. In our experiments, BMS-191095 visibly increased cellular survival when the calcium ionophore A23187 was used as the damaging agent. Concentrations of BMS-191095 required to decrease ionophore-induced cell injury (10-30 μM) were comparable to the concentrations used in other studies, in which the drug was shown to protect neuronal cultures against glutamate excitotoxicity and oxygen-glucose deprivation [30].

Most studies on the cytoprotective action of potassium channel openers have been performed with diazoxide. Many reports have shown that during stress conditions such as hypoxia-reoxygenation, cardioplegia or treatment with oxidants, diazoxide-mediated protection is accompanied by decrease in mitochondrial damage [39-43]. Diazoxide was shown to preserve mitochondrial structure, respiratory rates and ATP synthesis levels, to decrease ROS generation and to prevent mitochondrial calcium overload. To investigate whether the cytoprotective effects of BMS-191095 observed in C2C12 cells were related to modulation of mitochondrial function, we performed respiration measurements. At concentrations leading to cytoprotective effects, BMS-191095 visibly increased the respiration rate in C2C12 myoblasts. Similar effect was previously reported for other potassium channel openers such as diazoxide and pinacidil [11, 44]. This result indicates that in C2C12 cells mitochondria are a target of the drug's action.

However, in our experiments BMS-191095 failed to improve mitochondrial function after A23187 treatment, and mitochondrial damage was evidenced by a decrease in state 3 respiration rate. A decrease in state 3 respiration caused by A23187 was also reported for isolated rat liver mitochondria [45]. In that experiments the decrease was strongly diminished by presence of 2 mM Mg^{2+} in the incubation medium. This indicated that the decrease in state 3 respiration could result from A23187-mediated Mg^{2+} depletion of mitochondria (A23187 catalyzes both Ca^{2+} and Mg^{2+} transport accross biological membranes), which was most pronounced in magnesium-free medium. However, in intact cells the cytosolic Mg^{2+} concentration is estimated to be in the range of 0.5-1 mM [46]. Thus, it is not clear how much the A23187-mediated magnesium flux can contribute to the observed changes in respiration rate, besides the Ca^{2+} mediated mitochodnrial damage. The main conclusion however is that in C2C12 cells potassium channel opener BMS-191095 promoted cellular survival despite impaired mitochondrial function.

Moreover, the cytoprotective effect of BMS-191095 could not be reversed by 5-HD, an inhibitor of $mitoK_{ATP}$ channel. This questions the involvement of mitochondrial potassium channels in BMS-191095-mediated cytoprotection in C2C12 cells. It also stays in opposition to what was reported in several studies performed on ischemia-reperfusion models [22, 47, 48], where beneficial effects of this opener were abolished by 5-HD co-administration. On the other hand, 5-HD failed to prevent the BMS-191095-mediated neuroprotection in neuronal cultures under the conditions of oxygen-glucose deprivation or glutamate excitotoxicity [31]. Also in the study by Ahmad et al. [48] performed on heart muscle, cytosolic Akt phosphorylation observed upon BMS-191095 treatment was not 5-HD sensitive, while the opener-mediated cytoprotection was effectively abolished by this inhibitor. Such results suggest that BMS-191095 may have some channel-unrelated activities contributing to its protective properties. It is also not excluded that the efficacy of 5-HD-mediated $mitoK_{ATP}$ inhibition is tissue-dependent. 5-HD can be metabolized to its CoA derivative and then enter the β-oxidation pathway [19], so it is not clear if the inhibition is mediated by 5-HD itself or by one of its metabolites. Thus the efficacy of the inhibition may depend on cell type and metabolic state.

The channel-unrelated effects of the $mitoK_{ATP}$ opener diazoxide, such as succinate dehydrogenase inhibition and protonophoric properties, are well documented [17, 19, 20, 49]. In comparison to diazoxide, BMS-191095 is considered to be much more selective $mitoK_{ATP}$ opener [26], however it was so far much less intensively studied than diazoxide and its off-target effects could have been not identified yet.

To identify the potential site of action of BMS-191095 responsible for observed cytoprotection, we initially tried to determine the events that are crucial for the induction of cell death in the model applied in our study. A23187-induced cell death can result from the activation of calcium-dependent proteolytic enzymes such as calpains [50, 51], as well as from the opening of mitochondrial permeability transition pore (PTP), mitochondrial swelling and release of caspase-activating molecules from the mitochondrial intermembrane space [36, 52, 53]. To determine which mechanism is predominant in C2C12 cells, we checked the sensitivity of A23187-induced cell injury to inhibitors of calpains (PD 150606), caspases (Z-vad-FMK) and PTP (CsA). Our results show that A23187-induced injury in C2C12 myoblasts can be limited by preventing calpain activation, but not by inhibition of caspases or PTP. Calpain activity measurements confirmed that

concentrations of PD 150606 which demonstrated cytoprotective effects also visibly decreased calpain activity. In contrast to PD 150606, which is a direct inhibitor of calpain [54], BMS-191095 seems to prevent calpain activation indirectly, as it did not interfere with the activity of isolated calpain. One possible mechanism of BMS-191095 action is the limitation of A23187-induced increase in cytosolic calcium levels.

In C2C12 cells, the ionophore-induced elevation in cytosolic calcium had two phases: first, a transient but strong increase in Ca^{2+} levels resulting from the release of calcium from the endoplasmic reticulum, and second, a phase of mild Ca^{2+} elevation lasting for the next several minutes. The second phase seems to be related to calcium influx from the extracellular environment, as it was absent in calcium-free medium. Similar, short and ER-dependent elevation in cytosolic calcium upon addition of A23187 was reported in MH1C1 hepatoma cells [53]. The other literature data show however, that the kinetics of A23187-induced Ca^{2+} influx into the cytosol may vary, dependently on the type of the cells and on the applied ionophore concentrations [55-58]. For example, strong differences have been observed between poorly and highly metastatic melanoma cells [56]. The differences include: (i) the duration and the extent of the elevation in cytosolic Ca^{2+} levels, (ii) the contributions of different Ca^{2+} sources (calcium ions from extracellular space vs. Ca^{2+} from the intracellular stores) to this effect. This variability of the responses may reflect the differences in the calcium content of the ER as well as in efficiency of the mechanisms responsible for calcium removal from the cytosol (Ca^{2+} extrusion to the extracellular environment, Ca^{2+} re-uptake into the ER and mitochondrial Ca^{2+} uptake). In C2C12 cells calcium removal from the cytosol seem to rely mainly on Ca^{2+} extrusion through the plasma membrane, which in these cells appear to be more efficient than Ca^{2+} uptake into the ER. The thapsigargin-induced elevation of cytosolic calcium levels was prevented, if cells were previously treated with A23187. This indicates that in presence of A23187, calcium content of the ER is strongly decreased. The studies of calcium fluxes performed with the use of ^{45}Ca revealed, that in presence of A23187 there occur both: Ca^{2+} influx from the extracellular environment and Ca^{2+} extrusion from the cell, and that the second process can lead to a decrease in overall intracellular Ca^{2+} content, probably due to calcium-depletion of the ER [59, 60].

In A23187-treated C2C12 cells, BMS-191095 visibly affected the second, plasma membrane-dependent phase of cytosolic calcium elevation, indicating that the action of this potassium channel opener is related to an inhibition of Ca^{2+} influx from the extracellular environment rather than to an effect on Ca^{2+} release from intracellular stores. Measurements of calcium uptake by isolated sarcoplasmic reticulum indicated, that the SR-mediated removal of Ca^{2+} from the cytosol can even be slower in presence of BMS-191095 than in the absence of the drug.

Improved intracellular calcium handling was previously observed in the presence of diazoxide in a study performed on N2A neuroblastoma cells [61]. In that study, calcium influx into the cytosol was induced by purinergic stimulation, and the restoration of Ca^{2+} levels to initial values was faster in presence of diazoxide. However, similar to our study, a potential link between mitochondrial potassium channel activation and calcium fluxes was not evident.

In conclusion, as depicted in Fig. 10, our study confirms the cytoprotective properties of the potassium channel opener BMS-191095 in C2C12 cells. This action can be explained by the beneficial effects of the drug on the maintenance of intracellular calcium homeostasis under stress conditions. Whereas potassium channel opener-mediated protection was accompanied by a decrease in the proteolytic activity of calpains, the drug failed to prevent mitochondrial damage. The mechanism responsible for the observed effects of BMS-191095 on intracellular calcium levels remains unresolved and additional actions on non-mitochondrial targets cannot be excluded.

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