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Short communication

Cytoprotective action of the potassium channel opener NS1619 under conditions of disrupted calcium homeostasis

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Abstract:
Cytoprotective properties of potassium channel openers (KCOs) have been demonstrated in several models of cell injury, mainly in ischemia-reperfusion-induced damage of cardiac muscle. The mechanism responsible for the observed cytoprotection and the relative contribution of plasma membrane or inner mitochondrial membrane potassium channels regarding the beneficial effects exerted by KCOs remain unclear.
Our work demonstrates the cytoprotective properties of NS1619, an opener of large-conductance calcium-activated potassium channels (BK_{Ca} channels), using C2C12 myoblasts injured by calcium ionophore A23187 treatment. Application of two BK_{Ca} channel inhibitors, paxilline and iberiotoxin, abolished this cytoprotective effect. At concentrations of 10–100 μM, NS1619 increased the respiration rate and decreased mitochondrial membrane potential ($\Delta\psi$) in C2C12 cells in a dose-dependent manner. At a concentration of 0.2 μM, paxilline, which effectively abolished the protective effect of NS1619, failed to counteract the opener-induced mitochondrial depolarization and increase in cellular respiration. This result indicates that the NS1619-mediated increase in the survival rate of A23187-treated C2C12 cells occurs in a manner distinct from its effect on mitochondrial functioning and suggests that activation of BK_{Ca} channels in the plasma membrane is the mechanism responsible for cytoprotection by NS1619.

Key words: BK_{Ca} channel, mitochondria, cytoprotection, NS1619, calcium

Abbreviations: BK_{Ca} channel – large-conductance calcium-activated potassium channel, K_{ATP} channel – ATP-regulated potassium channel, KCO – potassium channel opener, LDH – lactate dehydrogenase, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OGD – oxygen-glucose deprivation

Introduction

It is well documented that the administration of certain potassium channel openers (KCOs) prior to ischemic insult results in a strong inhibition of ischemia and reperfusion-induced tissue damage [1]. This cytoprotective effect has been extensively studied in cardiac muscle, but it has been observed also in other tissues, including neuronal tissue [20]. Cytoprotective activity has been reported for numerous activators of ATP-regulated potassium channels (K_{ATP} channels), such as diazoxide, pinacidil and nicorandil [1]. More recently, it has been shown that activators of large-conductance calcium-activated potassium channels (BK_{Ca} channels) also demonstrate cytoprotective effects [22].

K_{ATP} channels and BK_{Ca} channels have been identified both in the plasma membrane and in the inner mitochondrial membrane in numerous cell types [20]. As both plasmalemmal and mitochondrial channels are targets of KCOs action, it is still somewhat controversial as to which of these channels is responsible for the beneficial effects of the openers [21]. Currently, the prevailing view is that KCO-mediated cytoprotection is related to the stimulation of potassium influx into the mitochondria. Potassium influx can lead to mild uncoupling of mitochondria, which results in changes in reactive oxygen species generation and limitation of calcium uptake into the matrix. This, in turn, helps to preserve mitochondrial function under stress conditions [13–15]. Conversely, activation of potassium channels located in the plasma membrane results in cell membrane hyperpolarization and reduction in the influx of Ca^{2+} into the cytosol, both of which can also protect against ischemic damage [21]. It is also possible that plasmalemmal and mitochondrial potassium channels coactively contribute to ischemic preconditioning [21].

NS1619 is a BK_{Ca} channel opener that has demonstrated cytoprotective activity in various models of cell injury, including cardiac tissue under conditions of ischemia and reperfusion [4, 19, 22], cardiomyocytes challenged with metabolic inhibition and anoxia [4], neuronal cell injury due to oxygen-glucose deprivation, glutamate exposure or hydrogen peroxide treatment [8, 9] and C2C12 myoblasts injured by H_2O_2 treatment [18]. In the present study, we demonstrate that NS1619 increases the survival of C2C12 cells under conditions of disrupted calcium homeostasis induced by the calcium ionophore A23187.

Materials and Methods

Reagents

NS1619, paxilline and A23187 were purchased from Sigma. Iberiotoxin was obtained from Bachem. Stock solutions of NS1619 (33 mM), paxilline (10 mM) and A23187 (10 mM) were prepared in DMSO, and iberiotoxin was dissolved in water. Immediately prior to carrying out our experiments, the appropriate chemical dilutions were prepared in Dulbecco's Modified Eagle's Medium (DMEM) and then used for cell treatment. The DMSO concentration in cell culture medium used for cell treatment did not exceed 0.3%. At such a low concentration, the solvent did not affect the viability of C2C12 cells.

Cell culture conditions

C2C12 murine myoblasts were obtained from European Collection of Animal Cell Cultures (ECACC). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and maintained in DMEM supplemented with 20% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For cell injury tests, cells between the 5^{th} and 15^{th} passage were seeded onto 96-well plates, and experiments were performed the following day. Chemical incubations were conducted in culture medium containing 0.1% FBS (culture medium was switched to the medium containing 0.1% FBS directly before the experiment).

Determination of cell injury levels

Cells grown in 96-well plates (about 6×10^3 cells per well) were treated for 6 h with the calcium ionophore A23187. NS1619 was added 15 min prior to ionophore administration, and iberiotoxin or paxilline was added 30 min prior to ionophore administration. Following treatment, cell survival was assessed using either the lactate dehydrogenase (LDH) release test or the MTT reduction assay.

LDH release was measured using the Cytotoxicity Detection Kit (Roche Molecular Biochemicals). After incubation with the tested chemicals, the incubation medium was collected, and cell debris was removed by centrifugation. In parallel, 0.1% Triton X-100 was added to each well to lyse the attached cells. LDH activity in the cell lysates and in the incubation medium was measured colorimetrically according to the instructions provided by the manufacturer. The amount of LDH released from the cells was then calculated using the formula: % of LDH release = (LDH_{medium}/LDH_{total}) × 100%, where 'LDH_{medium}' is the LDH activity detected in the incubation medium, and 'LDH_{total}' is the sum of the LDH activities detected in the incubation medium and in the cell lysates.

Cell survival was also assessed using the MTT reduction assay. Following chemical treatment, the cell culture medium was discarded, and 50 μl of DMEM containing 0.5 mg/ml of MTT [3-(4,5-dimethyl-

thiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well. After a 2 h incubation, 50 µl of lysis buffer (20% sodium dodecyl sulfate, 50% N,N-dimethylformamide, 2% acetic acid and 25 mM HCl) was added to each well, and the plates were incubated overnight at 37°C. MTT formazan formation was quantified by measuring the absorbance at 570 nm; 650 nm was used as a reference wavelength. The results were normalized to the values measured in the untreated controls for corresponding experiment.

Measurements of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi$) in C2C12 cells was assessed with the fluorescent probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide). Upon accumulation in mitochondria, JC-1 aggregates and undergoes a reversible change in fluorescence emission from green (535 nm) to red (595 nm). JC-1 accumulation is driven by the $\Delta\psi$; thus, $\Delta\psi$ can be estimated by determining the JC-1 F_{595}/F_{535} fluorescence emission ratio. Cells were plated on rectangular glass coverslips and incubated with the probe (10 µM JC-1 in DMEM containing 0.1% FBS) for 30 min at 37°C. Following an additional 30 min incubation in DMEM without JC-1, coverslips were placed in a 3-ml cuvette in HBSS-HEPES buffer with glucose (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 $MgSO_4$,10 mM HEPES and 5.5 mM glucose; pH 7.4). The cells were treated with the BK_{Ca} modulators in a manner similar to that used for the MTT tests, where there was a 15 min incubation with paxilline (or control cells were left untreated) followed by the addition of NS1619 (0, 10 or 30 µM). The JC-1 fluorescence emission spectra at λ_{ex} = 490 nm were measured three times for each sample: the measurements were taken before paxilline administration, directly prior to NS1619 administration (15 min after paxilline addition) and 15 min after NS1619 addition. The relative $\Delta\psi$ was expressed as the ratio of fluorescence at 595 nm to fluorescence at 535 nm (F_{595}/F_{535}).

Respiration measurements

For determination of the respiration rate, C2C12 cells were detached by trypsinization and suspended in DMEM containing 0.1% FBS, and then the oxygen consumption in the suspension was monitored with an oxygraph (Oroboros, Oxygraph-2k). At the beginning of each measurement, 6.75 µM oligomycin was added to obtain state 4 respiration. Then, NS1619 was titrated (where indicated, this was preceded by the addition of paxilline of iberiotoxin). The protein content of each of the samples was determined using the Lowry method. The oxygen consumption rate was calculated as nmoles of consumed O_2 per min per mg of protein. To compare the effects of the BK_{Ca} modulators on respiration rate, the results were normalized against the oxygen consumption rates detected in the presence of oligomycin only.

Statistical analysis

For statistical analysis, the mean values obtained from a minimum of 4 independent experiments were compared. The statistical significance of the results was determined using a paired two-sided Student's *t*-test.

Results

Treatment of C2C12 cells with calcium ionophore A23187 for 6 h resulted in a dose-dependent decrease in cell viability (Fig. 1A). In further experiments, 0.5, 1 or 2 µM concentrations of A23187 were used, and these doses resulted in approximately 40%, 65% and 90% decreases in cell viability, respectively.

As shown by both the MTT reduction assay and the LDH release test, NS1619 demonstrated a clear cytoprotective effect. This effect was most evident when 1 µM A23187 was used as a damaging agent (Fig. 1 B and C). In the presence of 2 µM A23187, only the highest concentration of NS1619 (100 µM) resulted in statistically significant cytoprotection. This concentration of the opener, however, decreased the viability of control cells by approximately 20%, so further experiments were performed using concentrations no higher than 30 µM of NS1619.

To examine whether the observed protection resulted from activation of calcium-regulated potassium channels, we investigated the influence of the BK_{Ca} channel inhibitors: paxilline and iberiotoxin on the beneficial action of NS1619. As depicted in Figure 2, both paxilline and iberiotoxin partially reversed the observed cytoprotective effects. Similar results were obtained with the LDH release test (data not shown).

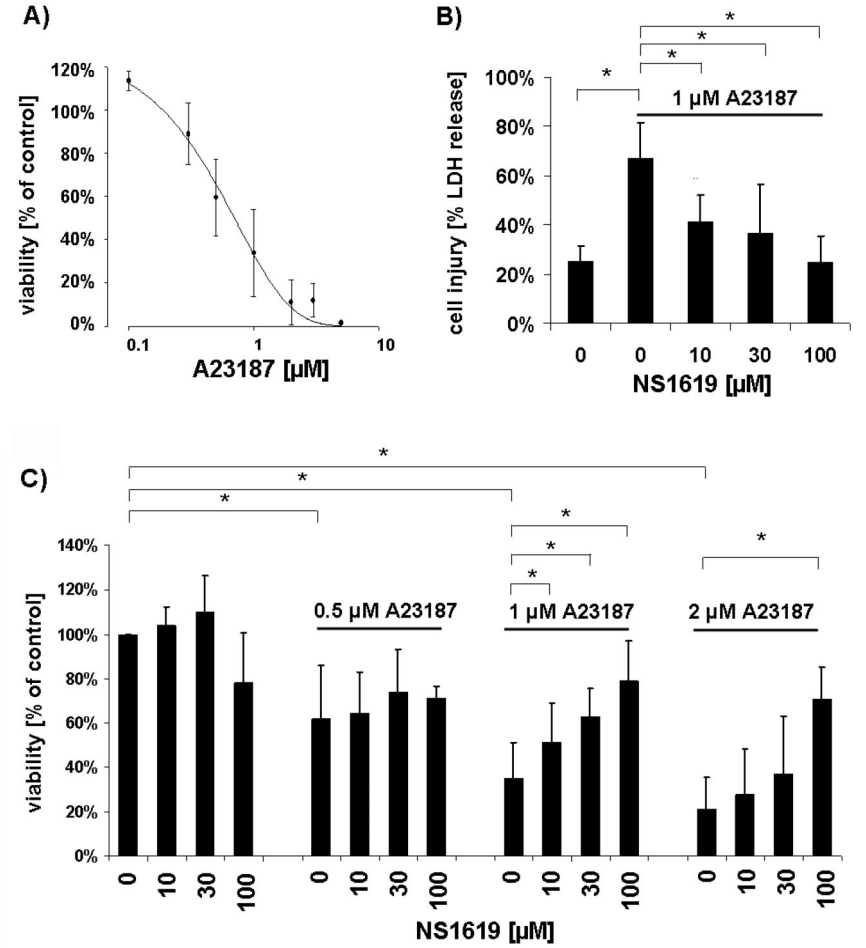

Fig. 1. Cytoprotective action of NS1619 in C2C12 cells injured by the calcium ionophore A23187. (**A**) Survival rate of C2C12 cells following a 6 h of exposure to different concentrations of A23187. Based on the obtained data, an IC_{50} of 0.89 µM was calculated. (**B**, **C**) Cells were pre-incubated with NS1619 for 15 min prior to the addition of A23187. After 6 h, cell injury was measured with either the LDH release test (**B**) or the MTT assay (**C**). Data are presented as the mean values ± SD from independent experiments performed in triplicate (n = 3–15 (**A**), n = 4 (**B**) or n = 13 (**C**)). * indicates $p \leq 0.05$

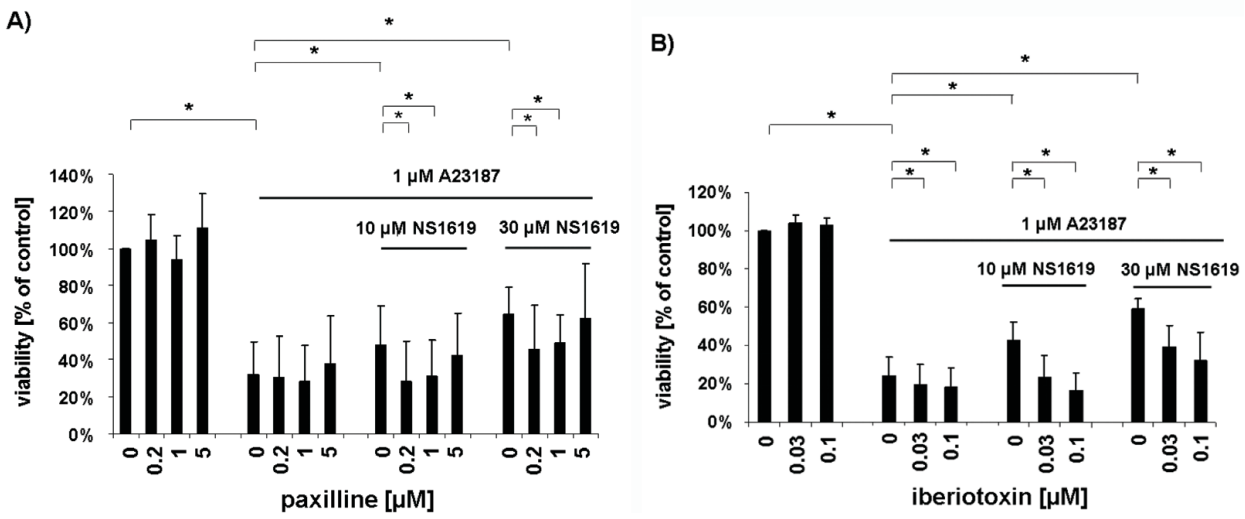

Fig. 2. The influence of the BK_{Ca} channel inhibitors paxilline (**A**) and iberiotoxin (**B**) on the cytoprotective effect of NS1619 in A23187-treated C2C12 cells. NS1619 was added 15 min after the addition of inhibitors. A23187 treatment was started after an additional 15 min interval. Following a 6 h incubation, cell injury was measured using the MTT test. Data are presented as the mean values ± SD from independent experiments performed in triplicate (n = 6 (paxilline) or n = 4 (iberiotoxin)). * indicates $p \leq 0.05$

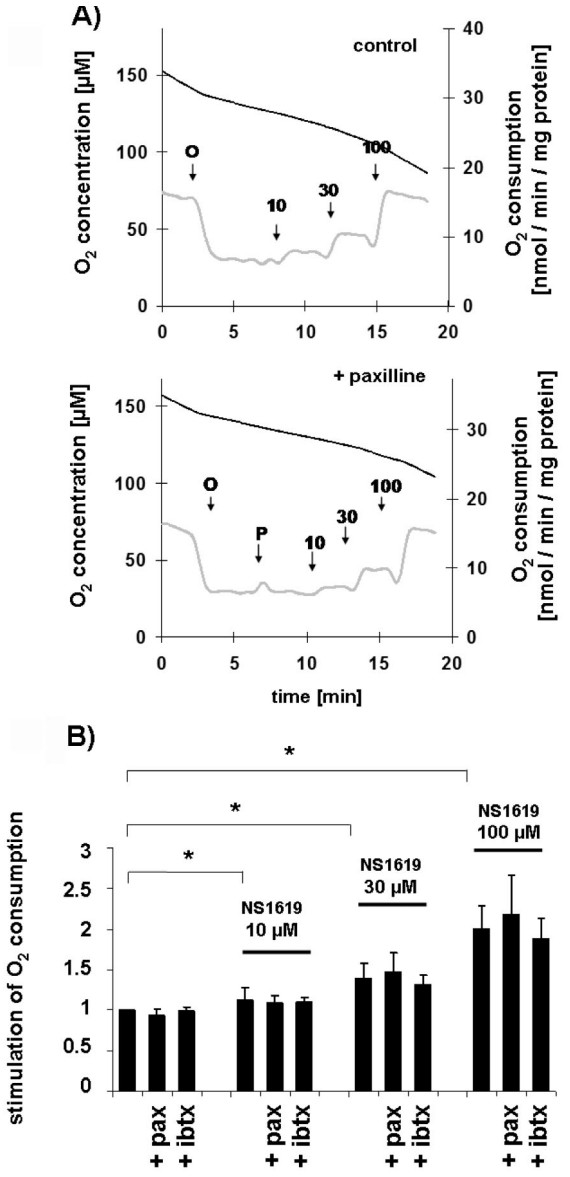

Fig. 3. Stimulation of the C2C12 cell respiration rate by NS1619 treatment is independent of BK_{Ca} channel inhibitor exposure. (**A**) Representative traces of oxygen consumption measurements. Black lines represent oxygen concentration (left axis), and grey lines represent oxygen consumption rate (right axis). Arrows indicate the addition of 6.75 μM oligomycin (O), 0.2 μM paxilline (P) and 10 μM, 30 μM and 100 μM NS1619 (marked as '10', '30' and '100'). (**B**) Stimulation of C2C12 cell respiration by NS1619 treatment in the absence and in the presence of BK_{Ca} inhibitors (0.2 μM paxilline ('+ pax') and 0.1 μM iberiotoxin ('+ ibtx')). The results are expressed as fold stimulation of O_2 consumption by NS1619, as compared to the rate detected in presence of oligomycin only (which was 4.7 ± 3.9 nmol O_2/min/mg protein). Data are presented as the mean values ± SD from independent experiments similar to the one presented in panel A (n = 4). * indicates $p \leq 0.05$

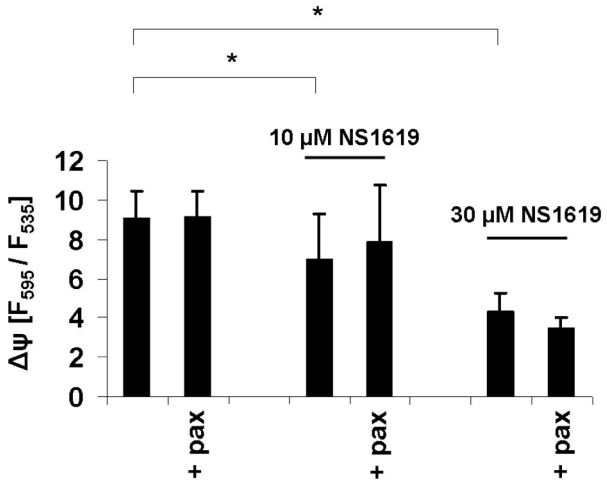

Fig. 4. NS1619-induced decrease in mitochondrial membrane potential in C2C12 cells. After a 15 min incubation with NS1619, mitochondrial membrane potential was measured with the fluorescent probe JC-1. The results are expressed as the ratio of JC-1 fluorescence intensity detected at λ_{em1} = 595 nm and λ_{em2} = 535 nm (λ_{ex} = 490 nm). This ratio reflects mitochondrial membrane potential. Where indicated, 0.2 μM paxilline was added 15 min prior to NS1619 administration. Data are presented as the mean values ± SD from independent experiments (n = 4). * indicates $p \leq 0.05$

These results confirmed that the observed increase in cellular survival was related to the activation of BK_{Ca} channels by NS1619. Interestingly, exposure to the lowest dose of paxilline (0.2 μM) resulted in the most significant effect in countering NS1619 action, suggesting the existence of an additional activity of paxilline that is likely channel-unrelated.

A potential involvement of the mitochondrial BK_{Ca} channels in the observed cytoprotection was assessed by measuring Δψ and respiration. The opening of mitochondrial potassium channels leads to a mild decrease in the Δψ, resulting in the stimulation of oxygen consumption by cells. Because stimulation of respiration is detectable only at sufficiently high Δψ, we performed the measurements of oxygen consumption in the presence of oligomycin. At the tested concentrations, NS1619 stimulated oxygen consumption in C2C12 cells. This effect was not sensitive to the presence of either 0.2 μM paxilline or 0.1 μM iberiotoxin (Fig. 3).

Similarly, the measurements of Δψ using the fluorescent probe JC-1 revealed that after a 15 min incubation of C2C12 cells with NS1619, the Δψ was decreased. This effect was not sensitive to 0.02 μM paxilline (Fig. 4). Paxilline alone did not induce the detectable changes in Δψ (data not shown). This result indicates that in our experimental model the reversal of the cytoprotective effect induced by BK_{Ca} channel inhibitors is likely to be unrelated to inhibition of mitochondrial potassium channels.

Discussion

The cytoprotective properties of NS1619 have been demonstrated under conditions of ischemia-reperfusion, oxygen-glucose deprivation (OGD) and glutamate excitotoxicity [4, 6, 8, 9, 19, 22]. In these models, cell injury is a consequence of multiple pathologic processes, including elevation of cytosolic calcium levels, increased generation of reactive oxygen species and ATP depletion [7, 14]. It remains unclear, however, as to how these events are affected by the administration of KCOs. Our results indicate that NS1619 improves the survival rate of C2C12 cells treated with the calcium ionophore A23187. Previously in our lab, similar results were obtained for the K_{ATP} channel opener BMS-191095 using the same cell injury model [12]. In this model, cell death results from an excessive calcium influx into the cytosol, resulting in calpain activation [12]. As these events are also observed during ischemia or glutamate excitotoxicity [7, 14], we conclude that the reduction in cell injury after NS1619 treatment is at least partially related to the counteraction of cellular mechanisms activated by the disruption of calcium homeostasis. Improved calcium handling in the presence of NS1619 was previously reported using neuronal cells, where pre-treatment with the opener was able to limit the increase in cytosolic calcium concentration observed upon administration of excitotoxic doses of glutamate [8]. In cardiomyocytes, NS1619 has been shown to prevent ouabain-induced mitochondrial calcium overload [17] under conditions of simulated ischemia and reperfusion [19].

Apart from opening the BK_{Ca} channel, NS1619 also induces channel-unrelated effects, such as the inhibition of the mitochondrial respiratory chain complex I [20]. Under certain conditions, inhibition of this complex can limit cellular damage [9]. To determine if the cytoprotection observed in C2C12 cells was caused by BK_{Ca} channel activation, we chose to investigate if this effect could be influenced by the specific channel inhibitors iberiotoxin and paxilline. Both inhibitors counteracted the beneficial effects of NS1619 in C2C12 cells, confirming the involvement of potassium channels in the cytoprotection induced by this opener. The modulation of NS1619-mediated protection in response to paxilline exposure was previously demonstrated in cardiac tissue injured by ischemia and reperfusion [19, 22], ouabain [17] and OGD [6]. In neuronal cells, however, under conditions of cell death induced by OGD, glutamate or H_2O_2, the protective effect of NS1619 was not abolished by either paxilline or iberiotoxin treatment [8, 9]. It is possible that the mechanisms underlying the beneficial properties of NS1619 may differ depending on the tissue type used in the study. It is worth noting, however, that in the experiments conducted by Gaspar et al. [8], a relatively high concentration of paxilline (20 μM) was used, whereas in the studies performed on cardiac tissue, the concentrations did not exceed 1 μM. In our study using C2C12 cells, paxilline concentrations above 1 μM were less effective in reversing NS1619-mediated cytoprotection than the lower concentrations. This finding indicates that additional attention is necessary regarding paxilline concentrations used in the studies on BK_{Ca} channel opener-mediated cytoprotection. Previous works demonstrated that paxilline inhibits the sarco/endoplasmic reticulum ATPase [2] and the inositol 1,4,5-triphosphate receptor [11] when used at concentrations around 5 μM or higher. Used at such concentrations, paxilline is likely to influence intracellular calcium homeostasis in a potassium channel-independent manner, which could complicate the interpretation of the results.

BK_{Ca} channels are present in the plasma membrane and in the inner mitochondrial membrane, and both types of channels can be activated by NS1619. Paxilline is an indole-diterpene that can pass through cell membranes due to its hydrophobicity [16]; therefore, it can effectively inhibit channels present in both the plasma membrane and the inner mitochondrial membrane. In contrast, iberiotoxin, a peptide consisting of 37 amino acids, is less likely to cross the cell membrane and, instead, acts by binding to the extracellular component of the plasma membrane-embedded BK_{Ca} channel [3]. In A23187-treated C2C12 cells, NS1619-mediated cytoprotection was abolished by treatment with either inhibitor, suggesting that the observed protection resulted from activation of the potassium channels located in the plasma membrane rather than from activation of the mitochondrial channels. However, since detailed studies confirming cell impermeability of iberiotoxin are lacking, further analysis of the BK_{Ca} modulators' influence on mitochondrial functioning in the context of C2C12 cells is required.

The expected consequence of opening of mitochondrial potassium channels is K^+ influx into the mitochondrial matrix resulting in mild depolarization of mitochondrial inner membrane, which can stimulate respiration. Previously, these effects were observed using NS1619, other potassium channel openers and the potassium ionophore valinomycin [5, 10]. In iso-

lated cardiac mitochondria, the stimulation of state 4 respiration induced by a 30 μM dose of NS1619 was abolished by paxilline [10]. This finding indicates that the respiration rate was dependent on the open state of the mitochondrial BK_{Ca} channel.

KCO-mediated depolarization of mitochondria decreases the efficiency of mitochondrial calcium uptake. This decrease could be beneficial under conditions of ischemia and reperfusion, during which mitochondrial calcium overload and consecutive megachannel activation can both function to trigger cell death. Indeed, the NS1619-mediated prevention of mitochondrial calcium overload was observed in cardiomyocytes challenged with ischemia and reperfusion [19] or treated with ouabain [17]. This finding indicates that mitochondrial depolarization may be responsible for the cytoprotective action of NS1619 in calcium ionophore-injured C2C12 cells. Measurements of $\Delta\psi$ revealed that $\Delta\psi$ is clearly decreased in C2C12 cells treated for 15 min with 10 μM or 30 μM of NS1619; however, this effect was not influenced by 0.02 μM paxilline treatment. Similar results were observed when measuring the influence of BK_{Ca} modulators on C2C12 cell respiration. NS1619 treatment increased the oxygen consumption rate in cells in a dose-dependent manner. The influence of NS1619 treatment on mitochondrial respiration was not counteracted by either 0.2 μM paxilline or 0.1 μM iberiotoxin dose, although these inhibitor concentrations did effectively abolish opener-induced cytoprotection. This result indicates that the cytoprotective effect of NS1619 is likely independent of its influence on mitochondrial function.

In conclusion, we have demonstrated that NS1619 exposure can prevent C2C12 cell injury under conditions of disrupted calcium homeostasis. This effect is likely due to the activation of BK_{Ca} channels that are located in the plasma membrane.

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