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Pharmacological and physiological stimuli do not promote Ca^{2+}-sensitive K^+ channel activity in isolated heart mitochondria

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Abstract

Objective: Mitochondrial calcium-activated K^+ (mitoK_{Ca}) channels have been described as channels that are activated by Ca^{2+}, inner mitochondrial membrane depolarization and drugs such as NS-1619. NS-1619 is cardioprotective, leading to the assumption that this effect is related to the opening of mitoK_{Ca} channels. Here, we show several weaknesses in this hypothesis.
Methods: Isolated mitochondria from rat hearts were tested for evidence of mitoK_{Ca} activity by analyzing functional parameters in K^+-rich and K^+-free media.
Results: NS-1619 promoted mitochondrial depolarization both in K^+-rich and K^+-free media. Respiratory rate increments were also seen in the presence of NS-1619 for both media. In parallel, NS-1619 promoted respiratory inhibition, as evidenced by respiratory measurements in state 3. Mitochondrial volume measurements conducted using light scattering showed that NS-1619 led to swelling, in a manner unaltered by inhibitors of mitoK_{Ca} channels, antagonists of adenosine triphosphate-sensitive potassium channels or inhibitors of the permeability transition. Swelling was also maintained when K^+ in the media was substituted with tetraethylammonium (TEA^+), which is not transported by any known K^+ carrier. Electron microscopy experiments gave support to the idea that NS-1619-induced mitochondrial swelling took place in the absence of K^+. In addition to testing the pharmacological effects of NS-1619, we attempted, unsuccessfully, to promote mitoK_{Ca} activity by altering Ca^{2+} concentrations in the medium and inducing mitochondrial uncoupling.
Conclusion: Our data indicate that NS-1619 promotes non-selective permeabilization of the inner mitochondrial membrane to ions, in addition to partial respiratory inhibition. Furthermore, we found no specific K^+ transport in isolated heart mitochondria compatible with mitoK_{Ca} opening, whether by pharmacological or physiological stimuli. Our results indicate that NS-1619 has extensive mitochondrial effects unrelated to mitoK_{Ca} and suggest that tissue protection mediated by NS-1619 may occur through mechanisms other than activation of these channels.

Keywords: Oxygen consumption; K^+ channel; Ischemia; Mitochondria; Preconditioning

1. Introduction

Myocardial cells present increased resistance to ischemic insults when previously exposed to brief, non-lethal ischemia, a phenomenon known as ischemic preconditioning [1]. This suggests that myocytes possess an evolutionarily selected, endogenous machinery of protection against ischemia. Ischemic preconditioning involves several redundant signaling cascades and end effectors (reviewed in [2,3]), a finding that attests to the importance of these mechanisms. A thorough understanding of the cardioprotective events

Abbreviations: NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; 5-HD, 5-hydroxydecanoate; CCCP, carbonyl cyanide *m*-chloro phenyl hydrazone; DNP, dinitrophenol; CsA, cyclosporin A; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; mitoK_{Ca}, mitochondrial ATP-sensitive K^+ channels; mitoK_{Ca}, mitochondrial calcium-activated K^+ channels; Pax; paxilline; TEA^+, tetraethylammonium ion; S.E.M., standard error of the mean; TMPD, *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylene-diamine

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involved in ischemic preconditioning will certainly contribute toward the development of newer therapeutic interventions designed to prevent tissue damage in ischemic diseases.

Mitochondrial adenosine triphosphate (ATP)-sensitive K^+ channels (mitoK_{ATP}) are an established part of the endogenous protective machinery mediating ischemic preconditioning. Indeed, ischemic preconditioning is prevented by mitoK_{ATP} channel antagonists [4–6] and mimicked by a variety of agonists [5,7,8]. When opened, these channels promote significant mitochondrial matrix expansion and mild inner membrane potential reduction [9]. These effects of K^+ entry through mitoK_{ATP} channels are thought to protect tissues by modulating the production of reactive oxygen species and avoiding the loss of cellular high energy phosphates (such as ATP and phosphocreatine) and the accumulation of matrix Ca^{2+}, resulting in the prevention of mitochondrial permeability transition (reviewed in [10,11]). All these protective mechanisms result from K^+ transport across the inner mitochondrial membrane, the primary consequence of mitoK_{ATP} opening. Indeed, despite the fact that some pharmacological regulators of mitoK_{ATP} present toxic effects, they have been shown to act on a selective K^+ transport pathway in isolated heart mitochondria [9].

Recently, a second type of K^+ channel, the mitochondrial calcium-sensitive K^+ channel (mitoK_{Ca}), has been identified as another possible mediator of ischemic cardioprotection. Patch clamp experiments show that the opening probability of this channel increases in response to Ca^{2+} and depolarization [12,13]. These stimuli are observed under physiological conditions such as high cardiac work, and pathophysiological conditions such as ischemia. Thus, it is possible that mitoK_{Ca} could be activated during ischemic preconditioning. Indeed, there is initial evidence that mitoK_{Ca} channels may be involved in protection mediated by preconditioning, based on effects of putative mitoK_{Ca} blockers [14]. Further evidence for the cardioprotective role of mitoK_{Ca} is based on the finding that 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS-1619), an activator of plasma membrane big conductance calcium-sensitive K^+ channels, leads to protection against ischemia in perfused hearts and cardiac cells [13–16]. However, the specificity of NS-1619 toward mitoK_{Ca} opening was not extensively verified. Indeed, although single-channel recordings of mitoK_{Ca} in glioma [12] and rat heart [13,17] mitoplasts have been conducted, there is no evidence NS-1619 activates currents under these conditions. Instead, the effect of NS-1619 was tested on mitochondrial K^+ uptake using a K^+-sensitive probe [13], an experimental setting which may be influenced by factors distinct from a regulated and selective K^+ entry pathway. Furthermore, there is some evidence in the literature that NS-1619 may have mitochondrial effects unrelated to K^+ transport, such as respiratory inhibition [18–20].

This work evaluated NS-1619 effects in isolated mitochondria, an experimental setting that allows for more detailed examination of bioenergetic effects and specificity of this drug. Surprisingly, we found that NS-1619 does not promote K^+ transport attributable to a channel or a specific proteinaceous cation transporter. Instead, this drug promotes non-selective ion transport across the inner mitochondrial membrane, in addition to respiratory inhibition. Furthermore, we demonstrate that measurable effects of mitoK_{Ca} opening cannot be obtained in isolated mitochondria by elevation of extramitochondrial Ca^{2+} or depolarization, the suggested physiological activators of this channel.

2. Materials and methods

2.1. Materials

All reagents used were analytical grade or better, and deionized water was used for all aqueous solutions. Respiratory substrates (pyruvate, malate, glutamate and succinate), ATP and EGTA stock solutions were prepared in water and buffered with the main cation used in the experimental media. NS-1619, paxilline and cyclosporin A stock solutions were prepared in DMSO; charybdotoxin and 5-hydroxydecanoate solutions (in deionized water) were prepared fresh the day of the experiment.

2.2. Mitochondrial isolation

All animal studies were approved by the *Comissão de Ética em Cuidado e Uso Animal* and conform with the *Colégio Brasileiro de Experimentação Animal* and the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health. Hearts were rapidly removed from adult (2 month) Sprague–Dawley rats weighing between 250 and 350 g, finely minced and homogenized in ice-cold buffer containing 300 mM sucrose, 0.1% BSA, and Na^+ salts of HEPES (10 mM) and EGTA (2 mM), pH 7.2. The suspension was then centrifuged at 800×*g* for 7 min, and the resulting supernatant was centrifuged at 9500×*g* for 10 min. The final pellet was resuspended in 300–500 μl of the same buffer. Mitochondrial protein concentrations were determined using the Biuret reaction. Respiratory control indexes were on average 24±7, using 5 mM malate plus 5 mM glutamate as substrates. Under these conditions, average state 3 and 4 oxygen consumption rates were 181±18 and 9.0±2.5 nmol min^{-1} mg^{-1}.

2.3. Mitoplast preparation

Mitochondria (1 mg/mL) were incubated under gentle stirring for 10 min in ice-cold medium containing 11 mM KCl and 0.7 mM HEPES, pH 7.2, then centrifuged at 12,000×*g* for 10 min. The pellet was resuspended in 20 mL of isolation buffer, centrifuged again at 12,000×*g* for 10 min, and resuspended in a small volume of isolation buffer. Rupture of the outer mitochondrial membrane was confirmed by verifying the stimulatory effect of 0.5 μM cytochrome *c* on respiratory rates.

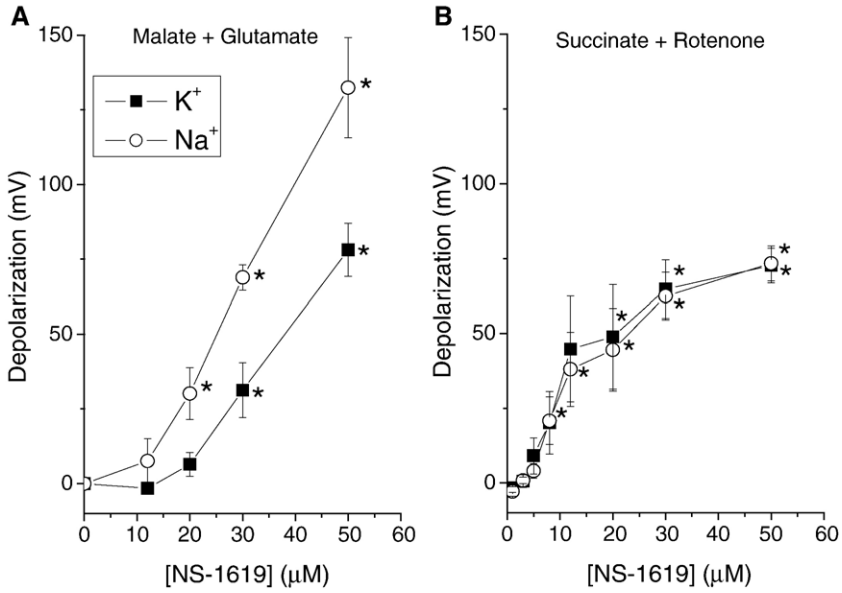

Fig. 1. NS-1619 depolarizes mitochondria suspended in both K^+- and Na^+-rich media. Rat heart mitochondria (0.25 mg protein/mL) were incubated in working buffer containing Na^+ (○) or K^+ (■) as the main cation, in the presence of 5 μM safranin O and 1 μg/mL oligomycin. In Panel A, 5 mM glutamate and 5 mM malate were used as substrates. In Panel B, 2 mM succinate and 1 μM rotenone were present. Membrane potentials were measured before and after the addition of NS-1619, and the average depolarization (in mV)±S.E.M. of 4–7 repetitions was plotted. *, $p<0.05$ in comparison to control.

2.4. Experimental conditions

Mitochondrial volume, oxygen consumption and membrane potential measurements were performed in a working buffer containing 2 mM $MgCl_2$ and K^+ salts of Cl^- (150 mM), phosphate (2 mM) and K^+-HEPES (5 mM), pH 7.2. Where indicated, experiments were also performed in buffers of similar composition, in which all K^+ salts were replaced by Na^+ or tetraethylammonium (TEA^+) salts. Further additions are mentioned in the figure legends. All experiments were conducted at 37 °C, with continuous stirring.

2.5. Mitochondrial swelling

Changes in 90° light scattering, reflecting changes in mitochondrial volume [21], were followed using a temperature-controlled Hitachi F4500 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm, with 2.5 nm slits. Light scattering decrease was calculated for each sample by taking the difference of scattered light between an early and a late time interval (3.5 to 6.5 s, and 220 to 230 s, respectively, after mitochondrial injection), and normalizing it to the scattered light at the early time interval. Light scattering decreases were calculated in the presence and absence of the pharmacological agents tested, both in K^+ or TEA^+ media, and the ratio was used as final data.

2.6. $NAD(P)^+$/NAD(P)H redox state

Fluorescence levels at 352 nm excitation and 464 nm emission, in which NAD(P)H, but not $NAD(P)^+$, fluoresces, were measured over time using a Hitachi F4500 spectrofluorometer.

2.7. Mitochondrial membrane potential estimation

Mitochondrial membrane potentials were estimated by following safranin O (5 μM) fluorescence [22] at 495 nm excitation and 586 nm emission on a Hitachi F4500 spectrofluorometer. A calibration curve was constructed using the K^+ ionophore valinomycin (0.1 ng/mL) and known K^+ additions, assuming matrix K^+ concentrations were 150 mM [23].

2.8. Measurement of mitochondrial respiration

Oxygen consumption was measured using a computer-interfaced Clark-type oxygen electrode from Hansatech Instruments, equipped with magnetic stirring.

2.9. Transmission electron microscopy

Mitochondria (0.6 mg protein/mL) were incubated for 240 or 150 s in working buffer containing either K^+ or TEA^+ as the main cation, respectively, in the presence of 1 μg/mL oligomycin, 5 mM malate and 5 mM glutamate. A shorter incubation time was used for TEA^+ because it is not extruded from the mitochondrial matrix, producing larger matrix volume increases, and eventually outer membrane rupture [24]. Suspended mitochondria were centrifuged at 16,500×*g* for 2 min and fixed first in 100 mM cacodylate buffer with 2% glutaraldehyde and then in 2% osmium tetroxide. The samples were dehydrated through a series of 70–100%

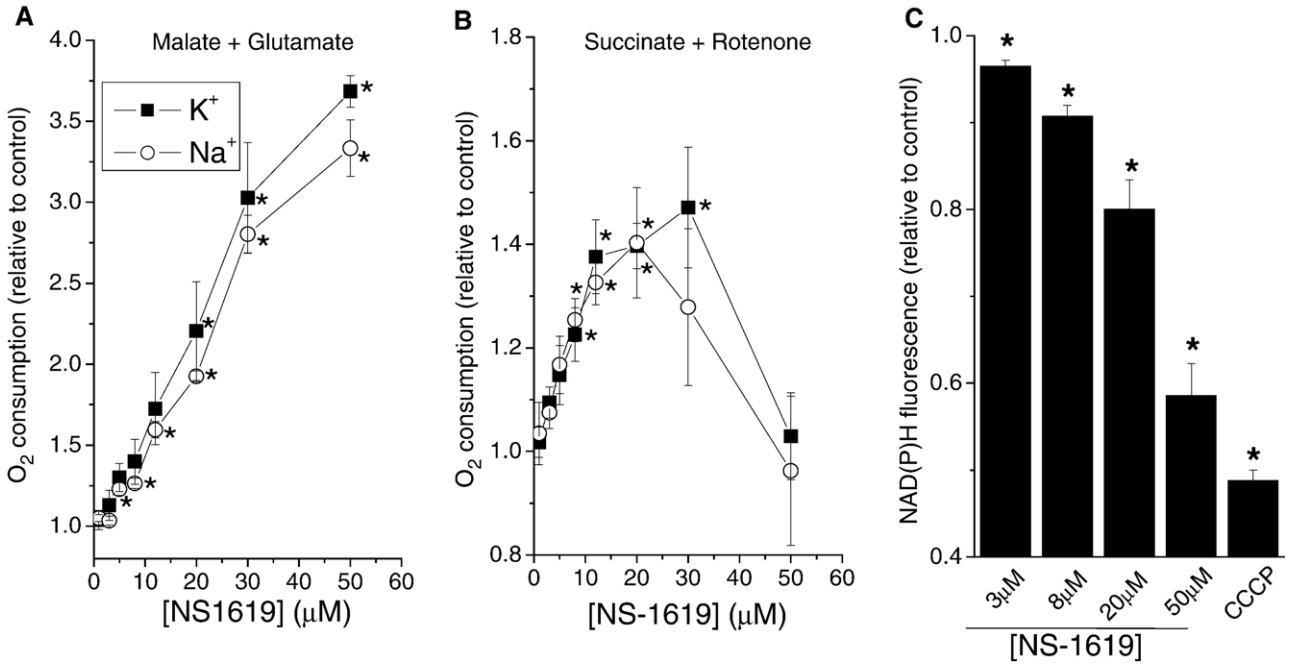

Fig. 2. NS-1619 increases non-phosphorylating respiratory rates. Mitochondria (0.3 mg protein/mL) were incubated in working buffer containing Na^+ (○) or K^+ (■) as the main cation, in the presence of 1 μg/mL oligomycin and (Panel A) 5 mM glutamate plus 5 mM malate, (Panel B) 2 mM succinate plus 1 μM rotenone or (Panel C) 2 mM succinate, 5 mM glutamate, 5 mM malate and 2 mM pyruvate. Changes in oxygen consumption (Panels A and B) or NAD(P)H fluorescence (Panel C) upon the addition of NS-1619 (as indicated) or 1 μM CCCP (Panel C) were recorded and plotted relative to control in the absence of NS-1619 and CCCP, as averages±S.E.M. of 3–6 repetitions. *, $p<0.05$ in comparison to control.

ethanol solutions and incubated in propylene oxide prior to infiltration with Spurr resin [25]. The samples then were embedded in 100% Spurr resin and polymerized at 72 °C. Ultrathin 70 to 80 nm sections were cut and stained with uranyl acetate and lead nitrate. Sections were examined with a Jeol Jem-1010 transmission electron microscope at 80 kV.

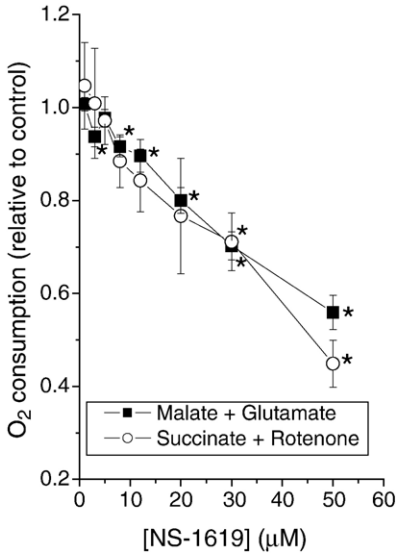

Fig. 3. NS-1619 promotes maximal respiratory rate inhibition. Mitochondria (0.3 mg protein/mL) were incubated in working buffer in the presence of 2 mM ADP and (○) 2 mM succinate plus 1 μM rotenone or (■) 5 mM glutamate and 5 mM malate. Respiratory rates were recorded before and after the addition of NS-1619, and the inhibitory effect was calculated relative to the basal oxygen consumption rate and presented as averages±S.E.M. of 3–13 repetitions. *, $p<0.05$ in comparison to control.

2.10. Data analysis

Experiments depict averages and standard errors of the mean (Figs. 1, 2, 3 and 4B) or representative results (Figs. 4A, 5 and 6) from at least three similar repetitions. NS-1619-induced depolarizations were evaluated by one-sample *t*-test comparisons against 0 mV. NS-1619-induced changes in control-normalized respiratory rates, light scattering decreases or NAD(P)H fluorescence were compared to 1, again by one sample *t*-tests. Since we were not interested in all pairwise comparisons, only in comparing treated groups to controls (in what is usually named planned comparisons) we used *t*-tests rather than ANOVA.

3. Results

Increases in inner mitochondrial membrane permeability to K^+ ions decrease the inner membrane potential and the H^+ electrochemical potential, since K^+ is positively charged and exchanged for H^+ by mitochondrial K^+/H^+ exchangers [11,26]. The extent of inner membrane depolarization is proportional to K^+ transport rates. In the case of mitoK_{Ca} channels, inner membrane potential changes should be pronounced, since the conductance and abundance of these channels is significant [12,13]. We measured the effects of NS-1619 on mitochondrial inner membrane potentials, in

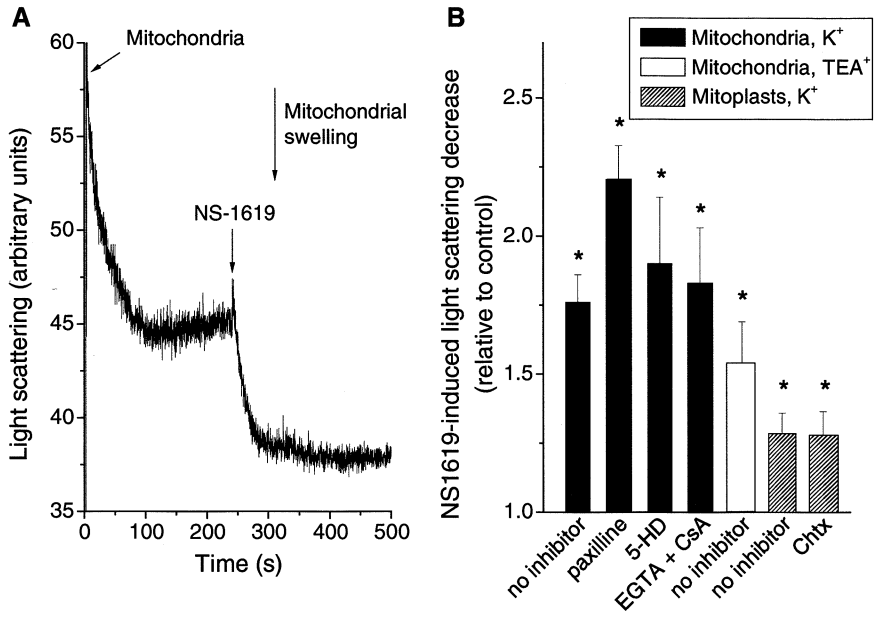

Fig. 4. NS-1619 promotes mitochondrial swelling. Mitochondria or mitoplasts (0.2 mg protein/mL) were incubated in working buffer containing K^+ or TEA^+, as indicated, as the main cation and 1 μg/mL oligomycin, 5 mM glutamate and 5 mM malate. In Panel A, 20 μM NS-1619 was added to K^+-rich buffer after initial mitochondrial volume stabilization. In Panel B, light scattering decreases in K^+ (full bars) or TEA^+ (empty bar) media promoted by 20 μM NS-1619 were measured, in the presence of no further addition (no inhibitor), 1 mM EGTA plus 1 μM cyclosporin A (EGTA+CsA), 5 μM paxilline or 300 μM 5-hydroxydecanoate (5-HD), as indicated. The striped columns represent experiments in which swelling induced by NS-1619 was followed in mitoplasts (see Materials and methods) in the presence or absence of 100 nM charybdotoxin (Chtx). Data represent averages±S.E.M. of 3–20 repetitions. *, $p<0.05$ in comparison with the respective (K^+ or TEA^+/mitochondria or mitoplast) control.

order to assess possible depolarizations promoted by mitoK_{Ca} (Fig. 1). Mitochondria were incubated in the presence of the ATP synthase inhibitor oligomycin, so changes in oxidative phosphorylation could not affect the measurements. Either malate plus glutamate (Panel A) or succinate (plus rotenone, Panel B) were used as respiratory substrates. We found that NS-1619 induced a concentration-dependent inner mitochondrial membrane depolarization, which was statistically significant at concentrations above 20 μM (malate plus glutamate) or 8 μM (succinate). This finding is in line with a previous description of mitochondrial depolarization induced by NS-1619 in glioma cells [18,19] and heart [20], and with the high conductance of mitoK_{Ca} [12,13]. However, the depolarization did not change if the main medium cation was K^+(■) or Na^+ (○), suggesting it does not reflect mitoK_{Ca} channel activity, since these channels are specific for K^+ [12].

Possible causes for the depolarization observed with NS-1619 could be respiratory inhibition and/or mitochondrial uncoupling, so we investigated the effects of this drug on mitochondrial respiratory rates, also in the presence of added oligomycin (Fig. 2A and B, note difference in scale). In the presence of malate plus glutamate as substrates (Panel A), NS-1619 induced a strong increase in respiratory rates which was statistically significant at concentrations as small as 5 μM and, at 50 μM, reached levels more than three times higher than controls. Again, the effect observed was not altered by the cation (Na^+, ○, or K^+, ■) used as osmotic support. Furthermore, increments in respiratory rates promoted by 20 μM NS-1619 (2.15±0.19, $n=6$) were unaltered by the presence of mitoK_{Ca} antagonist paxilline (5 μM, 2.14±0.23, $n=3$), indicating they are not attributable to the activity of this channel.

Increments in respiratory rates were also observed when succinate (plus rotenone) was used as a substrate (Panel B), at NS-1619 concentrations ranging from 3 to 30 μM. Interestingly, higher NS-1619 concentrations reversed the increments in respiratory rates observed at lower concentrations, suggesting the drug may not only lead to uncoupling, but may also promote respiratory inhibition (see below).

The uncoupling effect of NS-1619 was also confirmed by measuring mitochondrial NAD(P) redox state (Fig. 2C). We found that the addition of this drug to mitochondria energized by both succinate and NADH-linked substrates in the presence of oligomycin lead to significant oxidation of NAD(P)H, resulting in decreased fluorescence, which was significant at NS-1619 concentrations as low as 3 μM. The effect was dose-dependent and, at high NS-1619 concentrations, almost equivalent to that of the classical mitochondrial uncoupler CCCP.

As mentioned above, the results in Fig. 2B suggest NS-1619 may not lead only to mitochondrial uncoupling, but may also promote respiratory inhibition. Indeed, many lipophylic drugs such as NS-1619 are inhibitors of mitochondrial electron transport when used in high concentrations [9]. However, the data from Fig. 2B are not ideal to uncover a respiratory inhibition effect since maximal respiratory rates were not present, and experimental errors

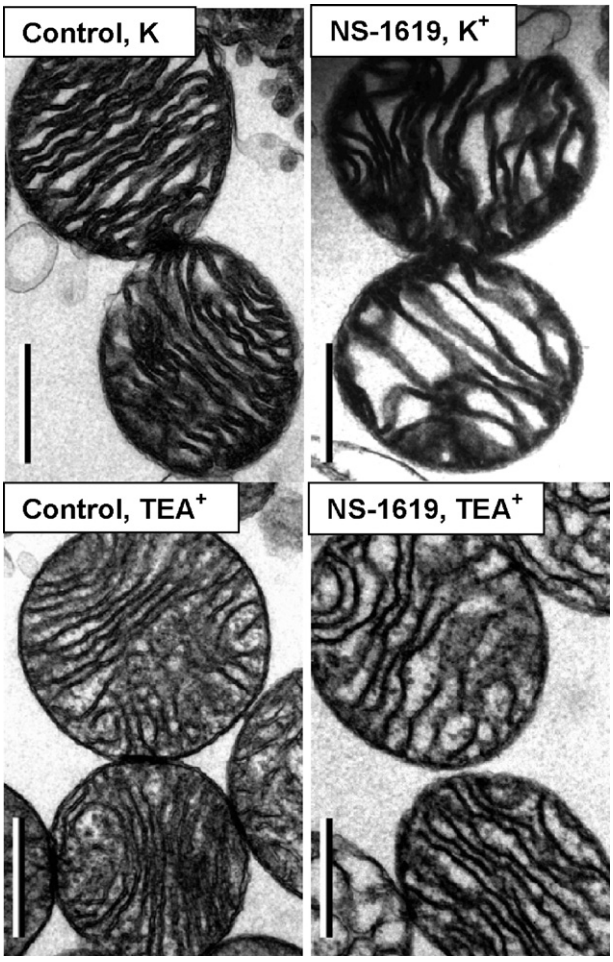

Fig. 5. NS-1619-induced changes in mitochondrial morphology. The figure depicts electron micrographs of mitochondria (magnification=30,000; scale bar length=500 nm) incubated in K^+ or TEA^+ media. In the rightmost Panels, 50 μM NS-1619 was present. Samples are typical areas of at least 5 images collected under the same experimental conditions.

were large. To investigate the respiratory effect further, we measured oxygen consumption in mitochondria in which maximum respiratory rates were induced by adding ADP (Fig. 3). Under these conditions, respiratory inhibition was promoted by NS-1619 at concentrations as low as 8 μM, with either malate plus glutamate (■) or succinate (○) as substrates. The similar extent of respiratory inhibition in the presence of substrates that reduce either complex I or II suggests that a downstream point in the electron transport chain, such as complex III or IV, is affected by this drug.

In order to investigate the site of respiratory inhibition further, we tested the effect of NS-1619 on respiration supported by 200 μM TMPD plus 2 mM ascorbate, which donate electrons directly to complex IV, bypassing complex III. Under these conditions, 50 μM NS-1619 reduced the maximum respiratory activity by 12.1±1.3% (n=4). Since 45% to 55% respiratory inhibition was observed with complex I or II-reducing substrates, this results suggests that, while complex IV contributes to the respiratory inhibition observed, at least part of the respiratory inhibition promoted by NS-1619 originates from effects of this drug on complex III. Altogether, our data show that, in addition to promoting uncoupling, NS-1619 inhibits mitochondrial respiration.

NS-1619 promoted mitochondrial uncoupling independently of the cation used in the media, suggesting it may promote non-selective inner membrane permeabilization instead of activating a cation transporter. To investigate this possibility, we measured light scattering of isolated mitochondria (Fig. 4), which is decreased as the organelles swell due to the uptake of ions and water [16]. Fig. 4A shows a typical light scattering trace over time. Mitochondria added to K^+-rich media swell rapidly, taking up K^+ ions lost during the isolation process [6], until they reach a steady state. We found that the addition of NS-1619 lead to further mitochondrial swelling, confirming that this drug increases inner membrane permeability to K^+, and not only to H^+, as indicated by the data in Figs. 1 and 2. Interestingly, the effect of NS-1619 (Fig. 4B) was also observed in buffer in which K^+ ions were substituted by TEA^+ (empty column), a cation which is not transported by any known mitochondrial carrier, and blocks several types of K^+ channels [27]. This indicates that the effect of NS-1619 is not ion selective, and cannot be ascribed to the activation of a K^+ channel. Indeed, the effect of NS-1619 was not prevented by mitoK_{Ca} antagonist paxilline (5 μM). The mitoK_{Ca} antagonist charybdotoxin was also ineffective in preventing NS-1619-induced swelling, even when used in mitoplast preparations (mitochondria devoid of outer membranes, striped columns), a condition in which this toxin is fully accessible to the inner mitochondrial membrane. Finally, mitoK_{ATP} inhibitors ATP (1 mM, data not shown) and 5-hydroxydecanoate (300 μM, Fig. 4B) or 1 mM EGTA plus 1 μM cyclosporin A, inhibitors of the mitochondrial permeability transition (a form of non-selective inner mitochondrial membrane permeabilization [28,29]) did not prevent swelling induced by NS-1619. These results indicate that the effect of this drug is to promote non-selective inner membrane permeabilization to ions in a manner independent of K^+ channels or the permeability transition.

In order to confirm our volume measurements using a more direct technique, we performed transmission electron microscopy on mitochondria incubated in the presence or absence of NS-1619 (Fig. 5). Normal mitochondrial conformations (Leftmost Panels) were altered by the addition of NS-1619 (Rightmost Panels), which caused largely enhanced matrix volumes and intercristal spaces, with decreased electron-density compared to controls. This effect occurred independently of the ion used as osmotic support, confirming that NS-1619 has significant effects on mitochondrial structure which are unrelated to selective K^+ transport.

Our results up to this point do not uncover any evidence of the activity of a mitoK_{Ca} channel, due mainly to undesirable effects of NS-1619. We thus decided to investigate possible mitochondrial effects of this channel

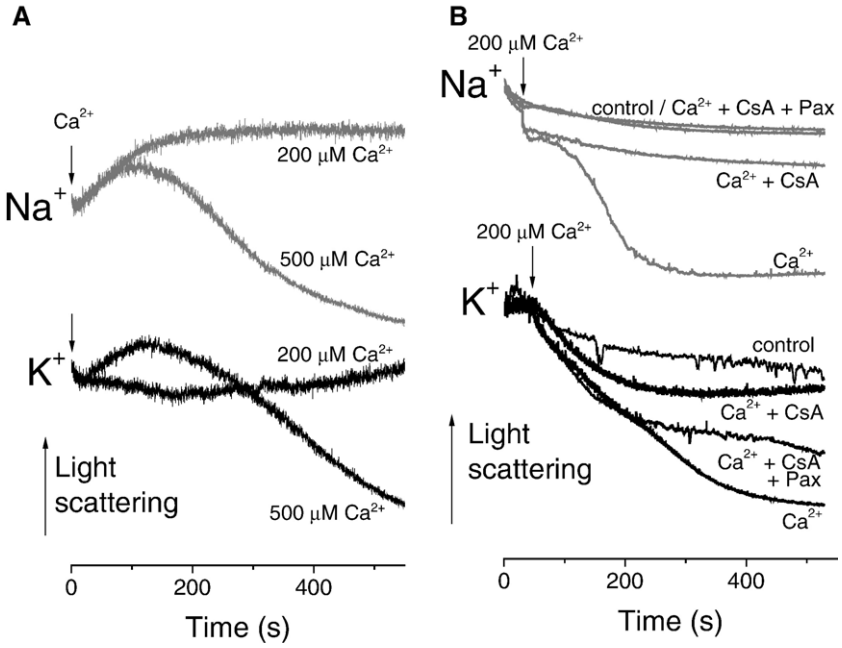

Fig. 6. Calcium and uncoupling do not promote selective K^+ transport in mitochondria. Mitochondria (0.25 mg protein/mL) were incubated in K^+ (black lines) or Na^+ (grey lines) working buffer in the presence of 1 μg/mL oligomycin, 5 mM glutamate, 5 mM malate. Light scattering was followed over time, in the presence of the Ca^{2+} concentrations indicated (Panel A). In Panel B, 1 μM dinitrophenol and 200 μM EGTA were present in all traces, and the following additions were made: none (control), 200 μM Ca^{2+} (Ca^{2+}), 2 μM cyclosporin A (CsA) and/or 5 μM paxilline (Pax). Ca^{2+} concentrations are expressed as estimated free Ca^{2+} calculated using MaxChelator software. DNP produced an average depolarization of 41.9 mV ± 3.3 mV in K^+ media and 36.0 ± 2.2 mV in Na^+ media. Traces were offset for easier visibility and represent typical experiments reproduced using at least three different preparations.

by stimulating its activity with putative physiological channel activators. Two different activating strategies were used: treating mitochondria with Ca^{2+} ions and uncoupling mitochondria with low doses of the mild protonophore dinitrophenol to decrease the inner membrane potential. Under these conditions, effects in K^+ and Na^+ media were compared, in search of K^+-specific transport. Fig. 6 shows representative data of a very large number of experiments conducted seeking a condition in which Ca^{2+} (with or without mitochondrial uncoupling, Panels B and A, respectively) lead to mitochondrial permeabilization exclusively in K^+-rich media (black lines). We found no evidence for such a condition, measuring mitochondrial swelling and varying Ca^{2+} concentrations. In all cases, when membrane permeabilization was observed (as indicated by a decrease in light scattering), it occurred also in Na^+-based media (gray lines), indicating it is attributable to non-selective permeabilization, as is typical of mitochondrial permeability transition. Indeed, the swelling observed was inhibited by mitochondrial permeability transition inhibitor cyclosporin A (CsA), but not mitoK_{Ca} antagonist paxilline (Pax), which causes slight swelling itself. These results are expected, since permeability transition is stimulated by Ca^{2+} and inner membrane depolarization [28,29].

A further attempt to verify the effects of mitoK_{Ca} in isolated mitochondria was to induce channel opening using β-estradiol [17], which has been reported to activate these channels at high concentrations (up to 10 μM; physiological concentrations usually do not exceed 2 nM [30]). In isolated mitochondria treated with 10 μM β-estradiol, light scattering measurements again indicated that increased cation permeability was not specific for K^+ (data not shown). Similar experiments were conducted using BK_{Ca} agonists Evans blue (25–50 μM), flufenamic acid (50–100 μM), GABA (2–200 μM), resveratrol (60 μM), or phloretin (50 μM) [31,32] as possible mitoK_{Ca} activators. In all cases, no specific K^+ transport was observed (data not shown).

4. Discussion

The data presented in this manuscript were obtained with the initial intent of identifying and quantifying effects of mitoK_{Ca} activity in isolated rat heart mitochondria. Using isolated mitochondria as an experimental model may present certain limitations in relation to intact cell or tissue samples, such as loss of possible regulating factors or damage induced by the isolation process. On the other hand, the use of isolated mitochondria allows for more stringent controls (such as changing the main ions in which the organelles are incubated) and a closer evaluation of bioenergetic functions (by using different substrates and respiratory states, for example). Indeed, using this experimental setup, we were able to uncover a myriad of undesirable actions of NS-1619, the main agonist used in the literature to study the cellular and whole heart effects of mitoK_{Ca} [13,15,16,33].

Interestingly, although NS-1619 has been widely used to uncover possible effects of mitoK_{Ca}, there is little evidence that it is an activator of mitoK_{Ca}, apart from the fact that this

drug activates other Ca^{2+}-activated K^+ channels (reviewed in [31,32]). Notably, in the most prominent publication related to this channel [13], the effects of NS-1619 were not tested in patch clamp experiments used to identify the activity of mitoK$_{Ca}$. Instead, NS-1619 was used in experiments measuring K^+ uptake into mitochondria using a fluorescent probe. The results obtained indicated that NS-1619 increased mitochondrial permeability to K^+, but do not determine if this permeability is specific for K^+. These findings are completely compatible with our data showing that NS-1619 increases mitochondrial permeability to K^+, Na^+ and TEA^+ (Figs. 1, 2, 4 and 5). Although NS-1619 is not capable of generating such a permeabilization in artificial phospholipid bilayers [18], mitochondrial inner membranes are extremely rich in proteins and present unique lipid compositions (such as the presence of cardiolipin [34]), which may allow for non-specific effects of this drug in mitochondrial membranes, increasing ion permeability.

In addition to uncovering NS-1619-induced non-specific inner membrane permeabilization, we found that it also promotes significant inhibition of mitochondrial respiration supported by NADH-linked substrates or succinate (Fig. 3). These results are in line with the previous finding that NS-1619 causes respiratory inhibition in glioma cells [18] and heart [19,20] and decreases respiratory control ratios in a manner insensitive to paxilline [19].

Due to the extensive mitochondrial toxicity of NS-1619, we attempted to study the bioenergetic effects of mitoK$_{Ca}$ by activating the channel physiologically. The strategies we adopted included adding Ca^{2+}, promoting inner membrane depolarization (Fig. 6), or testing the effects of a variety of signaling molecules previously described to activate these channels (as described in Results). Again, we found no evidence of selective K^+ transport in isolated mitochondrial preparations. Our inability to promote mitoK$_{Ca}$ channel opening, however, can be conciliated with patch-clamp studies of mitoplasts (mitochondria devoid of outer membranes) that show changes in the probability of the individual mitoK$_{Ca}$ channel opening promoted by depolarization and Ca^{2+} [12,13]. These studies were performed in patches at −60 to +40 mV potentials, while inner mitochondrial membranes commonly display electric potentials between −200 and −150 mV. Since polarization was found to decrease open probability, it is possible that physiological membrane potentials impair this channel's activity.

Thus, we were unable to observe pharmacological activation of mitoK$_{Ca}$ due to non-specific effects of NS-1619, and could not obtain physiological conditions in isolated mitochondria in which the activity of this channel could be observed. Indeed, considering the very large measured conductance and abundance of mitoK$_{Ca}$ [12,13], the effects of K^+ transport through this channel would be expected to lead to impaired oxidative phosphorylation, mitochondrial swelling, outer membrane rupture, release of intermembrane space proteins and, possibly, cell death. These undesirable effects are quite different from those promoted by K^+ transport through mitoK$_{ATP}$, which is very limited due to low conductance and abundance and does not impair oxidative phosphorylation nor lead to outer mitochondrial membrane rupture [9].

Both inner membrane permeabilization and respiratory inhibition observed in the presence of NS-1619 were obtained in a concentration range (3–50 μM) similar to the concentrations in which this drug has been shown to protect the heart against ischemic damage (3–30 μM [13,15,16]). This observation, added to the fact that heart preparations do not appear to have plasma membrane calcium-activated potassium channels [35], leads us to the hypothesis that the cardioprotective effects of this drug may be related to its non-specific effects and not to pharmacological activation of a target channel. Indeed, many drugs and conditions that promote mitochondrial uncoupling [36–40] or respiratory inhibition [41,42] have previously been shown to be cardioprotective. It is possible that, in a manner similar to ischemic preconditioning, moderate exposures to potentially damaging conditions can lead to signaling events that promote a more adapted cellular state to resist the damaging effects of ischemia. Furthermore, mitochondrial uncouplers may be protective against ischemia because they often reduce reactive oxygen species production [38–40,43].

In conclusion, our results show that NS-1619 has many effects unrelated to the activity of a mitoK$_{Ca}$ channel. This data uncovers the importance of careful controls when studying ion permeability of the inner mitochondrial membrane. Furthermore, our results suggest that alternative mechanisms for NS-1619-induced cardioprotection should be considered when studying the actions of this drug.

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