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Spermine selectively inhibits high-conductance, but not low-conductance calcium-induced permeability transition pore

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ABSTRACT

The permeability transition pore (PTP) is a large channel of the mitochondrial inner membrane, the opening of which is the central event in many types of stress-induced cell death. PTP opening is induced by elevated concentrations of mitochondrial calcium. It has been demonstrated that spermine and other polyamines can delay calcium-induced swelling of isolated mitochondria, suggesting their role as inhibitors of the mitochondrial PTP. Here we further investigated the mechanism by which spermine inhibits the calcium-induced, cyclosporine A (CSA) — sensitive PTP by using three indicators: 1) calcium release from the mitochondria detected with calcium green, 2) mitochondrial membrane depolarization using TMRM, and 3) mitochondrial swelling by measuring light absorbance. We found that despite calcium release and membrane depolarization, indicative of PTP activation, mitochondria underwent only partial swelling in the presence of spermine. This was in striking contrast to the high-amplitude swelling detected in control mitochondria and in mitochondria treated with the PTP inhibitor CSA. We conclude that spermine selectively prevents opening of the high-conductance state, while allowing activation of the lower conductance state of the PTP. We propose that the existence of lower conductance, stress-induced PTP might play an important physiological role, as it is expected to allow the release of toxic levels of calcium, while keeping important molecules (e.g., NAD) within the mitochondrial matrix.

1. Introduction

Permeability transition (PT) is a phenomenon of dramatically increased permeability of the inner mitochondrial membrane, which can lead to the loss of mitochondrial function and cell death [5,8,27,55]. The PT was first described by Hunter and colleagues [21] as a high-amplitude swelling of energized, isolated mitochondria in response to the addition of calcium. They concluded that large amounts of calcium accumulated in mitochondria induce an increase in the permeability of the inner mitochondrial membrane (i.e., PT), which becomes non-selectively permeable to ions and solutes. Later, electrophysiological studies established that PT is caused by opening of the channel PT pore (PTP) in the mitochondrial inner membrane [25,26,36,45,46]. The fully open PTP allows passage of molecules up to 1500 Da. Functional studies suggest that in addition to the fully open state, the PTP has a number of sub-conductance states, and can function in lower-conductance modes [26,47]. The low-conductance mode was proposed to be essential for the normal functioning of the cell under conditions of low calcium concentrations [22,23]. The high-conductance mode of the PTP is predominant during the exposure of the cell to certain stresses, like calcium overload and oxidative stress and is believed to be the primary cause of necrotic cell death [8]. The opening of the PTP can be selectively inhibited by the immunosuppressor cyclosporin A (CSA), which targets cyclophilin D [18]. Electrophysiological patch-clamp studies of native mitoplasts, as well as purified PTP components confirmed that the PTP has a number of stable sub-conductance states, of which a half-conductance state is predominant [34,46]. In fact, the PTP channel was initially termed as the Multi-Conductance Channel [25,26]. Although both high- and low-conductance states are present in patch clamp experiments, at the level of intact mitochondria during excessive calcium overload, however, only the high-conductance state of the PTP has been described.

Spermine is a biological organic polymer that has four primary amino groups. It is present in both prokaryotic and eukaryotic cells, where it is involved in the regulation of transcription [38], enzymatic activity and the cell cycle [54]. It has also been shown that spermine can be taken up by the mitochondria [50–52], where it plays a role in the regulation of enzymatic activity and free radical scavenging [37,40]. Furthermore, polyamines can inhibit calcium-induced mitochondrial swelling [31,32,39], suggesting their involvement in regulation of the PTP. Recent studies established that spermine can bind inorganic polyphosphate (polyP) with high affinity [44]. PolyP is a potent activator [1,42,43], and possibly a structural component of the calcium-induced PTP [34]. This raises the attractive possibility that the

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mechanism of spermine anti-PTP action occurs through disruption of polyP activation of the PTP.

Here, we demonstrate that spermine inhibits the high-conductance mode of PTP, which is required for induction of high-amplitude mitochondrial swelling in sucrose-based media. However, spermine did not inhibit the low-conductance mode PTP, which was defined as mitochondrial membrane depolarization and calcium release in the absence of high-amplitude swelling. These data are consistent with distinct regulation and/or nature of the different states of the PTP.

2. Experimental procedures

2.1. Animals

Sprague–Dawley rats were purchased from Charles River and housed in a climate-controlled environment with appropriate light: dark cycles. Rats were allowed to eat standard chow and water ad libitum. All procedures were approved by the Animal Care Committee of Dalhousie University and conformed to the standards of the Canadian Council on Animal Care.

2.2. Reagents

Tetramethylrhodamine methylester (TMRM) was purchased from Invitrogen. All other reagents were purchased from Sigma Aldrich.

2.3. Mitochondrial isolation

Rat liver mitochondria were isolated as described previously [13]. Briefly, the rat liver was homogenized in mitochondrial isolation buffer (300 mM Sucrose, 5 mM Tris–HCl, 2 mM EDTA, 0.5 mg/ml BSA, pH 7.4) and isolated by differential centrifugation.

2.4. Protein assay

Isolated mitochondria were resuspended in 2 ml of 1 M NaOH. Samples were homogenized and incubated at 4 °C with constant agitation for 30 min, then centrifuged at 12,000 ×*g* for 15 min. Protein concentration was determined in the supernatant of each sample with a modified Lowry method (Bio-Rad DC Protein Assay, Cat # 500–0116).

2.5. Mitochondrial PTP induction

PTP was induced by the addition of calcium to energized mitochondria. Briefly, energized isolated mitochondria (1 mg/ml of protein) in sucrose-based recording buffer (210 mM mannitol, 70 mM sucrose, 0.2 mM KH_2PO_4, 5 mM Tris–HCl, 0.8 μM rotenone, 5 mM succinate, pH 7.4) were treated either with 100 μM of $CaCl_2$ alone or in combination with 1 μM of CSA, or spermine (0.1–2 mM). The opening of the PTP was measured by three independent approaches: 1) calcium release from the mitochondria detected with calcium green 5 N (1 μM; excitation 506 nm, emission 532 nm); 2) mitochondrial membrane depolarization using TMRM probe (0.1 μM; excitation 546 nm, emission 590 nm) as previously described [13,41]. This method relies on the quenching of TMRM accumulated inside polarized mitochondria. When mitochondria become depolarized, TMRM is released into the media causing the overall increase in fluorescent signal; and 3) mitochondrial swelling measured as a decrease of light absorbance. The specific signal detected in these experiments is the intensity of the light passing through the recording cuvette. Under conditions of mitochondrial swelling, the mitochondrial matrix becomes less dense, and more light can pass through (i.e., the media becomes less light-absorbing) and thus the intensity of transmitted light increases. The intensity of light directly passing through the cuvette was recorded with both emission and detection wavelengths set at 540 nm with an in house modified Quantamaster-4 Spectrofluorimeter (PTI, Birmingham, NJ). In the mitochondrial swelling/light absorbance experiments, no fluorescent probe was used. Unless indicated otherwise, complete mitochondrial swelling was achieved by the addition of 5 μM of alamethicin. Data was analyzed with FelixGX software (PTI, Birmingham, NJ).

2.6. Electron microscopy

Following treatment with either calcium alone, calcium plus spermine or control without calcium and without spermine, samples were fixed in 2.5% glutaraldehyde in sucrose buffer and processed in the EM Facility Core (Dalhousie University). Briefly, the samples were centrifuged and rinsed 3 times in 0.1 M sodium cacodylate buffer and fixed in 1% osmium tetroxide for 2 h and, after dehydration, embedded in epon araldite resin. Approximately 100-nm thick sections were cut with an ultramicrotome and placed on 300 mesh copper grids, which were stained with 2% aqueous uranyl acetate, rinsed and treated with lead citrate, then rinsed and air dried. Images were captured with a Jeol Jem 1230 transmission electron microscope at 80 kV with Hamatsu ORCA-HR digital camera attached to the microscope.

2.7. Respirometry

Isolated rat liver mitochondrial respiratory oxygen flux (JO_2) was measured in high resolution, concurrent with fluorometric signal for calcium green 5 N, using the Oxygraph-2k with O2k-Fluorescence LED2-Module (OROBOROS Instruments, Innsbruck, AT). All mitochondrial samples were assessed in 2 ml of assay buffer, consisting of 210 mM of mannitol, 70 mM of sucrose, 0.2 mM of KH_2PO_4, 5 mM of Tris–HCl, and pH 7.4. The concentration of O_2 in the experimental chambers was maintained between 10 and 200 μM. All experiments were conducted at 25 °C. Instrumental background O_2 consumption was corrected using equations determined under the same parameters used for experimental data collection. In the presence or absence of 0.2 mM of spermine, respiration was initiated with 10 mM of glutamate and +4 mM malate. After JO_2 stabilization, PTP induction was initiated by addition of 50 μM $CaCl_2$, with calcium uptake and release confirmed by fluorometric signal. 1 mM of EGTA was added to determine whether PTP could be subsequently reversed by chelation of calcium (confirmed in fluorometric signal).

3. Results

3.1. Spermine inhibition of the PTP

In order to establish the appropriate conditions in which to study the mechanisms of action of spermine, isolated energized mitochondria (1 mg/ml of protein) were treated with different doses of spermine (10 to 200 μM) and challenged with either 100 μM or 200 μM of calcium. Calcium-induced PTP opening was first studied with mitochondrial swelling, detected as an increase of the intensity of transmitted light due to the decrease in absorbance at 540 nm [20]. Under these experimental settings, the maximal light intensity corresponds to the minimal absorbance of the media. Calcium-induced swelling was inhibited by spermine in a dose-dependent manner (Fig. 1A and B and Supplementary Fig. 1). In Fig. 1B the degree of inhibition was estimated from the value of light intensity at the end of experiment and normalized to light intensity in the presence of alamethicin (100%, completely swollen mitochondria) using the initial intensity of light as no swollen mitochondria (0%, completely intact mitochondria). Note that under the experimental conditions in Fig. 1A swelling was inhibited only partially. At concentrations higher than 200 μM, spermine also blocked the transport of calcium into the mitochondria (data not shown). Thus, higher concentrations of spermine were not used in our experiments. Notably, in experiments with 100 μM of calcium in the presence of high spermine concentrations we did not see any noticeable increase in swelling

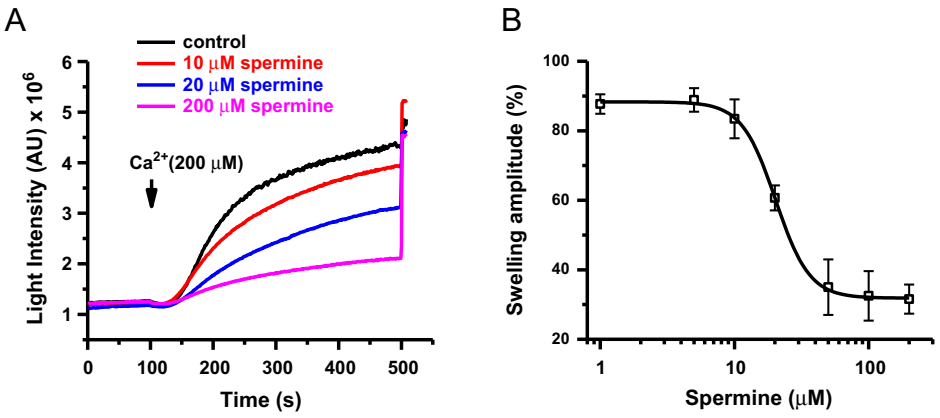

Fig. 1. Dose–response for spermine inhibition of mitochondrial swelling. Isolated mitochondria were incubated with different amounts of spermine (10–200 μM) and challenged with 200 μM of $CaCl_2$. A. Light absorbance assay traces in the presence of the different spermine concentrations. The Y-axis reading is the intensity of transmitted light. At the end of each experiment 5 μM of alamethicin was added to achieve complete swelling. B. Dose–response for the percent inhibition mitochondrial swelling with spermine treatment.

(compare Fig. 1A and Supplementary Fig. 1). It is possible that under the experimental protocol and amounts of calcium that were used in this section, spermine not only inhibited swelling but also inhibited membrane depolarization and calcium release. Because 200 μM of spermine produced a maximal inhibitory effect on the PTP without affecting the ability of mitochondria to uptake calcium, we chose this concentration of spermine to further investigate the mechanisms behind these observations.

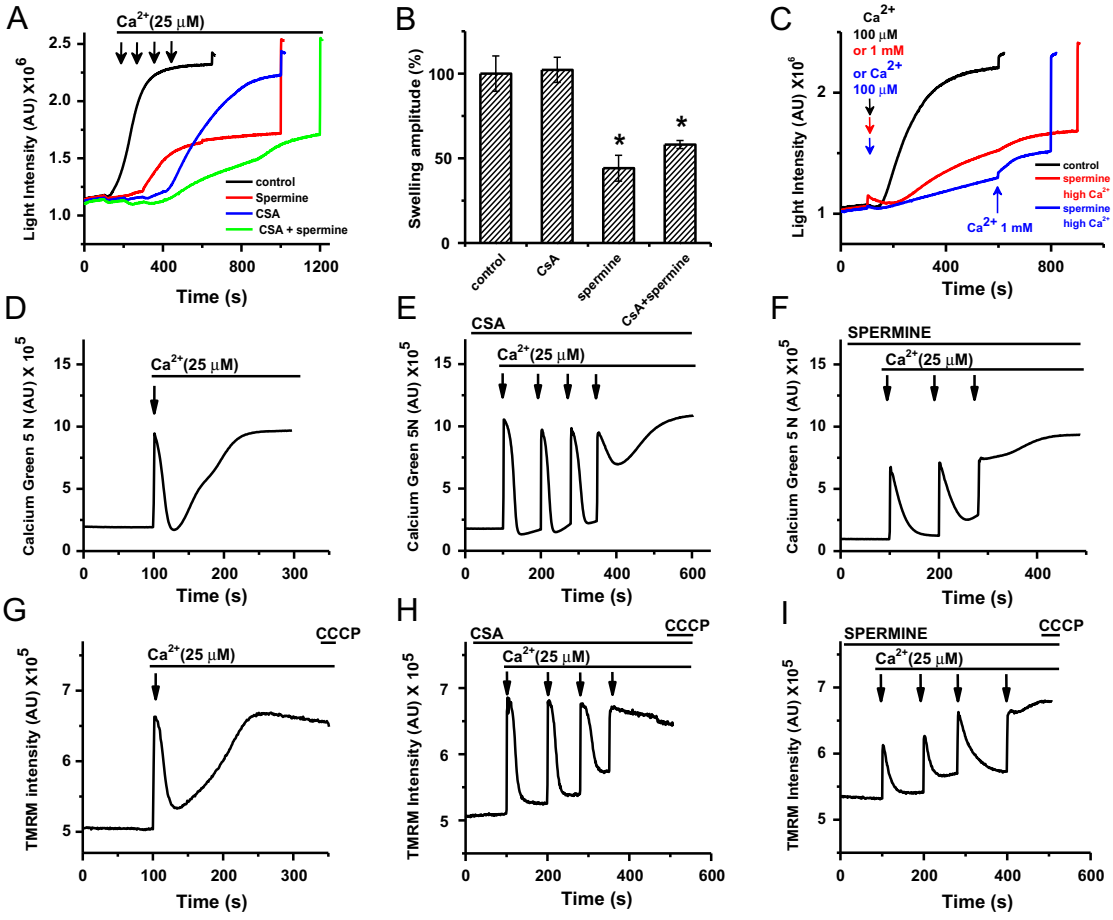

Fig. 2. Spermine inhibits swelling but allows calcium release and membrane depolarization. A. Mitochondrial swelling in the presence of CSA (1 μM) and/or spermine (200 μM). 5 μM alamethicin was added at the end of each experiment to establish the complete swelling. B. Degree of mitochondrial swelling as a percentage of maximum in the presence of alamethicin; *significantly different ($P < 0.05$) compared to control. C. Mitochondrial swelling following addition of the large amounts of calcium (1 mM, approximately 10 times higher than required for activation of PTP). Even with high amounts of calcium, swelling is only partial in the presence of spermine. 5 μM alamethicin was added at the end of each experiment to establish the complete swelling. D–F. Representative traces showing measurements of calcium-induced calcium release in control (D), CSA (E), and spermine-treated mitochondria (F). G–I. Representative traces showing measurements of the mitochondrial membrane potential in control (G), CSA (H), and spermine-treated (I) mitochondria; With the exception of panel C, all traces in this figure are from the same mitochondrial preparation — representative of 3 mitochondrial isolations, experiments done in triplicates for each individual isolation.

3.2. Spermine and CSA inhibit PTP through different mechanisms

Next, we compared the effects of spermine versus CSA on PTP inhibition. To test these effects, the PTP was assayed by measuring calcium-induced swelling (Fig. 2A), calcium release (Fig. 2D–F) and mitochondrial membrane potential depolarization (Fig. 2G–I). As illustrated in Fig. 2A (control trace), addition of 25 μM calcium induced near-complete mitochondrial swelling in untreated samples, with only moderate further swelling elicited by addition of pore-forming alamethicin. For isolated mitochondria treated with CSA (Fig. 2A, CSA trace), 4 additions of 25 μM calcium were required to induce swelling. Despite the requirement for greater calcium concentration, in the presence of CSA, the degree of swelling was comparable to control. In the presence of spermine, by contrast, the concentration of calcium which induced PTP, as evident from Fig. 2 F and I, caused only partial swelling (Fig. 2A, spermine trace, and Fig. 2B). Importantly, in control as well as in the presence of either CSA or spermine, mitochondria demonstrated behavior typical of the normal PTP detected by mitochondrial calcium release (Fig. 2 D–F) and membrane depolarization (Fig. 2 G–I). The observation that calcium challenge resulted in mitochondrial calcium release and membrane depolarization, but not swelling in spermine-treated samples, suggests that spermine prevents the high-amplitude swelling typical of high-conductance PTP. We should note that when moderate concentrations of calcium were used to induce swelling, the resulted degree of swelling varied to some extend likely due to the mode of calcium addition. For example when calcium was added in 25 μM of aliquots (rather than bulk addition) less amount was required to induce partial swelling (compare Fig. 2A, red trace with Supplementary Fig. 1 and blue trace Fig. 2C). Further, it is possible that sensitivity of mitochondria toward swelling can vary from preparation to preparation. These raise the possibility that partial swelling seen in our experiments was due to the relatively moderate calcium load and presence of subpopulation of intact mitochondria. To exclude the possibility that incomplete swelling was simply due to a remaining fraction of intact mitochondria in the sample, we also tested the effect of excessive calcium (1 mM) on spermine-treated samples. Even under these extreme conditions, swelling remained incomplete (Fig. 2C), rebutting the possibility of a remaining fraction of intact mitochondria.

3.3. Changes in mitochondrial morphology in the presence of spermine

The light scattering assay described in the previous section detects changes in the whole population of mitochondria suspended in the cuvette, but does not provide information about changes at the level of the individual organelle. To address this, we used electron microscopy to investigate morphological changes of the mitochondria under conditions identical to those described in the previous section. Results of these experiments are presented in Fig. 3. Isolated mitochondrial samples examined with electron microscopy were first monitored using a light absorbance assay to ensure that the mitochondria achieved fully developed PTP. Samples for electron microscopy were collected after treatment with either calcium alone, spermine plus calcium or vehicle, immediately prior to alamethicin addition (see Fig. 2A for the reference). In addition to characterizing the morphology of the mitochondria, we also performed quantitative analysis of the electron microscopy images by counting the number of mitochondria of each type found in the imaging field (see representative images of the field in Supplementary Fig. 1). We used 3 fields for each condition, and 2 independent mitochondrial isolations. In the mitochondrial morphology description below, we indicate the average (number of mitochondria ± S.D.) of each type found per field (images were taken at 10,000× and the fields were 196 μm^2). Control mitochondria appeared as relatively dense structures with easily recognizable cristae and double membranes (Fig. 3 A, D ($n = 3 \pm 2$), G ($n = 30 \pm 2$) and J, "no spermine, no calcium" column). Consistent with earlier reports, calcium-treated mitochondria that underwent high-amplitude swelling appeared as fully swollen, nearly transparent structures with only single membranes remaining (Fig. 3 "calcium, no-spermine" column, $n = 42 \pm 9$) and with only small fraction of population having double-membrane structure ($n = 3 \pm 1$). When PTP was activated in the presence of spermine, mitochondrial morphology differed substantially from the transparent structures observed in calcium-treated controls (Fig. 3). Although compared to normal mitochondria, the morphology of the spermine-treated mitochondria was substantially altered (Fig. 3), they still exhibited electronically dense matrix with some preserved internal crista structure (Fig. 3F, $n = 34 \pm 10$). In fact, some of the spermine-treated mitochondria exhibited morphological similarities with normal controls (Fig. 3I, $n = 10 \pm 1$), and only few of the mitochondria ($n = 5 \pm 2$) appeared to lose their structural integrity to the same degree as calcium-challenged mitochondria without spermine. Importantly, in the presence of spermine, the integrity of the outer mitochondrial membrane was maintained in both types of mitochondria, as evidenced by the presence of double membranes (see arrows, Fig. 3F, I and L). Overall, these data support the interpretation that spermine inhibits high-amplitude mitochondrial swelling.

3.4. Effect of ionic strength on spermine inhibition

Next, we investigated effects of spermine on mitochondrial swelling in a solution containing 150 mM of KCl in place of sucrose. Under these conditions, mitochondrial swelling during PTP formation is due to free diffusion of potassium and chloride ions. In this salt solution, spermine had no effect on mitochondrial swelling (Fig. 4A). This suggests that in the presence of spermine, the low-conductance PTP is not cation- or anion-selective. To determine whether the effects demonstrated in Fig. 4A are due to the ionic strength of the media, we used a recording solution containing methyl-D-glucamine ($NMDG^+$), a positively charged molecule similar in size to mannitol. Including $NMDG^+$, a large monovalent ion, increases the ionic strength of the media without permeating selective, low-conductance channels. Therefore, $NMDG^+$ will not contribute to mitochondrial swelling if a low-conductance mechanism is activated. When the buffer media contained only $NMDG^+$, not even control mitochondria underwent high-amplitude swelling (data not shown). Presumably, this was due to the combination of both the charge and size of $NMDG^+$, as $NMDG^+$ does not permeate even the high-amplitude PTP. However, when $NMDG^+$ buffer was mixed in equal volumes with sucrose–mannitol buffer to allow osmotic swelling when the PTP is open, mitochondrial swelling was observed in control mitochondria with 100 μM of calcium. Both spermine and CSA inhibited this calcium-induced swelling (Fig. 4B). These results support that the mechanism by which spermine prevents PTP-dependent mitochondrial swelling does not depend on the ionic strength of the solution.

3.5. Spermine inhibits swelling in uncoupled mitochondria

Although spermine inhibited mitochondrial swelling in the above experimental conditions, it was still only about 50% (as determined by the light scattering experiments) compared with control. We hypothesized that the degree of swelling in those cases might be related to the amount of the total mitochondrial calcium load and that the degree of swelling can be reduced if less calcium is used to trigger the PTP. To test this, we investigated mitochondrial swelling under conditions of mild uncoupling induced by CCCP. Under these conditions, significantly less calcium is required for induction of PTP. In the presence of CCCP, spermine inhibition of swelling (Fig. 5C and F) was stronger than previously described inhibition in the absence of CCCP (Fig. 2). Notably, under these conditions, mitochondria were depolarized and calcium was released from the mitochondria (Fig. 5D and E). These results suggest a relationship between calcium load and swelling, as less calcium is accumulated in uncoupled mitochondria. Indeed, when lower concentrations of calcium are required for induction of PTP, as

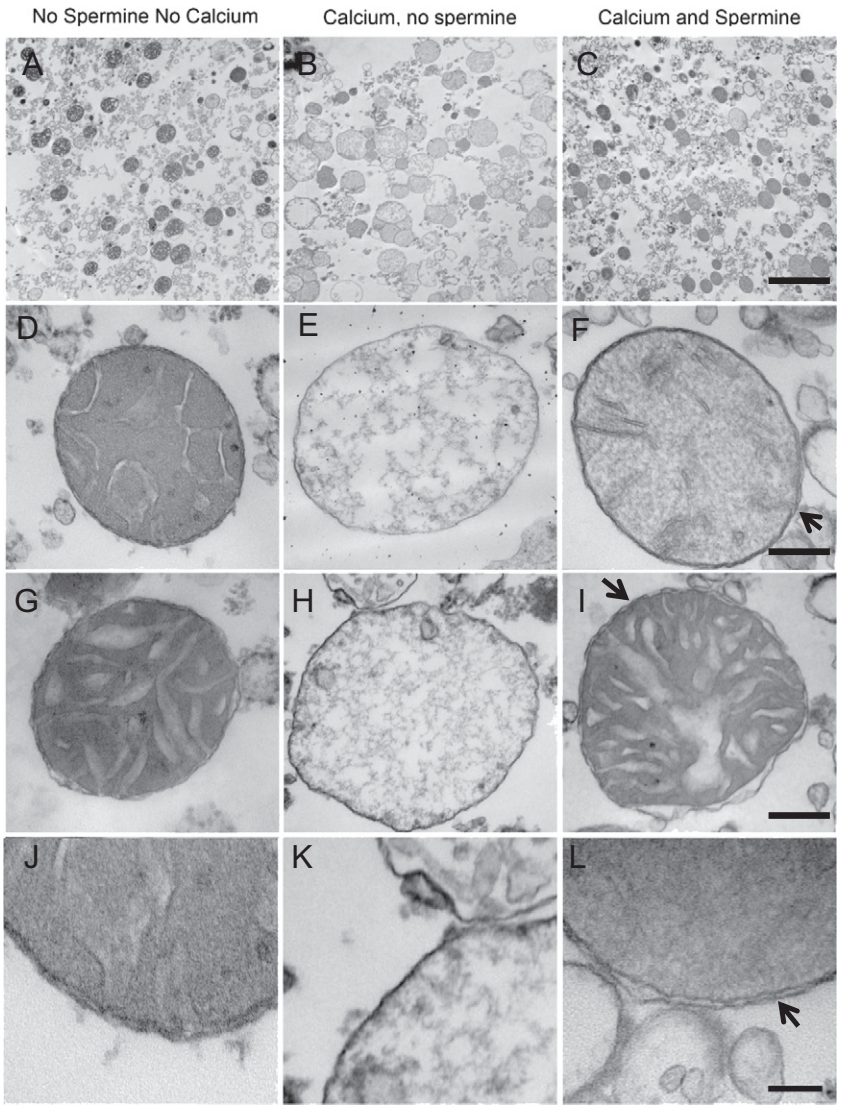

Fig. 3. Spermine inhibits swelling of mitochondria and partially preserves their morphology. Mitochondrial morphology was imaged via electron microscopy in untreated (Control panels A, D, G and J), calcium-treated (B, E, H and K) and spermine- and calcium-treated isolated mitochondria (C, F, I and L). The top three panels show low-magnification images (10,000×, scale bar = 2 μm). The panels below show that comparable to the unchallenged controls (D, G and J), mitochondria pre-incubated with spermine exhibit preserved double membranes, despite calcium challenge (F, I, L, arrows pointing at double-membranes). Mitochondria not treated with spermine are less dense in the matrix and lack double membranes after calcium treatment (E, H and K). Scale bars A–C, 2 μm; scale bars D–L, 100 nm; $n = 3$.

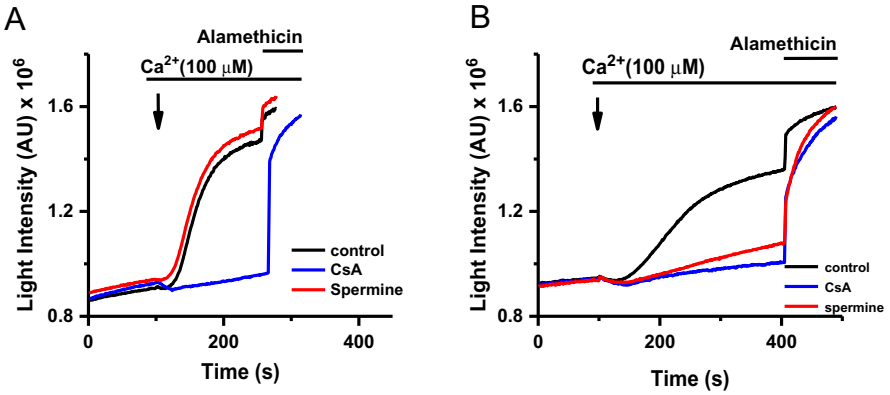

Fig. 4. Spermine does not inhibit swelling in KCl-based media, but does in the presence of $NMDG^+$. A. Spermine does not inhibit swelling in the buffer containing 150 mM of KCl. B. When sucrose was replaced partially by an ion larger in size and unable to cross intact mitochondrial inner membranes ($NMDG^+$), spermine inhibited swelling. n = 3; experiments done in triplicate.

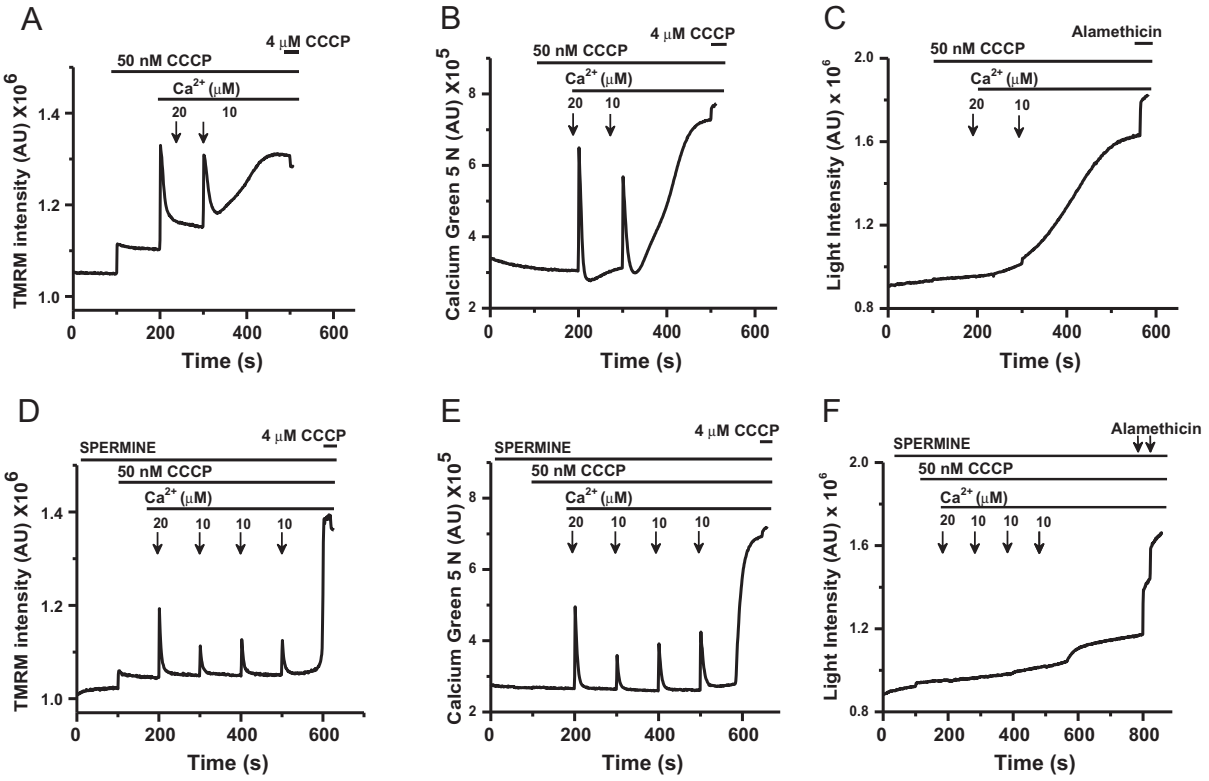

Fig. 5. Spermine inhibits swelling in mildly uncoupled mitochondria. Isolated mitochondria were treated with 50 nM CCCP, followed by calcium to induce the PTP in the absence (A–C) or presence of 200 μM spermine (D–F). Membrane potential (A and D), calcium release (B and E) and swelling (C and F) were analyzed. Compared to control (A and B), spermine delayed the opening of PTP (D and E); however, the same concentration of calcium induced only a small degree of swelling in panel F compared to control in panel C. Note that in the experiment illustrated in panel F, 2.5 μM of alamethicin was injected sequentially to the total final amount of 5 μM.

determined by membrane depolarization and calcium release, less swelling occurs.

3.6. Mitochondrial swelling in the presence of high phosphate and spermine

Next we investigated whether inhibition of swelling by spermine is related to its ability to increase the threshold of calcium-induced PTP activation. To study this, we monitored mitochondrial swelling in the presence of 1 mM phosphate. Under these conditions, spermine does not delay the onset of calcium-dependent PTP. Indeed, spermine did not inhibit calcium release (Fig. 6B) but almost completely inhibited swelling (Fig. 6A). These results suggest that spermine possesses dual inhibitory effects: one linked to the increase in mitochondrial calcium retention capacity, and the other to the inhibition of the high-conductance mode of PTP.

3.7. Mitochondrial respiration in the presence of spermine and calcium

Our data suggest that although spermine treatment did not prevent mitochondrial calcium release and membrane depolarization, the mitochondrial integrity was partially preserved (Fig. 3 F, I, L). In order to test whether this partial protection afforded by spermine is sufficient to maintain mitochondrial respiratory function in isolated rat liver mitochondria, the JO_2 under these conditions was investigated. Using high-resolution respirometry (Fig. 7B and D) coupled with simultaneous calcium green fluorescence monitoring (Fig. 7A and C), mitochondrial respiration was assessed during routine glutamate + malate oxidation, followed by calcium-induced PTP formation (calcium release) and closure (EGTA calcium chelation). The results demonstrate that although it took the spermine-treated mitochondria longer than control to release calcium (indicative of delayed PTP formation), the

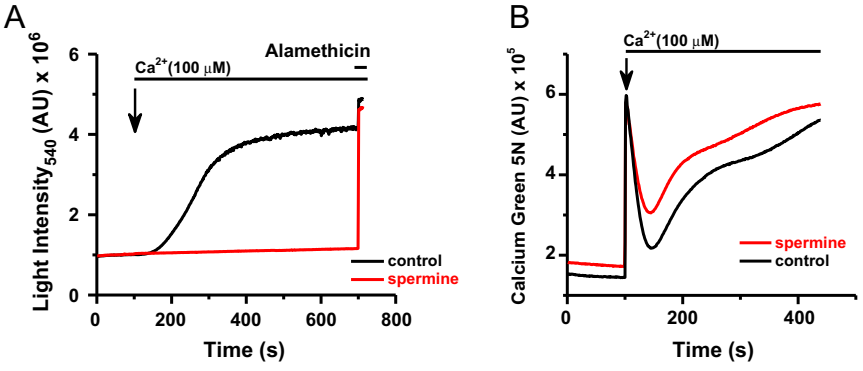

Fig. 6. In the presence of 1 mM phosphate, spermine inhibits swelling, but not the release of calcium. Isolated mitochondria were treated with 100 μM $CaCl_2$ in the presence (red trace) or absence (black trace) of 200 μM spermine. A. Mitochondrial swelling was inhibited when spermine was present. B. Spermine did not prevent calcium release from mitochondria.

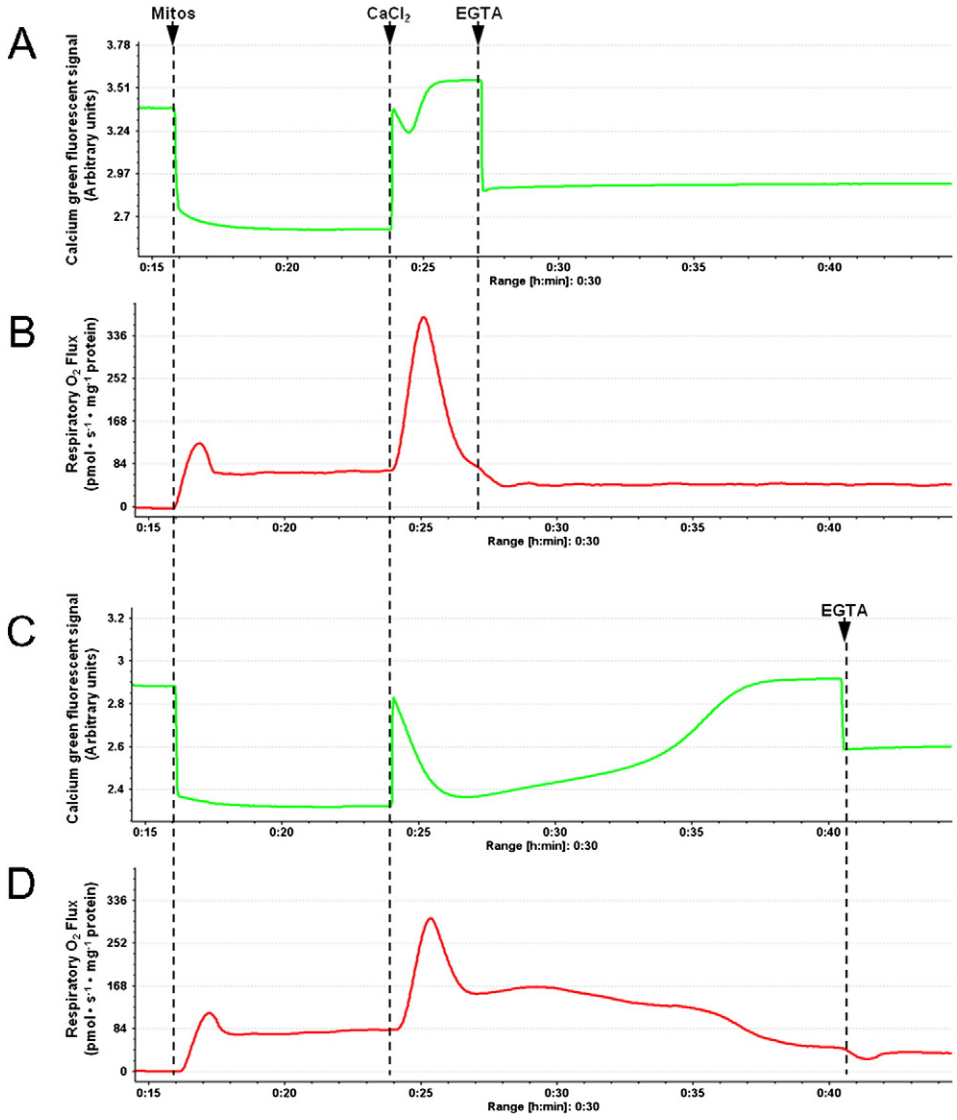

Fig. 7. Spermine does not preserve respiratory function following calcium-induced PTP formation. Isolated liver mitochondria ("Mitos") were injected into the oxygraph chamber containing 10 mM glutamate and +4 mM malate in physiological buffer solution alone (A and B) or containing 0.2 mM of spermine (C and D). Addition of 50 μM $CaCl_2$ led to calcium uptake (decrease in fluorescent signal for calcium green; A and C) and eventual calcium release (increase in calcium signal). Chelation of calcium with 1 mM EGTA did not restore respiratory function in any of the samples, as indicated by no increase in the respiratory oxygen flux (B and D).

subsequent JO_2 was negligible. Moreover, PTP closure with EGTA did not affect respiration in either condition (Fig. 7B and D). Overall, these data indicate that despite the prevention of swelling/PTP-related damage with spermine in our experimental conditions, mitochondrial respiratory function is not preserved.

3.8. DAPI inhibits swelling

According to our previously proposed model [34], in order to be functional, the PTP pore has to contain polyP, likely as a part of channel-forming poly-β-hydroxybutyrate/calcium/polyP complex. Also, PolyP is proposed to be an endogenous activator of the PTP [44]. Considering that DAPI, a fluorescent probe (Fig. 8A), is capable of binding polyP [3,49], we decided to test the effect of DAPI on mitochondrial swelling. Fig. 8C shows that DAPI inhibits mitochondrial calcium-induced swelling in a concentration-dependent manner. Importantly, this effect is likely not due to the ability of DAPI to inhibit calcium uptake. Indeed, as indicated in Fig. 8D, when the calcium-sensitive Calcium Green probe was used, both calcium uptake as well as calcium release still occurred.

4. Discussion

In the present study, the mechanisms of PTP inhibition by the polyamine spermine were investigated. We found that in addition to the well-established ability of spermine to reduce the calcium sensitivity toward PTP formation in mitochondria [31,48], spermine also blocks high-conductance PTP activity. This effect results in mitochondrial depolarization and calcium release from the mitochondrial matrix. Interestingly, spermine prevented mitochondrial swelling, even in the presence of calcium levels far exceeding those minimally required for PTP activation (Fig. 2 C). Indeed, electron microscopy confirmed intact double membranes in the majority of the spermine-treated mitochondria, as well as high-density matrices after the treatment with elevated calcium (Fig. 3C, F, I, and L). It is notable that even in the presence of the classical PTP inhibitor CSA, high levels of calcium induced swelling to the same degree as in control. This suggests that mechanism by which spermine inhibits the PTP differs fundamentally from most known PTP inhibitors (including CSA), which prevent or delay PTP opening, but do not affect the properties of the activated channel.

Results of the present study may have particular relevance to the proposed physiological roles of the PTP. A transient PTP opening can

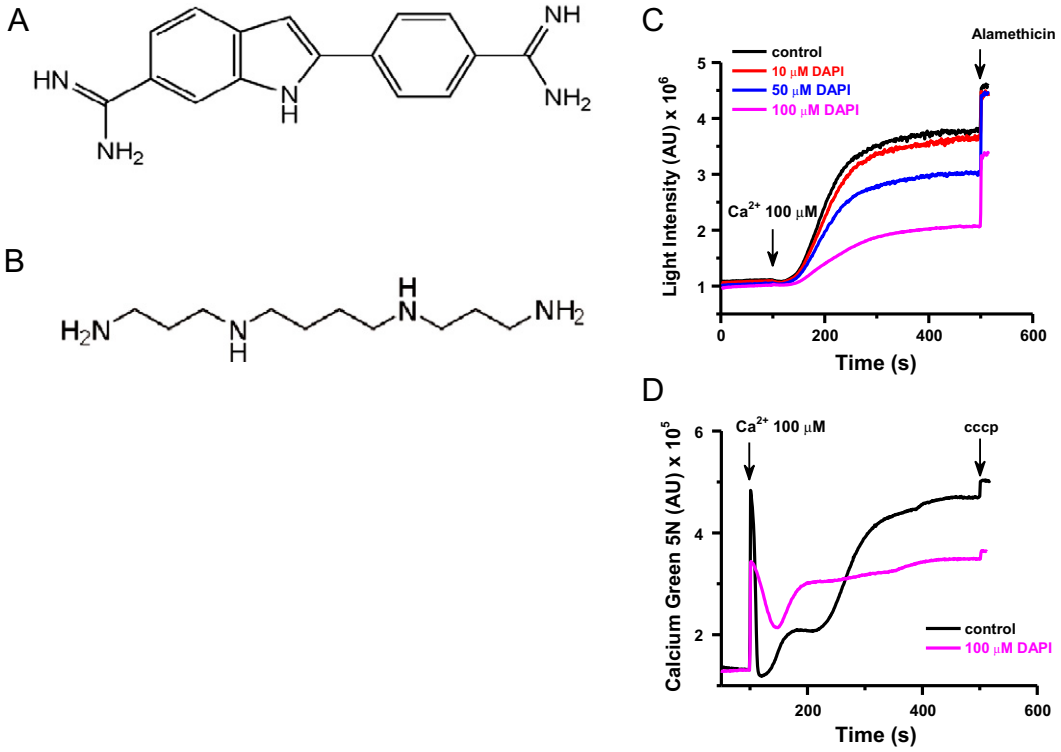

Fig. 8. Inhibition of the mitochondrial swelling by DAPI. A. Molecular structure of DAPI. B. Molecular structure of spermine. C. DAPI inhibits calcium-induced mitochondrial swelling. Isolated mitochondria were treated with different amounts of DAPI and challenged with 100 μM $CaCl_2$. Swelling was monitored spectrophotometrically at 540 nm. At the end of each experiment 5 μM of alamethicin was added to achieve complete swelling. D. Experiments confirming that DAPI did not inhibit mitochondrial calcium uptake and that calcium release still occurs.

be detected in intact cells [23,35]; such openings might play protective roles for example as calcium "release valves" [9]. In addition to accumulated calcium, profound mitochondrial damage can be triggered by increased mitochondrial production of reactive oxygen species (ROS) and related oxidative stress [10,14,29]. Thus, in addition to calcium, such transient opening of PTP could be protective by helping to decrease the amount of ROS inside the mitochondria. It is intriguing to consider the possibility that these transient PTP events can occur not only in the fully open, but rather in more nuanced, sub-conductance states. The benefits of such small-conductance states of the PTP should be apparent, considering that even a transient occurrence of the fully open state of the high-conductance PTP can disrupt mitochondrial function due to the release of physiologically important compounds from the matrix (e.g. NAD, glutathione, etc.). We should also note that the concentrations of spermine used in the present study are similar to those found in the cytoplasm [53]. Although the possibility that spermine protects mitochondria from high-conductance PTP is attractive, we were not be able to detect a protective effect of spermine on mitochondrial respiration — at least, not at the level of isolated liver mitochondria. In these functional experiments, even partial swelling led to the loss of respiration, similar to control. However, it is possible that in the case of transient PTP openings, protective effects will be more profound. Another interesting physiological implication is the possible occurrence of PTP and loss of mitochondrial function, without destruction of the outer membrane. Presumably, in such a situation where the release of mitochondrial pro-apoptotic factors (e.g., cytochrome *c*) is prevented, mitochondrial death could occur without the induction of apoptosis [27]. Indeed, the effects of spermine on PTP formation are considered to play an important role in apoptosis [53]. However, these options, though attractive, will require further investigation. We cannot say conclusively what the size of this lower conductance state of PTP is observed in our experiments. Taking into account that swelling still occurs to the full extent in KCl media, we can conclude that this state is not strongly selective between cations and anions. It is possible that this lower conductance state corresponds to the "half-conductance" state observed in patch clamp experiments, which is not highly selective [47]. We should note that initially, it was suggested that this half-conductance state might be linked to the presence of VDAC as an essential part of the pore. However, an essential role for VDAC in the PTP was not confirmed by experiments involving transgenic knock-out models [4,30]. In native PTP channels, not only sub-conductance states have been observed, they are also consistent with the newly proposed involvement of dimers of the ATP synthase PTP complex [15]. This further supports the notion that the presence of sub-state in PTP is not necessarily related to the VDAC activity.

The mechanisms by which spermine inhibits the PTP are not well-understood. It has been proposed that spermine may mask membrane surface charges, and in doing so, alter membrane surface potential [6]. This would explain the inhibitory effect against the onset of PTP. However, this is unlikely to result in the partial swelling observed in our light scattering experiments. Indeed, the effects of spermine were similar in the high and low ionic strength media (Fig. 4 B), suggesting that the mechanism of action does not depend on membrane surface charge.

Another potential explanation for spermine's mechanism of action is its recently discovered ability to bind inorganic polyphosphate (polyP). PolyP is proposed to be an endogenous activator of the PTP [44]. The molecular nature of PTP is not well-defined. PTP is suggested to be a multi-protein complex with the central part likely formed by dimers of ATP synthase [11,15] or by the channel within its C-subunit ring [2] (see also comment in [24]). Importantly, involvement of C-subunit in PTP has been demonstrated at the level of live cells [56]. However the view of the involvement of C-subunit ring is not consistent with a number of experimental observations (see [7,12,17] for details). Other membrane proteins have also been implicated as parts of the complex, such as ANT, VDAC and the phosphate carrier [19,33]. Recent review suggests that PTP pore might be formed at the interface between ATP synthase, ANT and Phosphate Carrier [19]. However, we should note that at least for calcium-induced PTP in isolated mitochondria none of these

proteins proved to be essential [4,16,28]. Furthermore the physical association (if any) between ATP synthase and abovementioned proteins remains to be established. Overall, the molecular identity of the components essential for pore formation has not been established yet, with no protein definitively assigned to be its pore part and is a subject of hot debate. On the other hand, according to our previously proposed model [34], in order to be functional, the PTP pore part does not require to contain any proteins but has to contain polyP, likely as a part of a channel-forming poly-β-hydroxybutyrate/calcium/polyP complex. We propose the possibility that increased spermine concentrations can lead to the binding of polyP and make it unavailable for binding to the other molecular components of PTP. This would result in the inability of the PTP channel to achieve a fully open conformation. While testing this hypothesis will require further investigation, it should be noted that the effects of spermine can be mimicked by the addition of DAPI (Fig. 8). DAPI is a fluorescent probe which is capable of binding polyP [3,49]. The fact that two molecules with significantly different chemical structures can cause similar inhibitory effects suggests that the mechanism of spermine action does not occur through interaction with some specific binding site, but rather some relatively non-specific target. Because it can assume multiple conformations, polyP could certainly fulfill the role of such target.

The present work demonstrates pharmacological regulation of the low- and high-conductance PTP. Different conductance states of the PTP have been previously identified utilizing electrophysiology techniques. Here, we demonstrate that PTP has at least two conductance states that can be discerned pharmacologically at the level of the intact mitochondria. Understanding the mechanisms through which spermine and similar compounds exert their effects on PTP formation and function may lead to the discovery and development of new treatments to prevent the damage produced by PT under pathological conditions. Furthermore, these results highlight the importance of selectively regulating a channel that can disrupt the mitochondria under certain circumstances, but may be an essential part of normal cell physiology.

Author contributions

EP, PAE and DAK conceived the study and designed experiments. PAE, AN, and CLK conducted experiments. PAE, AN, CLK, DAK and EP analyzed and interpreted the data. PAE and EP were the primary writers of the manuscript while all authors contributed to the critical interpretation and preparation of the manuscript. All authors approved the final manuscript.

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Appendix A. Supplementary data

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