Biochimica et Biophysica Acta 1847 (2015) 231–240

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

Pia A. Elustondo [a], Alexander Negoda [a], Constance L. Kane [b], Daniel A. Kane [b], Evgeny V. Pavlov [a,*]

[a] Department of Physiology and Biophysics, Dalhousie University, Halifax, NS B3H 4R2, Canada
[b] Department of Human Kinetics, St. Francis Xavier University, Antigonish, NS B2G 2W5, Canada

ARTICLE INFO

*Article history:*
Received 20 June 2014
Received in revised form 13 October 2014
Accepted 29 October 2014
Available online 1 November 2014

*Keywords:*
Mitochondrial permeability transition pore
Spermine
Calcium

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

* Corresponding author at: Department of Physiology and Biophysics, Dalhousie University, Halifax, NS, Canada. Tel.: +1 902 494 8980; fax: +1 902 494 1685.
*E-mail address:* Evgeny.Pavlov@dal.ca (E.V. Pavlov).

http://dx.doi.org/10.1016/j.bbabio.2014.10.007
0005-2728/

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