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Inhibition by spermine of the inner membrane permeability transition of isolated rat heart mitochondria

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The effect of spermine on the permeability transition of the inner mitochondrial membrane of isolated rat heart mitochondria was evaluated. The permeability transition was triggered using a series of agents (*t*-butyl hydroperoxide, phenylarsine oxide, carboxyatractylate, and elevated Ca^{2+} and inorganic phosphate concentrations), and was monitored via Ca^{2+}-release, mitochondrial swelling and pyridine nucleotide oxidation. By all three criteria, spermine inhibited the transition. A C_{50} of 0.38 ± 0.06 (SD) mM was measured for inhibition.

Spermine; Mitochondria; Permeability transition; Ca^{2+}; Swelling

1. INTRODUCTION

The aliphatic polyamines, spermine and spermidine, were discovered by Antony von Leeuvenhoek in 1674 in animal semen [1]. They are, in fact, present at millimolar concentrations in the cytosol of most eukaryotic cells [1]. Polyamines are required for essential cellular functions such as DNA, RNA and protein synthesis, and protein phosphorylation [2].

Spermine also protects mitochondria from damage. For example, spermine prevents loss of respiratory control and restores phosphorylation in heat-aged liver mitochondria [3]. Toninello [4] has reported that spermine blocks the collapse of the membrane potential and the release of Mg^{2+}, adenine nucleotides, and Ca^{2+} observed when mitochondria are exposed to damaging concentrations of inorganic phosphate (P_i) and Ca^{2+}.

Increased matrix Ca^{2+}, in combination with a triggering agent, which may be elevated P_i concentration, increased temperature, oxidative stress or one of a large number of chemicals, induces the inner mitochondrial membrane (i.m.) to undergo a permeability transition allowing entry/efflux of small (<1500 dalton) solutes. The permeability transition is Ca^{2+}-dependent and cyclosporin A (CsA)-sensitive. It is accompanied by uncoupling, collapse of the membrane potential, oxidation of pyridine nucleotides, loss of small matrix solutes including Ca^{2+}, swelling and a transition from the aggregated to the orthodox state (for a review see [5]).

The permeability transition reflects opening of a pore in the i.m. [6,7]. Cyclosporin inhibits pore opening [8], possibly by interacting with its intracellular receptor, cyclophilin, a peptidyl-prolyl *cis-trans* isomerase [9].

We propose that the protective effects of spermine on mitochondria reflect inhibition of the i.m. permeability transition. In these studies, the permeability transition of isolated rat heart mitochondria was monitored via Ca^{2+} retention, swelling and pyridine nucleotide [NAD(P)H] oxidation. A series of agents, *t*-butylhydroperoxide (*t*-BH), phenylarsine oxide (PhAsO), carboxyatractylate (CAT), elevated Ca^{2+} levels and high P_i concentration, thought to act by different mechanisms, was used to trigger the transition. By all criteria, spermine was a good inhibitor of the permeability transition in rat heart mitochondria.

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Abbreviations: BSA, bovine serum albumin, fraction V; CAT, carboxyatractylate; CsA, cyclosporin A; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; i.m., inner membrane; MOPS, 3-(*N*-morpholino)propane-sulfonic acid; PhAsO, phenylarsine oxide; P_i, inorganic phosphate; *t*-BH, *t*-butyl hydroperoxide.

2. MATERIALS AND METHODS

Heart mitochondria were isolated from male Sprague–Dawley rats (350–550 g) using a modification [10] of the procedure described by Sordahl [11]. Experiments were carried out at 30°C in a standard resin (Chelex-100)-treated buffer consisting of 100 mM sucrose, 50 mM KCl, 20 mM MOPS–KOH (pH 7.2), and 1.7 mM KH_2PO_4. All data reported are representative of multiple (≥3) experiments. Protein determinations were performed according to Lowry et al. [12] using BSA as standard.

2.1. *Mitochondrial swelling*

Changes in mitochondrial volume were measured qualitatively at 540 nm in an LKB Ultrospec II UV-Visible spectrophotometer [13].

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Mitochondria (0.2 mg/ml) were pre-incubated in standard buffer supplemented with 0.8 μM rotenone for 3 min prior to Ca^{2+} (100 nmol/mg protein) addition. Thirty seconds later the mitochondria were energized with 5 mM succinate. When appropriate, a triggering agent was added after a further 2.5 min.

2.2. *Pyridine nucleotide [NAD(P)H] oxidation*

Reduction and oxidation of NAD(P)H were followed in an SLM-Aminco DW-2000 spectrophotometer at the wavelength pair 340–370 nm [14]. The experimental protocol was identical to that used to monitor swelling except that a higher concentration of mitochondrial protein (0.6 mg/ml) was utilized. A differential extinction coefficient of 1.39 $M^{-1} \cdot cm$, determined by titration with NADH, was used in calculations.

2.3. *Ca^{2+} retention*

Ca^{2+} uptake and release were measured using a Ca^{2+}-selective electrode [10] and the protocol described above for mitochondrial swelling. The standard Ca^{2+} addition was, however, increased to 167 nmol/mg because the time course of Ca^{2+} release is significantly slower than that of swelling.

2.4. *Materials*

Sucrose, HEPES, EGTA, MOPS, BSA, $CaCl_2$, rotenone, protease type XIV, *t*-butyl hydroperoxide, carboxyatractylate, KH_2PO_4, phenylarsine oxide, and succinate were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine was from Aldrich (Milwaukee, WI) and Chelex-100 from Bio-Rad (Richmond, CA). Cyclosporin (OL 27-400) was the gift of Sandoz Research Institute, East Hanover, NJ. All other reagents were of the highest quality available. PhAsO was prepared as a 10 mM stock solution in DMSO.

3. RESULTS

All of the triggering agents tested induced the release of Ca^{2+} from isolated, preloaded rat heart mitochondria (Fig. 1A). Ca^{2+}-release occurred with a lag which was decreased by triggering agent; the amount of Ca^{2+} released was not altered. Spermine (2 mM) substantially delayed the Ca^{2+}-release triggered by each of the agents except elevated P_i concentration (Fig. 1B). Note that the sensitivity of the Ca^{2+}-electrode is altered when spermine is present, and electrode response time is markedly increased (not shown).

Spermine (2 mM) also blocked the swelling of isolated rat heart mitochondria induced by CAT, PhAsO, *t*-BH, elevated Ca^{2+}, and high P_i (Fig. 2). Inhibition of swelling induced by CAT, PhAsO, elevated Ca^{2+}, and *t*-BH was significantly greater than inhibition of swelling triggered by P_i ($P < 0.01$, Student's *t*-test).

The Ca^{2+}-dependent NAD(P)H oxidation associated with the i.m. permeability transition was likewise inhibited by spermine (Fig. 3). When heart mitochondria were added to buffer containing rotenone, reduction of NAD(P)H was observed (Fig. 3A, trace a). Further reduction resulted upon addition of succinate. Several minutes later, a rapid shift of NAD(P)H in the reduced direction occurred, indicating the exhaustion of O_2 [15].

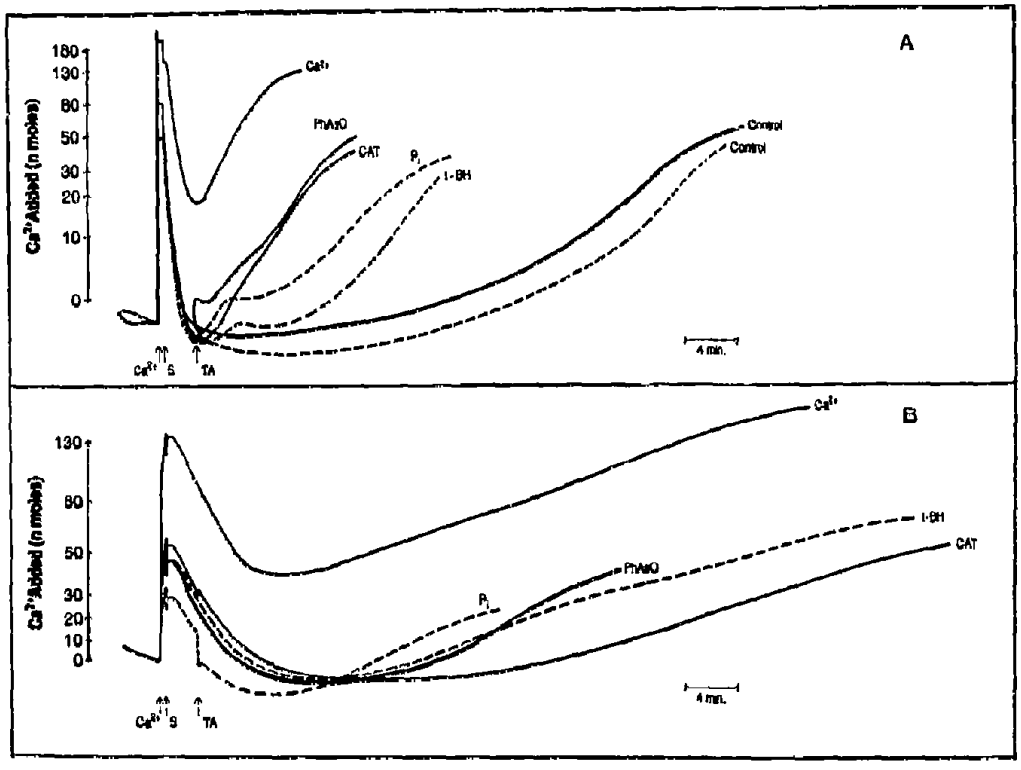

Fig. 1. Effect of spermine on Ca^{2+}-retention by isolated rat heart mitochondria. At the arrows, Ca^{2+} (167 nmol/mg mitochondrial protein), succinate (5 mM) and triggering agent were added. PhAsO (2.5 μM), Ca^{2+} (400 nmol/mg mitochondrial protein), CAT (10 μM), P_i (10 mM), and *t*-BH (600 μM) were used to trigger Ca^{2+} release. When Ca^{2+} was the triggering agent, the standard Ca^{2+} addition was increased to 400 nmol/mg. Solid and broken lines indicate data obtained from 2 different preparations of mitochondria. Experiments were carried out in the presence (B) or absence (A) of 2 mM spermine.

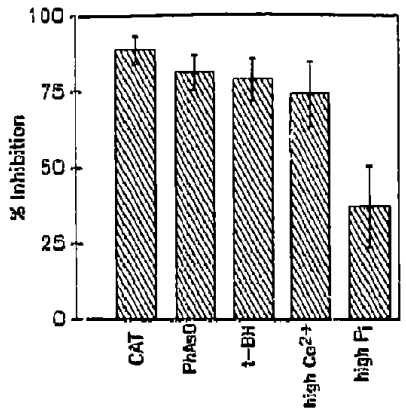

Fig. 2. Effect of spermine (2 mM) on rate of mitochondrial swelling. Conditions were as for Fig. 1 except the standard Ca^{2+} addition was reduced to 100 nmol/mg protein and the PhAsO concentration was 50 μM. The rate of swelling was quantified as ΔA_{540}/min. Data are the mean of 3 experiments. Swelling rates measured in the absence of spermine were 0.032 ± 0.003 (PhAsO), 0.017 ± 0.008 (CAT), 0.010 ± 0.002 (P_i), 0.036 ± 0.011 (Ca^{2+}) and 0.013 ± 0.004 (*t*-BH).

This was followed by a *slow* oxidation of NAD(P)H equivalent to 2.4 nmol NAD(P)H/mg protein at the time the run was completed. This slow NAD(P)H oxidation was Ca^{2+}-dependent (not shown) and reflects occurrence of the transition. It was blocked by spermine (trace b) and cyclosporin (trace c), a recognized inhibitor of the transition [8].

When 600 μM *t*-BH was used to trigger the i.m. permeability transition (Fig. 3B), NAD(P)H oxidation was biphasic (trace a). *t*-BH addition prompted the oxidation of a small amount of NAD(P)H. This process was unaffected by spermine or CsA. Following O_2 exhaustion, a second, *rapid* wave of NAD(P)H oxidation equivalent to 2.06 nmol/mg protein was observed. Spermine slowed (trace b) and cyclosporin eliminated (trace c) this second wave. It was also selectively eliminated by chelation of Ca^{2+} (data not shown). The occurrence of NAD(P)H oxidation linked to the permeability transition only after exhaustion of O_2 has been reported previously [15].

The dependence of transition inhibition on spermine concentration was determined by measuring swelling (Fig. 4). When elevated Ca^{2+} levels were used to trigger swelling, the C_{50} for inhibition was 0.38 ± 0.06 (SD) mM. Similar results were obtained when Ca^{2+}-retention was monitored (data not shown).

To further evaluate the mechanism of action of spermine, the effect of spermine on the Ca^{2+}-dependence of the Ca^{2+}-induced permeability transition was determined (Fig. 5A). Spermine (2 mM) decreased the rate of swelling observed at saturating Ca^{2+} concentrations; i.e. it was not competitive with Ca^{2+}. The C_{50} for Ca^{2+}-induced triggering was increased from 44 μM to 65 μM in the presence of spermine, as was the apparent cooperativity (Fig. 5B). The slope of the Hill plot increased from 3.5 to 4.5.

4. DISCUSSION

The experimental results outlined above demonstrate that spermine blocks the permeability transition of the inner mitochondrial membrane. This is consistent with the inhibition of Na^+-independent Ca^{2+} efflux from rat brain mitochondria by spermine measured by Rottenberg and Marbach [16]. Inhibition was generally independent of triggering agent, suggesting that spermine may act on the pore itself rather than at the level of the diverse biochemical processes postulated to modulate its opening.

Spermine was, however, a poor inhibitor of the transition triggered by high P_i, whether it was monitored via

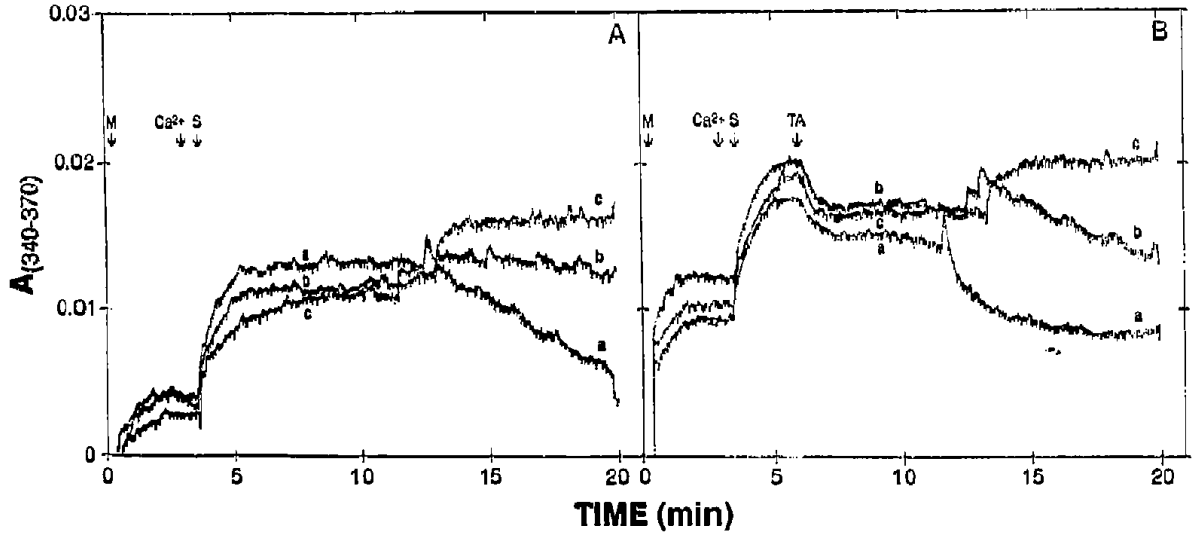

Fig. 3. Effect of spermine on NAD(P)H oxidation. At the arrows, mitochondria (M, 0.6 mg/ml), Ca^{2+} (100 nmol/mg mitochondrial protein), and succinate (S, 5 mM) were added. In Panel B, a triggering agent (600 μM *t*-BH) was also added. Traces show data obtained in the absence of inhibition (a), in the presence of 2 mM spermine (b), or with 1 μM CsA (c).

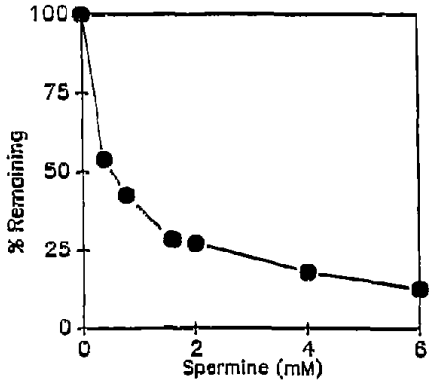

Fig. 4. Dependence of inhibition of mitochondrial swelling on spermine concentration. Ca^{2+} (400 nmol/mg mitochondrial protein) was used as triggering agent. 100% was equivalent to a swelling rate of 0.0375/min. Other details as in Fig. 2.

Ca^{2+}-release (Fig. 1), mitochondrial swelling (Fig. 2), or NAD(P)H oxidation (not shown). The binding of positively charged spermine (2 mM) to negatively charged P_i (10 mM) may effectively decrease spermine concentration.

Several mechanisms for spermine action can be proposed. First, it is possible that spermine decreases mitochondrial Ca^{2+} uptake. The results in Fig. 1 suggest that uptake is slowed, but the effects of spermine on electrode response make interpretation difficult. Numerous authors have reported that spermine plays an important role in the regulation of Ca^{2+} transport in mitochondria [17–20], but there is general agreement that spermine *increases* Ca^{2+} accumulation. The presence of spermine lowers the set point for mitochondrial buffering of extra-mitochondrial free Ca^{2+} [17,18]. Spermine has also been found to lower the apparent K_m of the electrophoretic, Ruthenium red-sensitive Ca^{2+} uniporter [17–19]. Internal markers have been used to confirm enhanced matrix Ca^{2+} accumulation in isolated rat heart, kidney and liver mitochondria [18–20].

It is possible that spermine enters the mitochondria and competes for the matrix Ca^{2+}-binding site on or near the pore. It has been reported that spermine is transported bidirectionally across the i.m. in both liver [21] and heart [22] mitochondria. Uptake is P_i-dependent but Ca^{2+}-independent and requires an elevated membrane potential (>180 mV) [4]. The K_d for spermine uptake by liver mitochondria (0.5 mM) [21] correlates well with the C_{50} for spermine inhibition of the permeability transition (0.38 mM), and is consistent with a matrix site of spermine action. The observation that spermine inhibition is not overcome by high Ca^{2+} concentrations (Fig. 5) would argue, however, against a competitive action of spermine at the Ca^{2+} site.

Finally, calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase [23], may be involved in the mechanism of spermine action. The cyclophilin–cyclosporin complex binds to and inhibits calcineurin [24]. Spermine inhibits calcineurin activity with a C_{50} of 1.2 mM [25]. Modulation of pore opening by protein phosphorylation–dephosphorylation reactions would then provide a single target for inhibition of the permeability transition by both CsA and spermine.

This work has identified spermine as an inhibitor of the mitochondrial permeability transition. The C_{50} for spermine inhibition of the permeability transition (0.38 ± 0.06 (SD) mM) falls into the range (0.3–2 mM) of spermine concentrations reported for the cytosol of quiescent cells [26]. Further elucidation of the mechanism

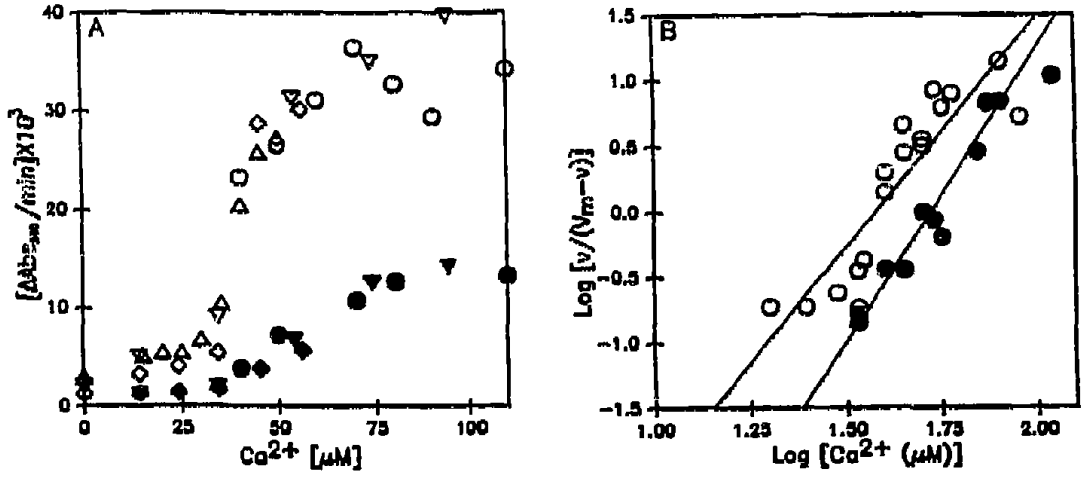

Fig. 5. Effect of spermine on the Ca^{2+}-dependence of mitochondrial swelling. Experiments were carried out in the absence (open symbols) or presence (filled symbols) of 2 mM spermine. Ca^{2+} was used as the triggering agent. Data from four mitochondria preparations (●, ◆, ▼, ▲) are combined. Maximal rates of swelling were 0.035/min (control) and 0.0145/min (2 mM spermine). Panel B is a Hill plot of the data.

of spermine action may, therefore, provide physiologically important insights into the transition.

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