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Polyamine Modulation of Mitochondrial Calcium Transport

II. INHIBITION OF MITOCHONDRIAL PERMEABILITY TRANSITION BY ALIPHATIC POLYAMINES BUT NOT BY AMINOGLUCOSIDES

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ABSTRACT. In this study, the effects of polyamines and analogous compounds on mitochondrial permeability transition were characterized to distinguish between these effects and those on mitochondrial Ca^{2+} uptake, which are described in an accompanying report (Rustenbeck *et al.*, Biochem Pharmacol 8: 977–985, 1998). When a transitional Ca^{2+} release from Ca^{2+}-loaded mitochondria was induced by an acute increase in Ca^{2+} concentration in a cytosol-adapted incubation medium (Ca^{2+} pulse), this process was inhibited, but not abolished by spermine in the concentration range of 0.4 to 20 mM. The aminoglucoside, gentamicin, and the basic polypeptide, poly-L-lysine, which like spermine are able to enhance mitochondrial Ca^{2+} accumulation (preceding paper), had no or only a minimal inhibitory effect, while the aliphatic polyamine, bis(hexamethylene)triamine, which is unable to enhance mitochondrial Ca^{2+} accumulation, achieved a complete inhibition at 4 mM. The conclusion that the Ca^{2+} efflux was due to opening of the permeability transition pore was supported by measurements of mitochondrial membrane potential, ATP production, and oxygen consumption. Mg^{2+}, a known inhibitor of mitochondrial membrane permeability transition, did not mimick the effects of spermine on mitochondrial Ca^{2+} accumulation, while ADP, the main endogenous inhibitor, showed both effects. However, a combination of spermine and ADP was significantly more effective than ADP alone in restoring low Ca^{2+} concentrations after a Ca^{2+} pulse. Two different groups of spermine binding sites were found at intact liver mitochondria, characterized by dissociation constants of 0.5 or 4.7 mM and maximal binding capacities of 4.6 or 19.7 nmol/mg of protein, respectively. In contrast to aminoglucosides, the aliphatic polyamine bis(hexamethylene)triamine did not displace spermine from mitochondrial binding sites. The total intracellular concentration of spermine in hepatocytes was measured to be *ca.* 450 μM and the free cytoplasmic concentration was estimated to be in the range of 10–100 μM. In conclusion, the enhancement of mitochondrial Ca^{2+} uptake by spermine is not an epiphenomenon of the inhibition of permeability transition. The physiological role of spermine appears to be that of an enhancer of mitochondrial Ca^{2+} accumulation rather than an inhibitor of permeability transition. BIOCHEM PHARMACOL **56**;8:987–995, 1998. © 1998 Elsevier Science Inc.

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An increased Ca^{2+} concentration in the mitochondrial matrix space in combination with a number of inducing agents or inducing conditions (for an overview see Ref. 1) may lead to an abrupt change in the permeability properties of the inner mitochondrial membrane. The loss of selective permeability, mitochondrial "permeability transition", has proved to be due to an opening of a large proteinaceous pore, as first postulated by Hunter and Haworth [2–4]. This pore is very probably identical to the mitochondrial megachannel [5, 6], which has a conductance of *ca.* 1.2 nS [7]. The calculated size of this channel corresponds to the size of the pore, which permits permeation of solutes with a molecular mass of up to 1500. The opening of both is inhibited by submicromolar concentrations of cyclosporin A [8, 9]. Exploration of the conditions which induce opening of the pore has led to the concept that this is a voltage-dependent channel regulated by divalent cations and matrix pH [10, 11]. It is still a matter of debate whether the permeability transition serves as a physiological Ca^{2+} release mechanism ([12]; for a recent review see [13]), but in the last few years a concept has been emerging according to which prolonged opening of the permeability transition pore is a decisive event in the course of apoptosis [14].

It has been shown that spermine inhibits the permeability transition of heart and liver mitochondria [15]. This effect is exerted at the cytoplasmic face of the inner mitochondrial membrane [16]. In addition to spermine,

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several other aliphatic polyamines were found to inhibit transition, spermidine and putrescine being less potent and efficient than spermine [17]. This structure–activity relation is reminiscent of the enhancement of mitochondrial Ca^{2+} accumulation by spermine and polyamine analogues which was characterized in the accompanying paper [18]. Thus, it appeared possible that the inhibition of permeability transition represents the underlying cause of the enhancement of mitochondrial Ca^{2+} accumulation by spermine.

We therefore investigated whether inhibition of permeability transition and enhancement of Ca^{2+} accumulation were separable effects and whether the former could also be produced by aminoglucosides and other polycationic compounds, which in a certain concentration range mimick the effect of spermine on mitochondrial Ca^{2+} accumulation [18]. By determining the binding of spermine to mitochondria and measuring the free cytoplasmic spermine concentration of hepatocytes, we tried to assess to what degree the mitochondrial effects of spermine are of physiological importance. The results suggest that the enhancement of Ca^{2+} accumulation by spermine is not identical to the inhibition of permeability transition and that aminoglucosides may exert detrimental effects on mitochondria by antagonizing rather than imitating the effects of natural polyamines such as spermine.

MATERIALS AND METHODS

Chemicals

Spermine, gentamicin, poly-L-lysine (MW 5,000–10,000) and dansyl chloride were obtained from Fluka. BHTA§ was from Aldrich. ATP, digitonin, dithiotreitol, Hepes, and SLO were from Sigma and ADP was from Boehringer. Solvents were of analytical grade or "for residue analysis" grade from E. Merck or purissimum grade from Fluka. HPTLC plates (silica gel 60, 10 × 20 cm) were from E. Merck. All other reagents of analytical grade were from E. Merck. Preparation of mitochondria, measurement of the free Ca^{2+} concentration with a Ca^{2+}-sensitive minielectrode, polarographic measurement of mitochondrial oxygen consumption, and polyamine chromatography have been described in the accompanying paper [18].

Preparation of Permeabilized Hepatocytes and Titrimetric Measurement of Free Cytoplasmic Spermine Concentration

Isolated hepatocytes were prepared by recirculating perfusion of rat liver with collagenase, filtration of the dissociated cells through nylon gauze, and purification by density gradient centrifugation [19]. Cells were kept in cell culture medium 199 (Sigma) and viability as assessed by trypan blue exclusion was higher than 85%. For permeabilization, hepatocytes were transferred into the incubation medium containing permeabilizing agents (see below) at a final concentration of 7×10^5 cells/incubation.

For permeabilization with digitonin, the incubation medium was supplemented with 660 μM EGTA and 20 μg/mL of digitonin. After addition of hepatocytes, a virtually complete permeabilization (>90%, as measured by trypan blue) was achieved during a 10-min incubation at 25°. Then, spermine was added at various concentrations and after 2 min, the incubation was terminated by centrifugation of the cells through an oil layer. The spermine content in the incubation medium and in the perchloric acid phase was determined by HPTLC of the dansylated derivative [18].

For permeabilization with SLO, the incubation medium was supplemented with 660 μM EGTA, 5 mM ATP, 4 mM dithiotreitol, and SLO (1250 units/mL). Permeabilization (*ca.* 80%, as measured by trypan blue) was achieved by incubation at 37° for 30 min. Thereafter, spermine was added and after 2 min, the incubation was terminated by centrifugation of the cells through an oil layer.

ATP Content

ATP production by incubated mitochondria was determined at the end of respiration measurements by a luminometric method using a commercial luciferase assay kit (Sigma).

RESULTS

To characterize the relation between the two main effects of spermine on mitochondrial Ca^{2+} transport, namely enhancement of Ca^{2+} accumulation and inhibition of permeability transition, two compounds were selected which resembled spermine in their ability to enhance Ca^{2+} accumulation, but which were structurally different: the aminoglucoside gentamicin and the basic polypetide poly-L-lysine. The concentration ranges at which the polyamines were tested were chosen according to the potencies known to stimulate Ca^{2+} accumulation [18]. Permeability transition was induced by injecting a defined amount of $CaCl_2$, a "Ca^{2+} pulse", into the incubation medium after mitochondria had taken up Ca^{2+} and established a steady-state Ca^{2+} concentration in the medium. The decrease in mitochondrial membrane potential by Ca^{2+} uptake [20] together with the pre-existing Ca^{2+} load regularly induced a rapid release of Ca^{2+} from control mitochondria, which most likely represents an opening of the permeability transition pore. With this experimental protocol, the effects on Ca^{2+} uptake velocity and Ca^{2+} accumulation could be observed as well as the effect on transitional Ca^{2+} release (Fig. 1A–D). In addition to spermine, gentamicin, and poly-L-lysine, BHTA, an aliphatic polyamine which does not enhance mitochondrial Ca^{2+} accumulation [21], was tested.

Spermine concentration-dependently inhibited the rate

§ *Abbreviations:* BHTA, bis(hexamethylene)triamine; SLO, streptolysin O; and TPP^+, tetraphenylphosphonium ion.

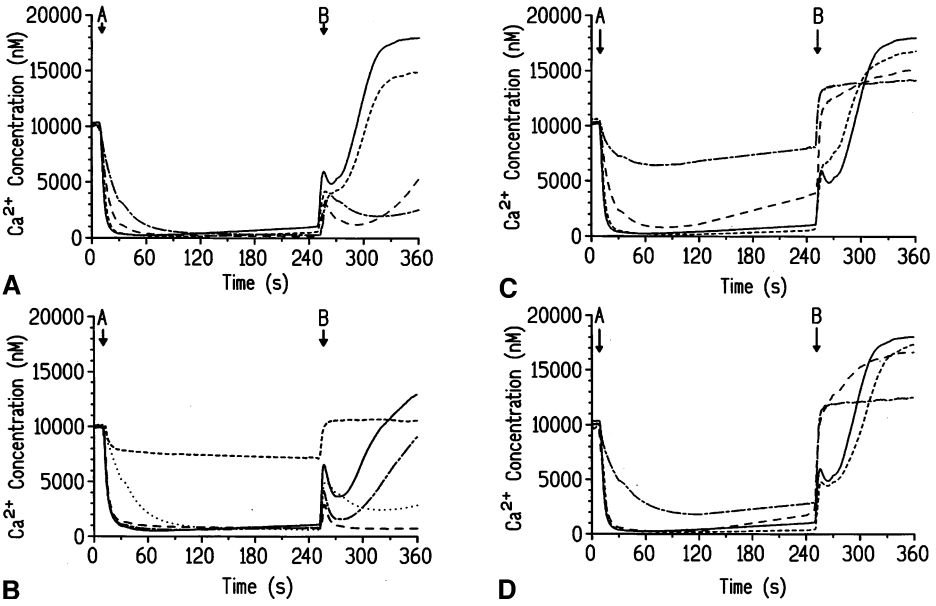

FIG. 1. Concentration-dependent effects of spermine and polyamine analogues on mitochondrial Ca^{2+} uptake and transitional Ca^{2+} release. Liver mitochondria were preincubated for 2 min in the incubation medium to activate energy metabolism and were then introduced (time point A) into the incubation chamber of the Ca^{2+} minielectrode. The initial concentration of the incubation medium in the chamber was 10 μM. Permeability transition was elicited by injection of a defined amount of Ca^{2+} (13.5 nmol/mg protein) with a microliter syringe (time point B). The following compounds and concentrations were used: (A) spermine: 0 mM = control (solid), 0.4 mM (short dash), 4 mM (long dash) and 20 mM (dash-dot-dash); (B) BHTA: 0 mM = control (solid), 1 mM (dash-dot-dash), 4 mM (long dash) and 20 mM (short dash); for comparison, the effect of 20 mM of spermine in this set of experiments is indicated (dots); (C) gentamicin: 0 mM = control (solid), 0.25 mM (short dash), 2 mM (long dash) and 10 mM (dash-dot-dash); and (D) poly-L-lysine: 0 μM = control (solid), 5 μM (short dash), 10 μM (long dash) and 100 μM (dash-dot-dash). All traces are mean values of 4 experiments, with the SEM ranges omitted for clarity.

of Ca^{2+} uptake. At the highest spermine concentration (20 mM), it took 2 min before the Ca^{2+} concentration in the medium was as low as in the control, but after 4 min, immediately before the addition of the Ca^{2+} pulse, the Ca^{2+} concentration in the presence of 20 mM spermine was clearly lower than in the control ($P < 0.01$, *t*-test). At 400 μM, spermine had only a small inhibitory effect on the onset of transitional Ca^{2+} release, but was strongly inhibitory at 4 and 20 mM. At these concentrations, uptake of the Ca^{2+} pulse was markedly reduced. However, even at 20 mM the protection by spermine against the onset of permeability transition was incomplete (Fig. 1A). BHTA had virtually no effect on the rate of Ca^{2+} uptake and did not enhance Ca^{2+} accumulation. When micromolar concentrations (100 to 1000 μM) were tested, a transient uptake of Ca^{2+} occurred after the Ca^{2+} pulse, but after a delay of approximately 1 min a rapid release of Ca^{2+} followed (data not shown). At 4 mM BHTA, however, the Ca^{2+} pulse was nearly completely taken up and no Ca^{2+} release occurred until the end of the incubation period (Fig. 2B). Up to a concentration of 4 mM, the velocity of Ca^{2+} uptake was only marginally reduced (compare with spermine, Fig. 1A), but at 20 mM BHTA, uptake of Ca^{2+} was completely blocked.

Gentamicin, at a concentration at which it moderately, but significantly ($P < 0.05$, *t*-test) enhanced Ca^{2+} accumulation (250 μM), did not inhibit Ca^{2+} release from the mitochondria following the Ca^{2+} pulse, but rather accelerated it (Fig. 1C). At 2 mM, the rate of Ca^{2+} uptake was greatly diminished, but in contrast to spermine at high concentrations, the Ca^{2+} retention capacity was impaired by gentamicin. Consequently, there was not even a transient uptake of Ca^{2+} after addition of the Ca^{2+} pulse. At the highest concentration (10 mM), a virtually complete block of Ca^{2+} uptake occurred. Here, the Ca^{2+} pulse resulted in a square wave-like increase in Ca^{2+} concentration. Under this condition, the maximal Ca^{2+} concentration after the Ca^{2+} pulse was lower than the concentration achieved by transitional Ca^{2+} release after a transient uptake of the Ca^{2+} pulse (Fig. 1C). The effects of poly-L-lysine were mainly similar to those of gentamicin. While there was a very slight protection against the transition at the lowest concentration (5 μM), a square wave-like increase was registered at the highest concentration (100 μM), even though the decrease in Ca^{2+} uptake velocity at the beginning of the experiments was less impressive than with gentamicin.

The divergent characteristics of action were confirmed when Ca^{2+} release from aged mitochondria was measured as an indicator of permeability transition. Ca^{2+} uptake was much slower in the presence of spermine but continued to the end of the incubation period, while in the presence of gentamicin and poly-L-lysine a faster release of Ca^{2+} than in controls occurred (Fig. 2). When mitochondrial membrane potential was measured by a TPP^{+}-sensitive electrode using the Ca^{2+} pulse protocol, it became clear that

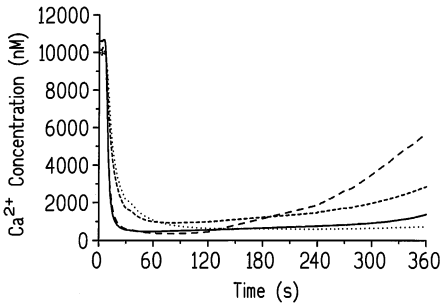

FIG. 2. Effect of high concentrations of spermine, gentamicin, and poly-L-lysine on Ca^{2+} release from aged mitochondria. A mitochondrial suspension which had been aged on ice for 3 hr was preincubated for 2 min in incubation medium to activate energy metabolism and then transferred into the incubation chamber of the Ca^{2+} minielectrode. The incubation medium had an initial Ca^{2+} concentration of 10 μM and contained spermine (4 mM, dotted trace), gentamicin (1 mM, short-dashed trace) or poly-L-lysine (10 μM, long-dashed trace). The traces are mean values of 4 experiments. Only spermine significantly decreased Ca^{2+} concentration below the control value (solid trace), this concentration being significantly increased in the presence of gentamicin and poly-L-lysine ($P < 0.01$, *t*-test at time point 360 sec).

the inhibition of transitional Ca^{2+} release by BHTA and spermine was accompanied by a preservation of mitochondrial membrane potential, while gentamicin and poly-L-lysine at high concentrations produced a nearly complete loss of membrane potential within the first 2 min (Fig. 3). This explains the block of Ca^{2+} uptake and the consequent lack of transitional Ca^{2+} release by high concentrations of these agents (Fig. 1, C and D).

To test whether an inhibition of the initial phases of permeability transition could be the underlying reason for

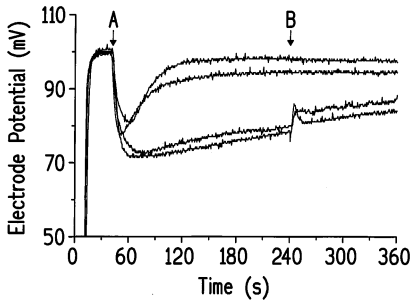

FIG. 3. Effect of high concentrations of spermine and polyamine analogues on mitochondrial membrane potential. The experimental protocol was the same as in Fig. 1, except that a TPP^+-sensitive electrode instead of a Ca^{2+}-sensitive electrode was used and that the incubation medium initially contained 8 μM TPP^+. A decrease in the electrode potential corresponds to an uptake of the lipophilic cation as a function of mitochondrial membrane potential. After addition of the Ca^{2+} pulse (time point B), there was a release and re-uptake of TPP^+ in the presence of BHTA (4 mM, first trace from bottom) and a limited release of TPP^+ in the presence of spermine (20 mM, second trace from bottom), while in the presence of gentamicin (10 mM, second trace from top) and poly-L-lysine (100 μM, first trace from top), TPP^+ was already completely released prior to the Ca^{2+} pulse. All traces are means of 3 experiments.

enhanced mitochondrial Ca^{2+} accumulation by polyamines, the effects of known inhibitors of permeability transition, Mg^{2+} and ADP, on mitochondrial Ca^{2+} uptake and retention were characterized. Because of a possible interaction of Mg^{2+} with membrane-bound Ca^{2+}, a preincubation protocol was used. Mitochondria were first preincubated for 2 min in the presence or absence of test agent and then introduced into the incubation medium set at an initial Ca^{2+} concentration of 10 μM to take up Ca^{2+} in the presence or absence of the same test agent. Mg^{2+} (1 mM) decreased the maximal velocity of Ca^{2+} uptake, but did not decrease the minimal Ca^{2+} concentration below control levels. However, Mg^{2+} diminished the net release of Ca^{2+} (Table 1). ADP (0.4 mM) had no influence on the rate of Ca^{2+} uptake, but decreased the minimal Ca^{2+} concentration in the medium. The Ca^{2+} concentration in the medium remained lower than in the control until the end of the incubation period (Table 1).

ADP (400 μM), when tested according to the Ca^{2+} pulse protocol, induced an uptake of a large portion of the Ca^{2+} pulse and, as expected, prevented transitional Ca^{2+} release. However, the steady-state Ca^{2+} concentration after the Ca^{2+} pulse remained elevated (Fig. 4). Spermine alone (400 μM) retarded but did not prevent transition (Fig. 4). In the combined presence of 400 μM spermine and 400 μM ADP, transitional Ca^{2+} release was prevented and free Ca^{2+} concentration after the Ca^{2+} pulse was reduced by more than 50% as compared to ADP alone (Fig. 4).

To ascertain that the spermine-induced inhibition of Ca^{2+} efflux after a Ca^{2+} pulse represents a protective effect on mitochondrial energy metabolism, ATP production and oxygen consumption were measured using the Ca^{2+} pulse protocol (incubation for 4 min, then addition of a Ca^{2+} pulse and registration for another 2 min). Spermine at 20 mM led to significantly higher ATP levels (193 ± 11% of a control value of 192 pmol × mg of protein^{-1}) and respiration rates (131 ± 9% of a control value of 8.75 nmol × mg of protein^{-1} × min^{-1}) at the end of the incubation, while the effect of 0.4 mM was not yet significant. With gentamicin, both at 0.25 or 10 mM, ATP levels and respiratory rates were below control levels at the end of the incubation (data not shown).

A previous measurement of spermine binding to intact, incubated mitochondria indicated that a saturation was reached below 1 mM [21]. Since the inhibition of permeability transition by spermine was substantial only above this concentration, the binding of spermine to mitochondria was measured over a concentration range of 0.1 to 10 mM. Binding of spermine to isolated incubated mitochondria reached an equilibrium within a very short time (half-maximal binding required 0.8 sec at 400 μM and 5.3 sec at 8 mM). Thus, an incubation time of 120 sec, the same time as for Ca^{2+} uptake measurements, appeared sufficient. Thereafter, mitochondrially bound spermine was separated from free spermine by oil-layer centrifugation. In agreement with our earlier data, a saturation was reached at *ca.* 1 mM spermine, but at 3 mM, the amount of mitochon-

TABLE 1. Effects of Mg^{2+} and ADP on the velocity of mitochondrial Ca^{2+} uptake and on mitochondrial Ca^{2+} accumulation

Test compound	Experimental condition			
	A	B	C	D
Maximal velocity of uptake (nmol × mg^{-1} × min^{-1})				
Mg^{2+}	198 ± 7 (100%)	198 ± 19 (100%)	150 ± 10 (76%)†	166 ± 13 (84%)*
ADP	184 ± 16 (100%)	169 ± 23 (93%)	182 ± 14 (99%)	182 ± 7 (99%)
Minimal Ca^{2+} concentration in incubation medium (nM)				
Mg^{2+}	361 ± 28 (100%)	377 ± 22 (104%)	356 ± 19 (99%)	358 ± 27 (99%)
ADP	399 ± 43 (100%)	364 ± 43 (91%)	317 ± 46 (79%)	252 ± 35 (63%)*
Ca^{2+} concentration in medium at t = 120 sec (nM)				
Mg^{2+}	559 ± 65 (100%)	585 ± 31 (105%)	398 ± 25 (71%)*	376 ± 27 (67%)*
ADP	534 ± 94 (100%)	401 ± 48 (75%)	318 ± 46 (60%)*	252 ± 35 (47%)†

To avoid an interference of Mg^{2+} with membrane-bound Ca^{2+}, a preincubation protocol was used to test the effects of Mg^{2+} and ADP on the velocity of mitochondrial Ca^{2+} uptake and on mitochondrial Ca^{2+} accumulation. Mitochondria which had been preincubated for 2 min at 25° were introduced into incubation medium which was set at an initial Ca^{2+} concentration of 10 μM and Ca^{2+} uptake was registered with a Ca^{2+}-sensitive minielectrode. Test agents were absent (A = control), present only in the preincubation (B), present only during Ca^{2+} uptake (C) or present in both (D). Data are means ± SEM of 5–6 experiments.

*$P < 0.05$, unpaired two-tailed *t*-test.

†$P < 0.01$, unpaired two-tailed *t*-test.

drially bound spermine again increased, reaching a second plateau at 6–8 mM. The saturation binding data could be fitted by a biphasic association curve with two different pseudo-Hill coefficients. Thus, there were two groups of interacting binding sites (Fig. 5). For the first group, a maximal binding capacity of 5.2 nmol × mg of $protein^{-1}$, a dissociation constant of 0.4 mM and a pseudo-Hill coefficient of 0.3 were determined. For the second group, the corresponding values were: 21.5 nmol × mg of $protein^{-1}$, 4.7 mM, and 8.8. The binding of spermine was not significantly decreased when the incubations were performed in the presence of 10 μM of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone. The presence of 2 mM BHTA and even 20 mM BHTA did not significantly influence the binding of spermine to metabolically active mitochondria in the concentration range of 0.1 to 10 mM (data not shown).

Free cytoplasmic spermine concentration was measured by a titrimetric procedure. Intact and digitonin-permeabilized hepatocytes were incubated for 2 min in a medium containing spermine in concentrations from 0 to 1000 μM. The incubation was terminated by centrifugation of the hepatocytes through an oil layer, and the amount of spermine in the incubation medium and in the perchlorate layer below the oil layer was determined. Spermine content

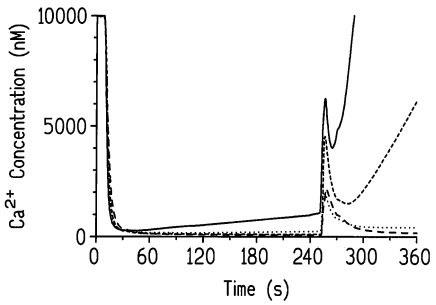

FIG. 4. Additive effects of ADP and spermine on transitional Ca^{2+} release from mitochondria. The experimental protocol was the same as in Fig. 1. The traces signify: solid = control; short-dashed = 400 μM spermine; dotted = 400 μM ADP; long-dashed = 400 μM ADP plus 400 μM spermine. All traces are means of three experiments. The difference between the traces in the presence of ADP and ADP plus spermine at the end of incubation is significant ($P < 0.01$, *t*-test).

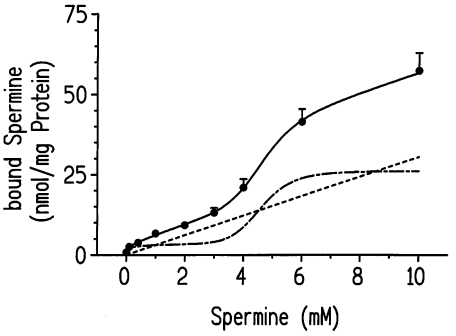

FIG. 5. Concentration-dependent binding of spermine to incubated intact mitochondria. A mitochondrial fraction was incubated for 2 min at 25° in incubation medium set at an initial Ca^{2+} concentration of 10 μM and containing the indicated concentration of spermine. After oil-layer centrifugation, the amount of mitochondrially bound spermine was determined chromatographically. The data are means of 6 experiments and were fitted to the function Y = AX/\F/(B/\F + X/\F) + CX/\G/(D/\G + X/\G) + EX, which is the addition of two saturation hyperbolas, each containing a pseudo-Hill coefficient, and of a linear term. Subtraction of the linear term (dashed trace) yields the specific binding of spermine (dash-dot-dashed trace) to the mitochondrial suspension.

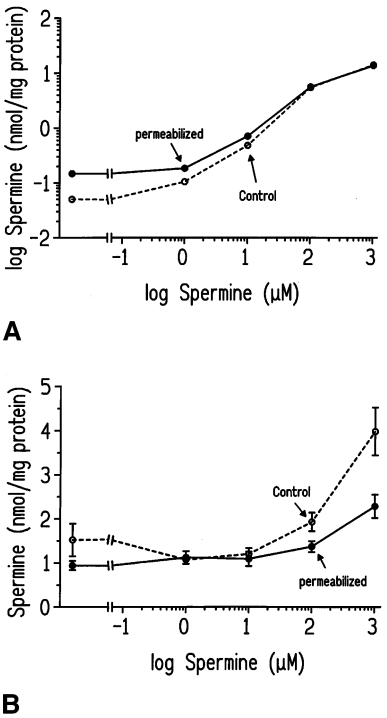

FIG. 6. Determination of free cytoplasmic spermine concentration in isolated, digitonin-permeabilized hepatocytes. After a permeabilization period of 10 min, spermine was added to the hepatocyte suspension to yield concentrations of 0, 1, 10, 100, or 1000 μM. After a 2-min incubation at 25°, hepatocytes were centrifuged through an oil layer and the spermine content in the media above and below the oil layer was determined. Intact hepatocytes served as controls. (A) Spermine content in the incubation medium of permeabilized (closed circles) or control (open circles) hepatocytes. Data are means of 4 experiments. At 0, 1, and 10 μM spermine, the spermine content in the medium of permeabilized hepatocytes was significantly ($P < 0.01$, *t*-test) higher than that of control hepatocytes, indicating spermine efflux from permeabilized cells. (B) Spermine content of sedimented permeabilized (closed circles) or control (open circles) hepatocytes. The data are means ± SEM of four experiments. At 100 and 1000 μM, the spermine content of intact cells was significantly ($P < 0.05$, *t*-test) higher than that of permeabilized cells.

in the medium of permeabilized hepatocytes was significantly higher than in the medium of intact cells up to a concentration of 10 μM (Fig. 6A), suggesting that an efflux of spermine from permeabilized cells had occurred. When the spermine content of the hepatocytes was determined by measuring the spermine content of the perchlorate layer, the unexpected observation was made that at high concentrations (100 and 1000 μM) the intact cells had a significantly higher content than the permeabilized cells (Fig. 6B). When these experiments were repeated with SLO-permeabilized cells, basically the same results were obtained (data not shown). To obtain a conventional estimate of the intracellular spermine concentration, the spermine content of hepatocytes (1.0 nmol/mg protein, Fig. 6B) was related to the intracellular volume. The volume of a single collagenase-isolated hepatocyte is given as 3.7 pL [22]. The hepatocytes in an incubation (7×10^5), which had an average protein content of 1.19 mg, can thus be assumed to represent a volume of 2.59 μL. From these data, a total intracellular concentration of 460 μM results. On the basis of a spermine binding of 95%, a free spermine concentration of 23 μM can be assumed.

DISCUSSION

The observations in this study suggest that the inhibition of mitochondrial membrane permeability transition by amino groups containing compounds is an effect distinct from the known enhancement of mitochondrial Ca^{2+} accumulation by these compounds. This conclusion is mainly based on the structure–activity relations: (1) compounds which like spermine enhance Ca^{2+} accumulation, but which are structurally dissimilar, do not inhibit the transition and (2) a compound structurally similar to spermine which inhibits the transition more efficiently than spermine does not enhance Ca^{2+} accumulation. Principally, a relation between effects on mitochondrial Ca^{2+} uptake and on permeability transition would not appear unlikely, because a slower Ca^{2+} uptake decreases membrane potential less and thus reduces the probability that the transition pore opens. In fact, an apparent block of Ca^{2+} uptake by high concentrations of gentamicin and poly-L-lysine also blocked the transitional Ca^{2+} efflux. However, a reduction in Ca^{2+} uptake velocity by spermine is unlikely to account for its inhibition of permeability transition, since the structurally related polyamine BHTA, which achieved a virtually complete inhibition of the transition, did not reduce the velocity of Ca^{2+} uptake at the relevant concentrations.

In addition, the concentration dependencies of both effects differed. While the inhibition of transition by spermine was at best moderate at 1 mM and maximal at 20 mM, the enhancement of Ca^{2+} accumulation was maximal below 1 mM [18]. Lapidus and Sokolove [15], who tested spermine under conditions more favorable for its potency (see below), gave an IC_{50} value of 380 μM for inhibition of transition, compared with an EC_{50} value of 50 μM for enhancement of Ca^{2+} accumulation [23]. In practice, it may not always be easy to distinguish between accumulation enhancement and inhibition of transition, particularly at high spermine concentrations which strongly retard Ca^{2+} uptake, given that with prolonged incubations the onset of permeability transition in the most susceptible part of the mitochondrial population can produce a net Ca^{2+} efflux in the control.

This reasoning applies to the effect of Mg^{2+}, which was included in this study as a cationic inhibitor of permeability transition. While Mg^{2+}, in contrast to polyamines, was not able to decrease the minimal Ca^{2+} concentration below control values, an improved Ca^{2+} retention in the presence of Mg^{2+} may well be due to an inhibition of the initial phases of transition. In contrast to Mg^{2+}, ADP not only showed an improved retention of Ca^{2+} but also a spermine-like decrease in the minimal Ca^{2+} concentration in the medium. However, this does not prove that an ADP-

induced inhibition of transition is responsible for this effect, because ADP is also known to have direct effects on mitochondrial Ca^{2+} uptake by action on the uniporter [24–26]. In comparison with Mg^{2+} and ADP, spermine seems unique in that it is the only compound which exerts a long-lasting effect. Spermine-preincubated mitochondria still showed an enhanced Ca^{2+} accumulation during an incubation in a medium without test agent [23], while the effects of a Mg^{2+} or ADP preincubation were immediately lost. The ability of ADP to prevent transitional Ca^{2+} release was unexpected in view of the report by Lapidus and Sokolove [27] that ADP protected strongly against P_i^-, but only minimally against Ca^{2+}-induced transition. The additive effect of spermine in decreasing Ca^{2+} concentration in the presence of ADP after a Ca^{2+} pulse may be more due to enhanced Ca^{2+} uptake than a prevention of transitional Ca^{2+} release.

Measurements of ATP production and oxygen consumption confirmed that inhibition of transitional Ca^{2+} efflux by a high concentration (20 mM) of spermine has a protective effect on mitochondrial energy metabolism, while a submillimolar concentration of spermine was only weakly effective. Under the same conditions, gentamicin decreased mitochondrial ATP production. Thus, the mitochondrial effects of spermine, but not of gentamicin, may in consequence be cytoprotective as suggested early on by Toninello *et al.* [28]. The requirement of millimolar concentrations of spermine to observe an inhibition of permeability transition is most likely due to our use of a cytosol-adapted medium [29] containing a high concentration of K^+ (120 mM) and P_i (5 mM). The low spermine concentrations described in earlier investigations as inhibiting permeability transition with high efficiency [15–17] were all obtained by use of a sucrose-based incubation medium, whereas K^+ was found to decrease spermine effectiveness [16]. The very small inhibitory effect of poly-L-lysine on transition is in marked contrast to the high efficiency reported by Rigobello *et al.* [30]. However, these authors showed that basic peptides which inhibited mitochondrial swelling did not inhibit glutathione release from mitochondria, which may be a more direct indicator of pore opening [31]. Thus, with respect to effects on permeability transition, basic peptides may be more similar to aminoglucosides than to aliphatic polyamines.

The binding experiments were designed to determine the concentration of spermine at its site of action under the same conditions as used for the Ca^{2+} transport measurements. The observation that the uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone, did not significantly reduce the amount of mitochondrially associated spermine indicates that only binding of spermine to the mitochondria was measured during the 2-min incubation period and not a membrane potential-dependent uptake of spermine into the matrix space, which is comparatively slow [32, 33]. The characteristics of both binding sites are similar to those recently published by Dalla Via *et al.* [34] except for a markedly lower affinity and a clearly more marked cooperativity. Again, the most likely reason for this difference is that these authors used a sucrose-based incubation medium with a low ionic strength, while in this investigation a cytosol-adapted medium with a high K^+ concentration was used.

The group of spermine binding sites with higher affinity comprises those sites for which a competition between spermine and aminoglucosides could be shown [18] and which may represent phospholipid headgroups [35, 36]. The lack of competition between spermine and BHTA for this first group was not surprising in view of the inability of BHTA to influence the rate of Ca^{2+} uptake and enhance Ca^{2+} accumulation. However, one would have expected a competition between BHTA and spermine for the second group of binding sites, since both compounds inhibited permeability transition at millimolar concentrations and BHTA was the more potent compound. It thus remains an open question as to whether spermine and BHTA inhibit permeability transition by different mechanisms or whether the binding site which mediates the inhibition of transition is only a small fraction of the low-affinity spermine binding sites.

The intracellular concentration of spermine and its precursors is often stated to be in the millimolar range [e.g. 15, 37]. However, data on the intracellular concentration of spermine are quite variable, depending on cell type and methodology (for an overview, see [38]). Furthermore, as spermine is bound to a high degree to intracellular polyanions [39], free cytoplasmic concentrations may rather be in the micromolar range. Such low micromolar concentrations of spermine have been postulated to regulate inward rectifying K^+ channels [40]. A fundamental problem for the measurement of free cytoplasmic spermine concentration is that no specific indicator exists. Usually, the cellular content of spermine is determined chromatographically, the total concentration is estimated from cellular volume [38] and the free concentration by taking into account the degree of binding to anions.

Because of the inherent drawbacks of this procedure, we attempted a direct determination of free cytosolic spermine by titrating the medium of permeabilized hepatocytes. The underlying assumption was that at a lower concentration of spermine in the medium than in the cytosol an efflux of spermine from the permeabilized hepatocytes would take place, while at a higher concentration the hepatocytes would bind spermine from the medium. The measurement of the spermine contents in the incubation medium showed that at spermine concentrations up to 10 μM, a significant efflux from permeabilized cells had indeed occurred, suggesting that the free cytoplasmic spermine concentration was higher than 10 μM. However, the measurement of the cellular spermine content after oil-layer centrifugation did not permit us to determine an upper limit of free spermine concentration, because intact (control) hepatocytes paradoxically had higher spermine contents than permeabilized hepatocytes when the spermine concentration in the medium was high (100 and 1000 μM). Principally, an active

accumulation of spermine by intact hepatocytes could account for this phenomenon, which was independent of the permeabilization technique; however, in view of the short incubation time, the low temperature and the spermine content, which was similar to that of mitochondria incubated at the same spermine concentrations (100 or 1000 μM), a loss of spermine from the permeabilized hepatocytes during oil-layer centrifugation appears more probable.

The result of a "conventional" calculation of the total spermine concentration in hepatocytes, 460 μM, is in good agreement with earlier determinations of total spermine concentration in liver tissue [41]. The estimation of a free concentration of 23 μM, based on a 95% binding of spermine, fits to the experimentally determined lower limit of free spermine concentration, 10 μM. If we take the lower limit of the cytoplasmic spermine concentration (10 μM) and the total spermine concentration (460 μM) in hepatocytes to define the range of physiologically relevant spermine concentrations, it appears that spermine may well influence the velocity and extent of mitochondrial Ca^{2+} uptake, but is unlikely to be a regulator of permeability transition in its own right. In conjunction with ADP, however, it may permit the mitochondria to take up Ca^{2+} from Ca^{2+} oscillations in the cytosol and participate in the re-establishment of low cytosolic Ca^{2+} concentrations without triggering permeability transition. Thus, the presence of spermine could influence the process of Ca^{2+}-induced Ca^{2+} release from mitochondria, which is gaining attention as a generator of cytoplasmic Ca^{2+} oscillations [12, 42]. A competition between the natural polyamines and aminoglucosides for mitochondrial binding may well disturb mitochondrial Ca^{2+} handling and contribute to aminoglucoside toxicity. On the other hand, when polyamine concentrations are substantially elevated, as is the case with many tumor cells and in regenerating tissue [43, 44], the ability of spermine and spermidine to inhibit permeability transition may become important. Permeability transition has recently been linked to the induction of apoptosis [14], and it may be possible that high, but not normal polyamine concentrations in the cytosol could thus have an anti-apoptotic effect.

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