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Polyamine Modulation of Mitochondrial Calcium Transport

I. STIMULATORY AND INHIBITORY EFFECTS OF ALIPHATIC POLYAMINES, AMINOGLUCOSIDES AND OTHER POLYAMINE ANALOGUES ON MITOCHONDRIAL CALCIUM UPTAKE

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ABSTRACT. In this study, the regulation of mitochondrial Ca^{2+} transport by polyamines structurally related to spermine and by analogous polycationic compounds was characterized. Similar to spermine, a number of amino groups containing cationic compounds exerted a dual effect on Ca^{2+} transport of isolated rat liver mitochondria: a decrease in Ca^{2+} uptake velocity and an enhancement of Ca^{2+} accumulation. In contrast to the effects of spermine and other aliphatic polyamines, however, the accumulation-enhancing effect of aminoglucosides, basic polypeptides, and metal–ammine complexes turned into an inhibition of Ca^{2+} accumulation at higher concentrations. Within groups of structurally related compounds, the potency to decrease Ca^{2+} uptake velocity and to enhance Ca^{2+} accumulation correlated with the number of cationic charges. The presence of multiple, distributed cationic charges was a necessary, but not sufficient criterion for effects on mitochondrial Ca^{2+} transport, because cationic polyamines and basic oligopeptides which did not enhance mitochondrial Ca^{2+} accumulation could be identified. Spermine was not able to antagonize the blocking of Ca^{2+} uptake by ruthenium red, but rather showed an apparent synergism, which can be explained as a displacement of membrane-bound Ca^{2+} by spermine. The aminoglucosides, gentamicin and neomycin, but not the inactive polyamine bis(hexamethylene)-triamine, inhibited the binding of spermine to intact mitochondria. Apparently, the binding of spermine, gentamicin, and a number of polyamine analogues to low-affinity binding sites at mitochondria, which have low, but distinct structural requirements and which may correspond to phospholipid headgroups, indirectly influences the activity state of the mitochondrial Ca^{2+} uniporter. The ability of aminoglucosides to displace spermine from the mitochondria and to inhibit mitochondrial Ca^{2+} accumulation may contribute to the mitochondrial lesions, which are known to occur early in the course of aminoglucoside-induced nephrotoxicity. BIOCHEM PHARMACOL **56**;8:977–985, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. spermine; polyamines; aminoglucosides; calcium; rat liver mitochondria

An endogenous cytoprotective role of the polyamine, spermine, has been proposed by several authors, based on the effects of spermine on mitochondrial metabolism and ion transport [1, 2]. On the other hand, spermine has been reported to be cytotoxic, an effect apparently elicited by spermine directly [3], and it has been suggested that prevention of toxicity may be the major reason for the elaborate control of polyamine metabolism [4]. Even if only the actions of spermine on mitochondria are considered, there is an obvious conflict: the stimulatory effect of spermine on mitochondrial Ca^{2+} uptake [5, 6], which has become generally accepted as physiologically relevant [7], is mimicked by aminoglucosides [8], the well-known toxicity of which has been linked to their effects on mitochondrial energetics and ion transport [9, 10]. Typically, the stimulation of mitochondrial Ca^{2+} uptake by spermine leads to an approximate 50% reduction in the steady-state Ca^{2+} concentration in the incubation media of isolated mitochondria [5, 6] and of permeabilized cells [11].

A major hindrance to clarifying the role of spermine is that the active principle by which spermine influences mitochondrial Ca^{2+} transport is not known. There is a long-standing controversy in polyamine research as to which degree the multiple positive valency of the polyamines is responsible for the bewildering multitude of their biological effects [12]. The sequence of potency and efficacy of the natural polyamines with regard to their effects on mitochondrial Ca^{2+} uptake is the same as in many other experimental systems: putrescine $\ll$ spermidine $<$ spermine [6]. This sequence may result from an increasing chain length of the molecules and steric interaction with specific

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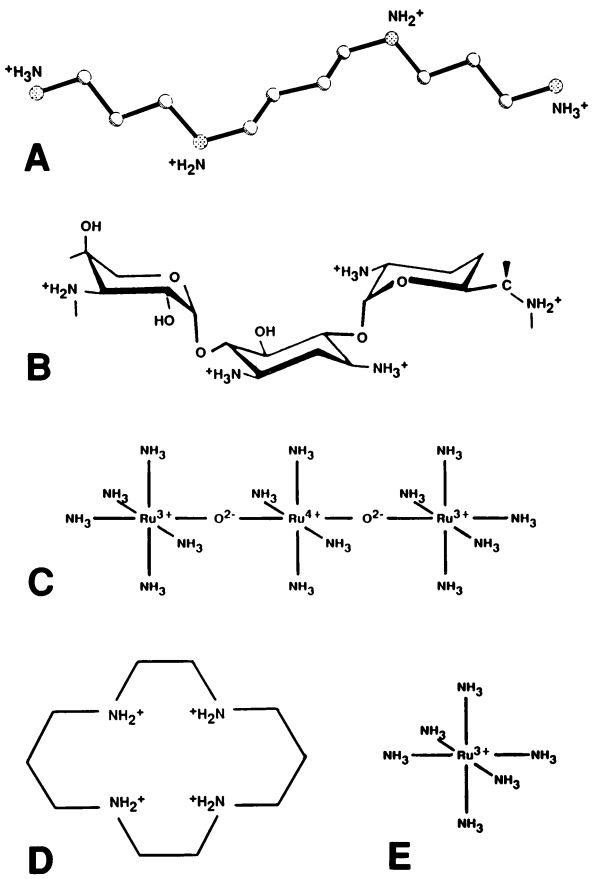

FIG. 1. Chemical structures of representative polyamines. (A) Aliphatic polyamines: crystal structure of spermine (as tetrahydrochloride salt); (B) aminoglucoside antibiotics: conformational structure of gentamicin C1; (C and E) metal–ammine complexes: structural formula of ruthenium red and ruthenium(III) hexammine; (D) cyclic polyamines: structural formula of tetraazacyclotetradecane. The formulas were drawn to approximately the same scale, the distance between the terminal N-atoms of spermine corresponding to *ca.* 15 Å. Ammine ligands (in C and E) are formally neutral, but are polarized due to the strong dipole character of ammonia, with the negative partial charge being directed towards the central atom and the positive partial charge directed outwards.

target structures as well as from the concomitant increase in charge from +2 to +4. The apparent importance of multiple cationic charges for the stimulatory effects of spermine on mitochondrial Ca^{2+} uptake appears paradoxical in view of the fact that the well-known inhibitor of mitochondrial Ca^{2+} uptake, ruthenium red, is also a polycation.

We therefore selected groups of structurally diverse polycationic compounds with known or presumed activity on mitochondrial Ca^{2+} transport to compare their actions with those of spermine and spermidine, putting particular emphasis on the charge versus structure issue. These groups (Fig. 1) were:

A) aliphatic polyamines, which differ from spermine [*N*,*N*′-bis(aminopropyl)-diaminobutane] in the lack of one aminopropyl group (spermidine), the narrower spacing of the amine groups in the molecule (triethylenetetramine, tetraethylenepentamine, and pentaethylenehexamine), or the wider spacing of the amine groups (BHTA§);
B) aminoglucoside antibiotics, which consist of 3–4 glycosidically linked amino sugars and aminocyclitols and have charges between +3 (streptomycin) and +6 (neomycin);
C) metal–ammine complexes, one being ruthenium red (charge +6), the classical inhibitor of mitochondrial Ca^{2+} uptake [13] but also of other ion transport systems [14, 15], which consists of two ruthenium pentammine complexes bridged by oxygen atoms to a ruthenium tetrammine complex in the middle of the molecule [16]. The other compounds were hexammine complexes of ruthenium or cobalt;
D) basic polypeptides, consisting of the protamine, salmine sulfate, and poly-L-lysine (molecular mass of both compounds 5,000–10,000);
E) cyclic polyamines such as tetraazacyclotetradecane and tetraminobenzene, which are comparatively rigid structures as compared to spermine, where the positive charges are distributed along a flexible chain.

By determining which actions are common to all of the above polycations and which are specific for spermine, we intended to define the essential structural features of spermine and its mechanism of action more precisely. The question as to whether the mitochondrial actions of spermine may have cytotoxic or cytoprotective consequences led us to investigate the effects of spermine and of selected polyamine analogues from the above list on the mitochondrial permeability transition. These are described in the accompanying report [17].

MATERIALS AND METHODS

Chemicals

Spermine, spermidine, gentamicin, streptomycin, triethylenetetramine, poly-L-lysine (MW 5,000–10,000), protamine (salmine sulfate, MW 5,000–10,000), ruthenium red, tetraazacyclotetradecane, and dansyl chloride were obtained from Fluka. Tetraethylenepentamine, pentaethylenehexamine, BHTA, ruthenium(III)-hexammine, ruthenium(II)-hexammine, and cobalt(III)-hexammine were from Aldrich. Neomycin, tobramycin, ATP, and Hepes were from Sigma. 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.

§ *Abbreviation:* BHTA, bis(hexamethylene)triamine.

Preparation of Mitochondria and Mitoplasts

Liver mitochondria from Wistar rats were prepared in a medium consisting of 210 mM mannitol, 70 mM sucrose, 20 mM Hepes (pH 7.0) and 0.5 mM EGTA by differential centrifugation as described earlier [18]. Mitoplasts were prepared from isolated mitochondria by incubation in digitonin (0.12 mg/mg of protein) for 15 min [19]. The protein content of the samples was determined by a bicinchoninic acid (BCA)-based protein assay kit (Pierce).

Electrode Measurements

The free Ca^{2+} concentration of the incubation medium (125 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, and 5 mM succinate, adjusted to pH 7.0) of the mitochondria was measured by a purpose-built Ca^{2+}-sensitive minielectrode containing the ionophore ETH 1001. Incubations were performed in a microincubation chamber (40 μL volume) as described [20]. The electrode potential was measured by a pH meter which was connected to an analogue-digital converting board (DAS 8PGA, Keithley–Metrabyte) hosted in a microcomputer. After calibration of the electrode with Ca^{2+} buffers [21], data were acquired and converted into Ca^{2+} concentrations in real time using the Labtech Notebook software (Laboratory Technologies Corp.).

Oxygen Consumption

The oxygen consumption of incubated mitochondrial fractions was determined polarographically by a Clark-type electrode in a thermostated glass vessel at 25° [22].

Polyamine Analysis

At the end of a 2-min incubation using the same conditions as for the Ca^{2+} transport measurements, the mitochondria were separated from the incubation medium by centrifugation (2 min × 8700 *g*) through an oil layer (1-bromodecane/di-isononylphtalate 1:1.7, by vol.) into 6.6% perchloric acid. The polyamines in the perchloric acid phase were dansylated according to Seiler [23], except that reaction time and temperature were 15 min and 56°, respectively. The dansylated polyamines were extracted with toluol and separated by HPTLC with *in situ* quantitation as recently described [24].

Determination of Polyamine Net Charge

A titrimetric technique was employed as described by Josepowitz *et al.* [25]. A solution containing a known amount of polyamine was prepared and HCl added until all amino groups were protonated as visible from the titration curve. The millimoles of NaOH required to establish a pH value of 7.0 divided by the millimoles of polyamine in solution gave the number of neutralized amino groups per molecule. Subtracting this number from the maximal number of charges per molecule gave the net charge.

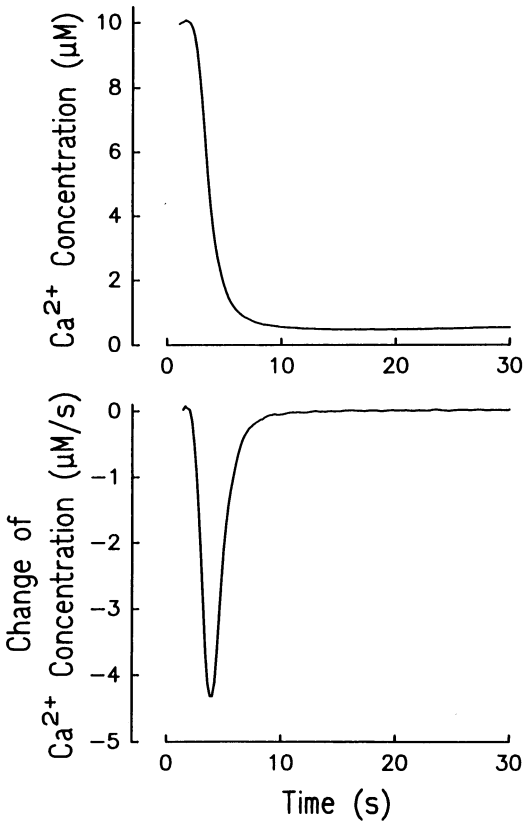

FIG. 2. Calculation of the maximal velocity of mitochondrial Ca^{2+} uptake as a measure of Ca^{2+} uniport activity. The Ca^{2+} concentration curve during mitochondrial Ca^{2+} uptake (upper trace) was transformed into its first derivative, $d[Ca^{2+}]/dt$ (lower trace). The peak value of this curve, representing the maximal velocity of Ca^{2+} concentration decrease in the incubation medium, was taken as the maximal velocity of mitochondrial Ca^{2+} uptake.

RESULTS

In the presence of spermine, the decrease in Ca^{2+} concentration in the incubation medium by uptake into mitochondria appeared slower, but longer-lasting than in controls [18]. Thus, the activity of test compounds was evaluated by two parameters: 1) the minimal Ca^{2+} concentration achieved during a 2-min incubation period; and 2) the maximal velocity of Ca^{2+} concentration decrease. The latter parameter was obtained by calculating the first derivative of the recorded curve of the Ca^{2+} concentration versus time. (Fig. 2).

To exclude the possibility that interactions of spermine with the outer mitochondrial membrane contributed to the effects on Ca^{2+} uptake, we tested whether mitoplasts and mitochondria reacted in the same way to the presence of spermine. While control mitoplasts had a slightly higher velocity of Ca^{2+} uptake than control mitochondria (376 ± 4 vs 310 ± 5 nmol/mg × min, 10 μM initial Ca^{2+}

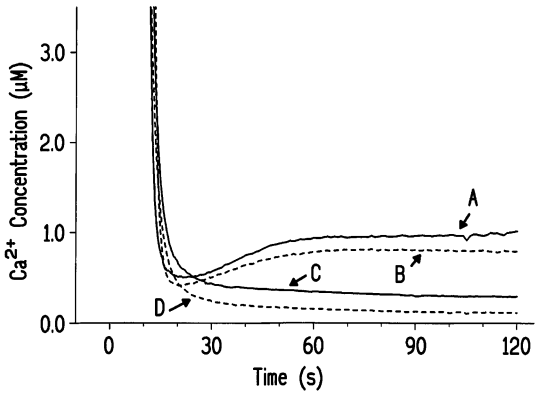

FIG. 3. Effect of spermine on mitochondrial Ca^{2+} uptake in the presence of potassium chloride (solid lines) or choline chloride (dashed lines). The concentration of spermine was 400 μM, the initial Ca^{2+} concentration was set at 10 μM, and the experiment was started by adding 1 μL of the mitochondrial fraction. In the presence and absence of potassium, spermine (C; D) significantly ($P < 0.05$, *t*-test) reduced the maximal velocity of Ca^{2+} uptake and the minimal Ca^{2+} concentration in the incubation medium as compared with controls (A; B).

concentration), the minimal Ca^{2+} concentration in the incubation medium was not significantly different, nor was the effect of 400 μM spermine with both preparations (data not shown). The role of K^+, which was present in high concentration in the cytosol-adapted incubation medium, was investigated by measuring the Ca^{2+} uptake of intact mitochondria in a medium in which KCl was replaced by an equimolar concentration of choline chloride. As shown in Fig. 3, the typical effects of spermine were also visible when KCl was exchanged for choline chloride, ruling out the possibility that changes in Ca^{2+} transport were secondary to an influence of spermine on K^+ uptake. Thus, the effects of all test agents were measured using intact mitochondria and a cytosol-adapted incubation medium with a high K^+ concentration.

When tested according to the above-described experimental protocol, both spermine and ruthenium red reduced the maximal velocity of Ca^{2+} uptake (Fig. 4). In contrast, spermine over the whole concentration range consistently enhanced Ca^{2+} accumulation (measured as a decreased minimal Ca^{2+} concentration of the medium), and ruthenium red consistently inhibited Ca^{2+} accumulation (measured as an increased minimal Ca^{2+} concentration of the medium). A number of compounds (e.g. neomycin) showed a biphasic effect: enhancement of Ca^{2+} accumulation at lower concentrations turning into an inhibition at higher concentrations (Fig. 4).

In Table 1, an overview is given of the ability of metal–ammine complexes, basic peptides, aminoglucosides, and aliphatic polyamines to exert a spermine-like effect on mitochondria (to enhance Ca^{2+} accumulation and decrease maximal Ca^{2+} uptake velocity). Among the aliphatic polyamines, spermine and pentaethylenehexamine most effectively enhanced Ca^{2+} accumulation. Aliphatic polyamines did not inhibit Ca^{2+} accumulation at higher

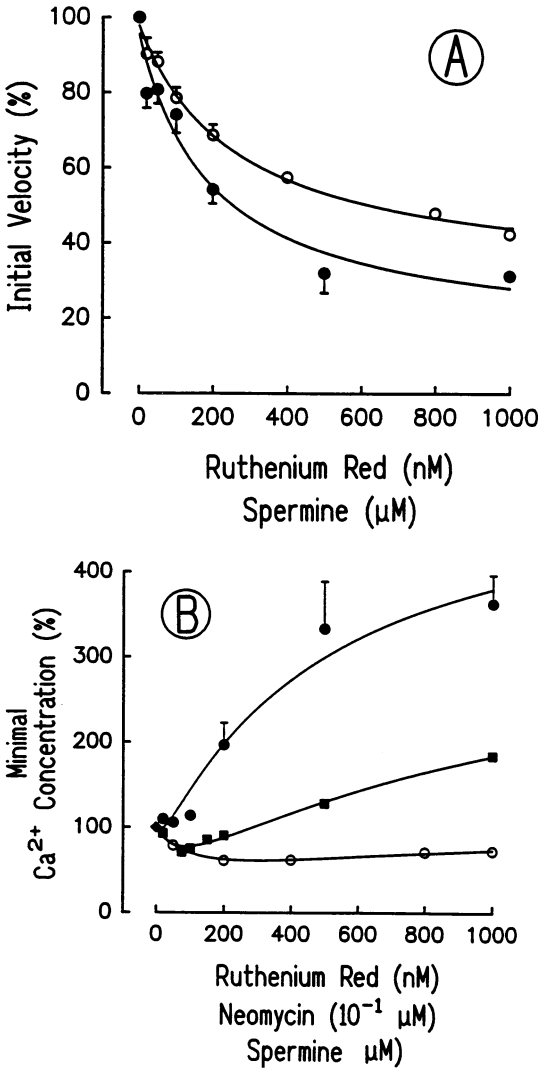

FIG. 4. Effects of polyamines and polyamine analogues on velocity of Ca^{2+} uptake and on Ca^{2+} accumulation by isolated rat liver mitochondria. (A) Concentration-dependent inhibition by ruthenium red (closed circles) and spermine (open circles) of the initial velocity of Ca^{2+} uptake. The initial Ca^{2+} concentration of the incubation medium was 2 μM. Values are means ± SEM of four experiments. The maximal velocity of Ca^{2+} uptake by control mitochondria (82 ± 2 nmol × mg^{-1} × min^{-1}) was set as 100%. Both ruthenium red and spermine significantly inhibited the initial velocity of Ca^{2+} uptake ($P < 0.01$, ANOVA). Note that the concentrations are given in nM for ruthenium red and in μM for spermine. (B) Concentration-dependent effects of ruthenium red (closed circles), spermine (open circles), and neomycin (closed squares) on the minimal extramitochondrial Ca^{2+} concentration achieved during a 2-min incubation as a measure of mitochondrial Ca^{2+} accumulation. The minimal Ca^{2+} concentration achieved by control mitochondria (0.45 ± 0.02 μM) was set as 100%. Spermine significantly decreased the minimal Ca^{2+} concentration, while ruthenium red significantly increased this parameter. Neomycin at low concentrations (up to 15 μM) decreased and at higher concentrations (from 50 μM upwards) increased the minimal Ca^{2+} concentration. Note the different concentration scales for all three compounds.

concentrations. The aminoglucoside, gentamicin, was at least as effective as spermine in enhancing Ca^{2+} accumu-

TABLE 1. Inhibition of the initial velocity of Ca^{2+} uptake and stimulation of the Ca^{2+} accumulation of isolated rat liver mitochondria by polyamines and polyamine analogues

Test agent	Initial velocity		Enhancement of accumulation	
	IC_{50} (μM)	Maximal effect (% inhibition)	EC_{50} (μM)	Maximal effect (% decrease)
Metal–ammine complexes				
Ruthenium red (+6)	0.19 ± 0.02	86 ± 2		no Ca^{2+} accumulation
Ruthenium(III) hexammine (+3)	5.2 ± 0.4	94 ± 1	1.1 ± 0.2	11 ± 3
Cobalt(III) hexammine (+3)	7.5 ± 0.8	93 ± 2	1.6 ± 0.3	9 ± 3
Ruthenium(II) hexammine (+2)	10.9 ± 1.1	96 ± 1	1.8 ± 0.2	12 ± 3
Basic peptides				
Protamine (*ca.* +26)	1.2 ± 0.1	93 ± 1	0.9 ± 0.1	22 ± 1
Poly-L-lysine (*ca.* +32)	5.2 ± 0.8	60 ± 4	2.1 ± 0.2	39 ± 4
Aminoglucosides				
Neomycin (+6)	6.7 ± 0.8	94 ± 2	3.3 ± 0.3	28 ± 2
Tobramycin (+5)	113 ± 16	70 ± 4	21 ± 4	46 ± 3
Gentamicin (+5)	148 ± 23	80 ± 3	28 ± 1	49 ± 3
Streptomycin (+3)	252 ± 56	73 ± 3	88 ± 13	36 ± 3
Aliphatic polyamines				
Pentaethylene hexamine (+6)	164 ± 22	68 ± 3	26 ± 4	45 ± 5
Spermine (+4)	189 ± 19	75 ± 2	45 ± 5	45 ± 6
Tetraethylene pentamine (+5)	248 ± 19	75 ± 3	40 ± 10	37 ± 3
Triethylene tetramine (+4)	274 ± 68	49 ± 4	90 ± 16	23 ± 2
Spermidine (+3)	233 ± 60	36 ± 6	176 ± 16	23 ± 3

Given are the half maximally inhibitory concentrations (μM) and the maximal effects (% inhibition of control initial velocity of Ca^{2+} uptake) for the inhibition of Ca^{2+} uptake velocity and the half maximally effective concentrations (μM) and the maximal effects (% decrease in control minimal Ca^{2+} concentration in the incubation medium) for the enhancement of Ca^{2+} accumulation. Values are means ± SEM of 4–6 experiments each. All effects were significant, except for those of the metal–ammine complexes on Ca^{2+} accumulation, which were only marginally significant (P between $P = 0.08$ and $P = 0.05$).

lation, but like neomycin, this effect turned into an inhibition at higher concentrations (>500 μM). The polypeptides also had a concentration-dependent biphasic effect on Ca^{2+} accumulation which was remarkably potent. Even the mononuclear metal–ammine complexes produced a slight increase in Ca^{2+} accumulation within a small concentration range, but no such increase could be seen with the trinuclear complex, ruthenium red. All test agents decreased the maximal velocity of Ca^{2+} uptake. Here, a significant correlation existed between high potency (low IC_{50} value) and high inhibitory efficacy ($r = 0.70$, $P = 0.004$). A good correlation existed between the IC_{50} values for inhibition of Ca^{2+} uptake velocity and the EC_{50} values for enhancement of Ca^{2+} accumulation ($r = 0.78$, $P < 0.001$). There was no correlation between potency and efficacy to enhance Ca^{2+} accumulation, apparently due to the biphasic effect of aminoglucosides, basic polypeptides, and metal–ammine complexes. Cyclic polyamines were ineffective except for tetraazacyclotetradecane, which produced a small enhancement of Ca^{2+} accumulation at millimolar concentration (data not shown). Similarly, the basic oligopeptides, tri- and tetralysine, were not markedly effective (data not shown), nor was BHTA, an aliphatic polyamine with a close similarity to spermine and spermidine [24].

The cationic charges given in Table 1 refer to the state of complete protonation. However, protonation could not be expected to be complete under our experimental conditions. To delineate the charge–activity relation more precisely, we measured the net charge of selected compounds at pH 7.0, which is the value of the incubation medium. As shown in Table 2, aliphatic polyamines and ruthenium red are protonated to *ca.* 90%, while aminoglucosides have only two-thirds of their maximal charge at pH 7.0. The

TABLE 2. Net charge of aliphatic polyamines, aminoglucosides and ruthenium red at pH 7.0

Test agent	Maximal charge	Net charge at pH 7.0	% of maximum
Spermine	+4	+3.69 ± 0.05	92.3
Triethylenetetramine	+4	+3.29 ± 0.04	82.3
Tetraethylene pentamine	+5	+4.34 ± 0.03	86.8
Tetraazacyclotetradecane	+4	+3.92 ± 0.04	98.0
Tobramycin	+5	+3.39 ± 0.04	67.8
Neomycin	+6	+4.09 ± 0.24	68.2
Ruthenium red	+6	+5.23 ± 0.12	87.2

Charge was determined with a titrimetric procedure as described by Josepowitz *et al.* [25], except that the titration was continued until pH 7.0, because this was the value of the incubation medium of the Ca^{2+} transport experiments. Values are means ± SEM of four determinations.

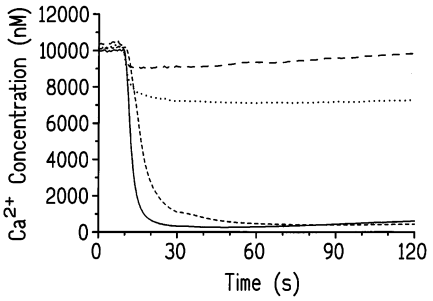

FIG. 5. Paradoxical synergism between ruthenium red and spermine. Incubation medium containing the test compounds was set at an initial Ca^{2+} concentration of 10 μM and Ca^{2+} uptake was started by injection of a mitochondrial suspension into the incubation chamber of the Ca^{2+} minielectrode. The traces are means of four experiments. The minimal Ca^{2+} concentration achieved in the presence of 1 μM ruthenium red plus 400 μM spermine (long-dashed trace) was significantly ($P < 0.05$) higher than that in the presence of ruthenium red alone (dotted trace). The effect of 400 μM spermine alone is shown by the short-dashed trace and control Ca^{2+} uptake is shown by the solid trace.

slightly lower protonation of triethylenetetramine compared with spermine is compatible with the shorter span of methylene groups in triethylenetetramine and, consequently, a smaller +i-effect. However, this rather small difference is not sufficient to explain the marked differences in potency and efficacy between these two compounds.

Since ruthenium red was the only purely inhibitory compound with respect to Ca^{2+} accumulation, we tested whether spermine was able to antagonize the inhibition of Ca^{2+} uptake by ruthenium red. In the presence of 400 μM spermine, the inhibition of Ca^{2+} uptake by 0.25, 1 or 2 μM ruthenium red was not attenuated, but rather increased. Apparently, the presence of spermine led to an increased Ca^{2+} concentration in the medium by a mechanism different from the blocking of the Ca^{2+} uniporter (Fig. 5). Mg^{2+} is known to inhibit mitochondrial Ca^{2+} uptake and in an earlier investigation, we found that Mg^{2+} antagonized the spermine-induced enhancement of Ca^{2+} accumulation [18]. When spermine (400 μM) was present together with Mg^{2+} (1 or 5 mM), the decrease in Ca^{2+} uptake velocity by these agents was clearly additive, even at the higher concentration of Mg^{2+}. However, a net Ca^{2+} uptake continued during the whole incubation period and at the end of a 6-min incubation, there was no longer a significant difference between the Ca^{2+} concentration in the presence or absence of Mg^{2+} (data not shown).

Earlier, we had concluded that spermine did not really decrease the rate of mitochondrial Ca^{2+} uptake but only displaced Ca^{2+} from low-affinity sites at the membranes, thereby retarding the decrease in Ca^{2+} concentration in the medium [18]. The observation which had led to this conclusion, namely that the maximal velocity of Ca^{2+} uptake was faster than control when spermine was present only in a preincubation, slower than control when spermine was present during Ca^{2+} uptake, and the same as control when spermine was present in both incubations, could be reproduced with selected polyamine analogues such as gentamicin, neomycin, poly-L-lysine, and even ruthenium(III) hexammine, but not with ruthenium red, which inhibited Ca^{2+} uptake under all conditions (data not shown).

To assess whether spermine and polyamine analogues influence the Ca^{2+} uniporter exclusively when they enhance Ca^{2+} accumulation or whether they might also interfere with the generation of the driving force of mitochondrial Ca^{2+} uptake, the oxygen consumption of mitochondria was measured. The oxygen consumption in the absence of ADP was reduced by 400 μM spermine to 53.4 ± 2.7%, by 250 μM gentamicin to 49.1 ± 4.3%, and by 5 μM poly-L-lysine to 46.0 ± 4.7% of control (9.2 ± 0.4 nmol O_2 min^{-1} × mg of $protein^{-1}$). However, BHTA and Mg^{2+} also significantly reduced oxygen consumption to 60.1 ± 7.5 or 60.0 ± 4.1% of control (all values are means ± SEM of five experiments), which was not significantly different from the effect of the active polyamines. ATP generation under the same conditions was not reduced by spermine.

By chromatographic determination of the amount of spermine which was bound by incubated, intact mitochondria, we tested whether the similarity between the effects of aminoglucosides and spermine was due to common binding sites at mitochondria. The binding of spermine (40 or 400 μM) to a mitochondrial fraction was measured as a function of gentamicin concentration. Spermine at a concentration of 40 μM was completely displaced from its binding sites by gentamicin with a biphasic characteristic (Fig. 6A). In the presence of 400 μM spermine, the displacement was incomplete, but again a biphasic curve resulted. The displacement of spermine by neomycin had similar characteristics, but neomycin was a more potent competitor than gentamicin by a factor of *ca.* 200 (Fig. 6B). In the presence of 40 μM spermine, neomycin reduced the mitochondrial spermine content below endogenous levels. Among the aliphatic polyamines, spermidine slightly, but significantly ($P < 0.05$, ANOVA) decreased spermine binding (data not shown), while BHTA was ineffective as shown recently [24].

DISCUSSION

It has been known for some time that the enhancing effect of spermine on mitochondrial Ca^{2+} uptake is shared by a number of polycationic compounds. In a recent review comparing the various biological effects of aliphatic polyamines and aminoglucosides, it was concluded that the effects of these polycationic compounds on mitochondrial Ca^{2+} transport were probably identical [7]. In the present study, it is shown that marked differences exist. This resolves hitherto conflicting data and indicates that distributed polycationic charge, a common property of both groups of compounds, is not sufficient to define the essential structural features of spermine effects on mitochondrial Ca^{2+} transport. It must be emphasized that the effect of the

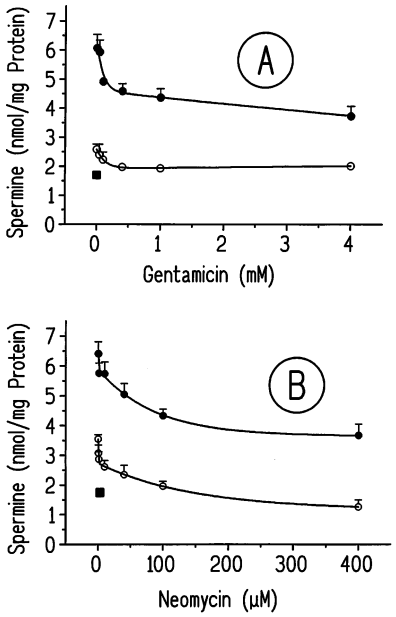

FIG. 6. Displacement of mitochondrially bound spermine by aminoglucosides. (A) Binding of spermine to a mitochondrial fraction from rat liver as a function of the gentamicin concentration. Mitochondrial fractions were incubated for 2 min at 25° in incubation medium containing 40 μM (open circles) or 400 μM (closed circles) of spermine and gentamicin in the range of 0.04 to 4 mM. Incubations were stopped and bound spermine separated from free by oil-layer centrifugation. Bound spermine was determined chromatographically. A curve was fit to the data according to the biphasic function Y = A^DX + B^EX + C. The IC_{50} values for displacement of 40 μM spermine were 0.13 and 53.32 mM, while the corresponding values for 400 μM spermine were 0.07 and 10.78 mM. The data are means ± SEM of five experiments, with the square indicating the endogenous spermine content of incubated mitochondrial fractions. (B) Binding of spermine to a mitochondrial fraction from rat liver as a function of the neomycin concentration. Conditions were the same as in (A), except that instead of gentamicin neomycin was present in the range of 0.4 to 400 μM. The IC_{50} values for displacement of 40 μM spermine (open circles) were 0.36 and 101.48 μM, and the corresponding values for 400 μM spermine (closed circles) were 0.45 and 59.95 μM. The data are means ± SEM of 5 experiments, the square indicating the endogenous spermine content of incubated mitochondrial fractions.

polyamines in this study is not simply an activation of mitochondrial Ca^{2+} uptake; rather, there is a dual effect: a decrease in the velocity of Ca^{2+} uptake and an increase in Ca^{2+} accumulation capacity as has been described for spermine in an earlier report from this laboratory [18]. The conflict between Åkerman's observation that spermine inhibits Ca^{2+} uptake [26] and Nicchitta and Williamson's observation that spermine stimulates Ca^{2+} uptake [5] as well as the corresponding conflict between data by Sastrasinh *et al.* [27], who found an inhibition of Ca^{2+} uptake by gentamicin and data by Kröner [8], who found a stimulation, is very probably due to this duality. When techniques which have a low time resolution are used (e.g. 0.1 sec^{-1} with $^{45}Ca^{2+}$ measurements), it may not be possible to distinguish an increased maximal rate of Ca^{2+} uptake from a prolonged net uptake [8].

A displacement of Ca^{2+} by spermine and other polyamines from low-affinity binding sites occurs with model phospholipid membranes [28] and intact mitochondria [29, 30] and may complicate the interpretation of Ca^{2+} transport measurements. A displacement of Ca^{2+} from mitochondrial membranes may well explain the paradoxical observation that spermine apparently enhanced the blocking action of a maximally effective concentration of ruthenium red and the variability of the effect of polyamines on the maximal velocity of Ca^{2+} uptake, which depended on the order of exposure of the mitochondria to polyamine or Ca^{2+}. However, the observation that the velocity of Ca^{2+} uptake is decreased when a Ca^{2+} "pulse" is added to spermine-incubated mitochondria in steady state (see accompanying paper, [17] requires the assumption that Ca^{2+} uptake into the mitochondria is indeed slowed by spermine.

By which mechanism do polyamines enhance Ca^{2+} accumulation (i.e. decrease the steady-state Ca^{2+} concentration in the extramitochondrial space)? An "allosteric" activation of the Ca^{2+} uniporter as was initially suggested by several authors [5, 31, 32] appears unlikely for two reasons: 1) the maximal velocity of Ca^{2+} uptake was not increased ([18]; this investigation); and 2) the structural requirements which could be defined fit more easily to an interaction with membrane phospholipids (see below) rather than to a polyamine-binding regulatory site on the uniporter protein. Alternatively, an increase in the driving force could be considered. An increase in membrane potential by a few millivolts was in fact described earlier [5, 6]. Such a small effect might already account for the increased Ca^{2+} accumulation, because a 50% reduction in free extramitochondrial Ca^{2+} concentration in steady-state (e.g. from 0.6 to 0.3 μM) led to an increase in the total mitochondrial Ca^{2+} load of only 1–2% in our system. However, BHTA, which does not increase Ca^{2+} accumulation [24], was at least as efficient as spermine in increasing mitochondrial membrane potential (see accompanying paper, Ref. 17). Thus, an effect on membrane potential may be necessary, but not sufficient.

Since all polyamines which enhanced Ca^{2+} accumulation also reduced the velocity of Ca^{2+} uptake, while the inactive polyamine, BHTA, influenced neither of these parameters, a relation between these two effects seems likely. However, a reduction in uptake velocity as such is not necessarily coupled to an enhanced Ca^{2+} accumulation, since Mg^{2+} also inhibits the velocity of mitochondrial Ca^{2+} uptake, probably by binding directly to the uniporter [33], but does not enhance Ca^{2+} accumulation [17]. There is a similarity between the inhibition of uptake velocity by Mg^{2+} and spermine in that both abolished the "rebounding" of Ca^{2+} uptake, which has been interpreted as a Ca^{2+}-dependent activation of the Ca^{2+} uniporter [34]. In view of the low structural specificity of the polyamine effects, it appears that spermine and polyamine analogues inhibit the activity of the Ca^{2+} uniporter indirectly, mediated by binding to low-affinity, low-specificity binding sites, probably membrane phospholipids (see below), whereby

the activity of the channel components of the Ca^{2+} uniporter [35] might be affected. The enhancement of Ca^{2+} accumulation could then result from changes in voltage-sensing of the channel or an inhibition of a Ca^{2+}-dependent inactivation mechanism of the channel. Alternatively, binding of polyamines and analogues at low-affinity binding sites might affect additional ion transport systems which are not influenced by Mg^{2+}. A decreased matrix Ca^{2+} concentration [36] as a result of this action would allow more Ca^{2+} to flow down the concentration gradient via the uniporter channel. The biphasic effect of aminoglucosides and basic polypeptides on Ca^{2+} accumulation capacity (stimulation at low and inhibition at high concentration) originally suggested a ruthenium red-like direct block of Ca^{2+} uptake superseding the indirect stimulation of Ca^{2+} accumulation. However, the experiments described in the accompanying paper [17], which demonstrate that effects of polyamines on mitochondrial Ca^{2+} uptake and on mitochondrial permeability transition are separable, also revealed that aminoglucosides and basic polypeptides at high, but not at low concentrations, decrease mitochondrial membrane potential, thus abolishing the driving force for Ca^{2+} uptake.

In spite of its widespread use, the essential structural feature of ruthenium red as an inhibitor of a number of ion transport systems and ligand at Ca^{2+} binding proteins [37] has not yet been defined. The results obtained with the mononuclear metal–ammine complexes suggest that any stable complex of a metal ion with multiple ammine ligands is able to exert a strong inhibitory action on mitochondrial Ca^{2+} uptake. Depending on the positive charge of the central atom, which may equally well be a cobalt atom instead of a ruthenium atom, a polarization of the ammine ligands is induced, which may thus correspond functionally to protonated amine groups with regard to the interaction with cation transport systems or anionic phospholipids.

The observation that not only the sum of charges but also steric factors determine the structure-activity relation of aliphatic polyamines and polyamine analogues (e.g. compare the effects of tetravalent triethylenetetramine with those of spermine or of trivalent BHTA with those of spermidine) is compatible with an interaction of these compounds with membrane phospholipids (see also [30, 38]). Studying the interactions between organic polycations and model phospholipid membranes, Chung *et al.* [39] noticed that both spermine and gentamicin behaved as tetravalent point charges. They concluded that spermine and gentamicin lie flat on the bilayer surface, thus bringing into contact all of their positive charges with negatively charged phospholipid headgroups. It is conceivable that short distances between the amine groups (e.g. in triethylenetetramine) reduce the number of charge interactions and thus the biological activity. On the other hand, an increase in hydrophobicity by elongation of the carbohydrate backbone (as in BHTA) may induce an insertion of the molecule into the hydrophobic core of the membrane bilayer. In terms of this model, the inefficiency of tetralysine and the efficiency of poly-L-lysine, which is roughly comparable to that of hexavalent neomycin, would best be explained by assuming that only a minor portion of the protonated amine residues of the basic peptides can interact with the membrane surface charge.

The hypothesis that spermine and aminoglucosides share common binding sites at the mitochondrial membrane was substantiated by competition binding experiments. Indeed, binding of spermine and aminoglucosides to phospholipids of cellular membranes and mitochondria has already been described [25, 40]. Here, we demonstrate a competition between spermine and aminoglucosides which is consistent with the effects on mitochondrial Ca^{2+} uptake. The ability of gentamicin to completely displace mitochondrially bound spermine (down to the endogenous spermine content) in the presence of 40 μM spermine, but only incompletely with 400 μM, suggests that spermine at the higher concentration binds to sites which have a higher affinity for aliphatic polyamines than for aminoglucosides, perhaps because of their hydrophobic stretches. The observation that in the presence of the low, but not of the high spermine concentration, neomycin reduced the spermine content of the mitochondria below endogenous levels suggests that neomycin induces a release of spermine from the matrix space, but cannot do so when spermine concentrations are high.

In summary, the results obtained in this study have led us to set up a unifying hypothesis to explain not only the effects of spermine on mitochondrial Ca^{2+} transport, but also of a number of organic and inorganic polycationic compounds. A primary effect at cation-binding phospholipid headgroups would explain the comparatively low potency of the polyamines, the charge–activity relationship and also the low, but distinct structural requirements. The observation that aminoglucosides at high concentrations displace spermine from mitochondrial binding sites and inhibit Ca^{2+} uptake by reducing membrane potential suggests that the well-known toxicity of this group of compounds may involve an interference with mitochondrial functions, due to an antagonism of spermine function rather than an imitation. To delineate more clearly how the mitochondrial effects of aliphatic polyamines and aminoglucosides relate to their cytoprotective or cytotoxic properties (see the introductory section), the effects of spermine, gentamicin, and selected polyamine analogues on mitochondrial permeability transition were investigated and described in the accompanying paper [17].

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