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Polycations induce the release of soluble intermembrane mitochondrial proteins

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Abstract

The release of proapoptotic proteins from the intermembrane space of mitochondria is an early critical step in many pathways to apoptosis. Induction of the mitochondrial permeability transition pore (PTP) was suggested to be the mechanism of the release of soluble mitochondrial intermembrane proteins (SIMP) in apoptosis. However, several studies suggested that proapoptotic proteins (e.g. Bax and Bid) can induce the release of SIMP (e.g. cytochrome *c* (cyt *c*) and adenylate kinase 2 (AK2)) in vivo and in vitro independent of PTP. We have found that a number of structurally diverse polycations, such as aliphatic polyamines (e.g. spermine and to a lesser extent spermidine), aminoglycosides (e.g. streptomycin, gentamicin and neomycin), and cytotoxic peptides (e.g. melittin), induce the release of SIMP from liver mitochondria, in vitro. All the polycations released AK2 together with cyt *c*, suggesting that rupture of the outer membrane is a common mechanism of cyt *c* release by these polycations. Several polycations (e.g. spermine, spermidine and neomycin) induced SIMP release without inducing significant swelling, and this release was not inhibited significantly by the PTP inhibitor cyclosporin. In contrast, under the same conditions, streptomycin and melittin induced swelling and SIMP release that was inhibited strongly by cyclosporin. Gentamicin-induced swelling and release of SIMP were partially inhibited by cyclosporin. The affinity of polyamines to the anionic phospholipids of the mitochondrial membranes (spermine = neomycin > gentamicin > streptomycin = spermidine) correlated roughly with their ability to induce PTP-independent release of SIMP, which suggests that the binding of polycations to the anionic phospholipids of the outer mitochondrial membrane facilitates the rupture of this membrane. However, some polycations facilitated the induction of PTP, possibly by binding to cardiolipin on the inner membrane. This dual mechanism may be relevant to the induction of SIMP release in apoptosis.

Keywords: Mitochondria; Cytochrome *c*; Permeability transition pore; Polyamine; Aminoglycoside; Apoptosis

1. Introduction

Apoptosis is a genetically programmed cell death process that efficiently disposes of injured, dysfunctional, or superfluous cells [1]. Many cellular events trigger both proapoptotic and antiapoptotic signals, and the integration of these contrasting signals determines whether the cell proceeds to execute apoptotic death or recovers. Two large families of proteins that

Abbreviations: cyt *c*, cytochrome *c*; AK, adenylate kinase; AIF, apoptosis inducing factor; SIMP, soluble intermembrane protein(s); PTP, permeability transition pore; ROS, reactive oxygen species; $\Delta\Psi m$, mitochondrial membrane potential; CL, cardiolipin; PI, phosphatidylinositol

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play a central role in the integration of apoptotic signals are the caspases, a group of cysteine proteases that are activated during apoptosis [2], and the Bcl-2 family that include both proapoptotic and antiapoptotic proteins [3]. The mitochondrion is the site of integration of several proapoptotic and antiapoptotic signals [4]. The soluble intermembrane proteins (SIMP) are released from the mitochondrial intramembrane space during apoptosis, and several of these proteins enhance apoptosis (e.g. cytochrome *c* (cyt *c*), apoptosis inducing factor (AIF) and procaspases). For example, the activation of caspase-3, which commits the cell to the execution of apoptosis, is enhanced by the release of cyt *c*, which activates caspase-9, which in turn activates caspase-3.

Proapoptotic proteins of the Bcl-2 family (e.g., bax, bak and bid) translocate to the mitochondria during apoptosis and induce the release of cyt *c*, while antiapoptotic proteins of the Bcl-2 family (e.g. Bcl-2 and Bcl-X(L)), are anchored to the outer mitochondrial membrane, and inhibit the release of cytochrome *c* [5]. The mechanism(s) by which the proapoptotic proteins of the Bcl-2 family induce cytochrome *c* release from mitochondria have not been elucidated as yet. It is also apparent that other signals (e.g. Ca^{2+}, nitric oxide and reactive oxygen species (ROS)) may induce the release of proapoptotic proteins from the mitochondria, independent of the proapoptotic Bcl-2 proteins [6,7].

The induction of the mitochondrial permeability transition pore (PTP) results in large amplitude swelling, outer membrane rupture, and the release of cyt *c* and other SIMP from isolated mitochondria [8]. Several investigators reported that in apoptosis PTP activation, detected as the collapse of the mitochondrial membrane potential ($\Delta\Psi m$), preceded the release of SIMP, and that the release was inhibited by PTP inhibitors (reviewed in [4,9]). However, other investigators reported that cyt *c* release in apoptosis preceded the collapse of $\Delta\Psi m$, and was not inhibited by PTP inhibitors [10,11]. Moreover, the caspase inhibitor zVAD.fmk inhibits the collapse of $\Delta\Psi m$ in some apoptotic systems, without inhibiting the release of cyt *c* [12]. There are conflicting reports regarding the participation of PTP in bax-induced release of cyt *c* from mitochondria [9,12–15]. However, it appears that a subclass of the Bcl-2 family, which only contains the homology domain BH3 (e.g. bid and bik) induce cyt *c* release without inducing PTP [15–17].

Many cytotoxic peptides produced by insects, bacteria and other organisms contain clusters of positive charges that destabilize membranes [18]. Ellerby et al. [19] showed recently that a short synthetic peptide that carries a cluster of six D-lysine residues ($_D$(KLAKLAK)$_2$) caused mitochondrial swelling and release of cyt *c* (detected by activation of caspase-3 in a cell free system), and induced apoptosis in endothelial cells that were tricked into accumulating the peptide. This activity was attributed to direct disruption of the mitochondrial membranes. However, it is known that mastoparan, another positively charged cytotoxic peptide, induces PTP in isolated mitochondria [20], and it is therefore not clear whether the release of cyt *c* in the study of Ellerby et al. [19] resulted from the activation of PTP, or from direct destabilization of the outer membrane. Another polycation, the ubiquitous aliphatic polyamine spermine, was shown to induce apoptosis when loaded into HL60 cells [21] by inducing the release of cyt *c* from the mitochondria [22]. Since spermine is known to inhibit PTP [23], the release of cyt *c* by spermine may also depend on direct destabilization of the outer membrane. Most proapoptotic proteins carry clusters of positively charged residues. Bax, which carries a cluster of positively charged residues in its membrane insertion domain, was shown to destabilize the structure of planar phospholipid bilayers [24]. Bcl-2, in which these residues are replaced by negatively charged residues, did not affect membrane stability. Similarly, Bid and tBid (the active form of bid) destabilized phospholipid membranes and released proteins encapsulated within liposomes [17]. These findings suggest that direct destabilization of the outer membrane phospholipid bilayer by Bax and BH3-only proteins could contribute to their ability to induce SIMP release.

In this study we investigated the effects of a number of polycations of diverse structure on the release of SIMP (e.g., cyt *c* and adenylate kinase (AK)) from isolated mouse liver mitochondria. We found that these polycations cause the rupture of the outer mitochondrial membrane and release SIMP from mitochondria by both PTP-dependent and -independent mechanisms. The results suggest that the same polycation may induce either or both pathways, and sug-

gest that the interaction of the charged polycations with the anionic phospholipids of the inner and outer mitochondrial membranes may play a critical role in this process.

2. Materials and methods

2.1. Preparation of mouse liver mitochondria

Mitochondria were prepared from minced mouse liver by conventional differential centrifugation. Briefly, the minced liver was homogenized in a sucrose (0.25 M)/EGTA (0.5 mM)/HEPES (12 mM, pH 7.4)/BSA (0.5%) buffer, and first centrifuged at low speed ($500\times g$) for 10 min. The pellet was suspended again in the isolation medium and centrifuged at $1000\times g$ for 10 min. The two supernatants were combined and centrifuged at $8000\times g$ for 10 min. The mitochondrial pellet was resuspended, discarding the lysosomal layer from the top and the red blood cell layer from the bottom, and centrifuged once more at $8000\times g$ for 10 min. The pellet of purified mitochondria was suspended in a small volume of sucrose (0.3 M)/HEPES (3 mM, pH 7.4) solution and kept on ice. All the experiments described in this study were conducted in a medium that contained 200 mM KCl, 10 mM K-HEPES (pH 7.4), 5 mM K-succinate, 2 mM K-P_i, and 0.1% BSA.

2.2. Determination of cyt c release by Western blot

Both the amount of cytochrome *c* in the supernatant and in the mitochondrial pellet was determined by Western blot. Each sample was subjected to SDS-PAGE (Laemmli system: 12% acrylamide/6% acrylamide stacker), then electroblotted to nitrocellulose membranes in Tris-glycine-methanol transfer buffer for 50 min at 100 V. The blots were washed, developed and detected by chemiluminescence using Western Light Plus reagents (Tropix). The primary monoclonal antibody was mouse anticytochrome *c* (7H8.2C12, Pharmingen) used at a dilution of 1/1000. Band intensities were determined by scanning the X-ray films developed after exposure to the chemiluminescent blots and analyzing the optical densities using a Molecular Dynamics imaging system.

2.3. Assay for the amount of adenylate kinase that is released from the mitochondria

Adenylate kinase was estimated using an enzymatic assay – the initial rate of conversion of ADP to ATP. 20 μl of each sample were mixed with 30 μl of 20 mM tricine-HEPES (pH 7.6), 0.2% BSA. The solution was warmed to 30°C, then 7 μl Mg-ADP solution (43 mM $MgCl_2$/27 mM ADP, pH 7.6) were quickly mixed in, and 10 μl aliquots were removed at 0 (first quenched before ADP addition), 30 and 60 s after ADP addition, and quenched by mixing with 3.3 μl 30% trichloroacetic acid. The mixtures were centrifuged 5 min in a microfuge and 12 μl of the supernatant was neutralized with 3.6 μl of 3:2 1 M NaOH:0.2 M tricine-HEPES, pH 7.8, then diluted 1000-fold with luciferase assay buffer for ATP determination. To determine ATP, 100 μl of a diluted sample was mixed with 100 μl of luciferase-luciferin solution (containing about 200 ng/ml luciferase and 0.2 mM luciferin. After 5–10 min at room temperature, the luminescence of the sample and ATP standards was measured in a scintillation counter.

Mitochondrial swelling was determined from the absorbance (i.e. light scattering) of the mitochondrial suspension measured at 540 nm. Oxygen consumption was determined with an oxygen electrode (YSI).

3. Results

3.1. Estimation of cyt c release from liver mitochondria from its effect on the rate of respiration

Several recent studies have shown that induction of PTP by calcium, or other inducers, results in the release of many proteins from the intermembrane space, including cyt *c* and adenylate kinase (AK), apparently by a rupture of the outer mitochondrial membrane (cf. [8]). To be able to measure the kinetics and the extent of the release of cyt *c* and AK that was induced by polycations we modified the conventional PTP assay conditions. PTP is usually assayed in a low salt medium, but since cyt *c* binds to membranes in a medium of low ionic strength, it was necessary to assay the rate and extent

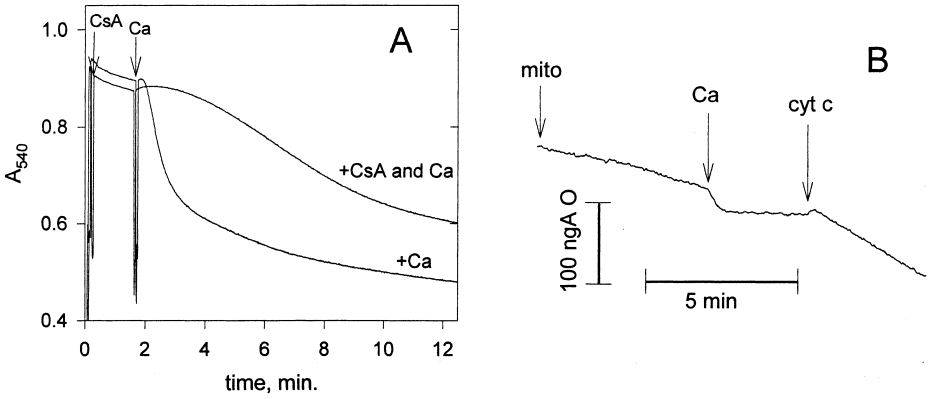

Fig. 1. Assay for the kinetics of the release of cyt *c* from mitochondria using an oxygen electrode. Mitochondria (1 mg/ml) were incubated in the standard assay medium (Section 2). 360 nmoles Ca^{2+}/mg protein were added to trigger mitochondria swelling, rupture of the outer membrane, and the release of cytochrome *c*. Cyclosporin when added was 1 μM. Panel A shows the kinetics of the swelling that was induced by the addition of Ca^{2+}, and the inhibition by cyclosporin, when added before Ca^{2+}. Panel B shows the kinetics of the inhibition of respiration after the addition of Ca^{2+}, and the stimulation of respiration by the addition of cyt *c* (0.16 mg/ml).

of the release of cyt *c* in a medium of high ionic strength. During incubation of isolated mitochondria at high temperature, contaminating phospholipases slowly release free fatty acids, which activate PTP [25]; to inhibit this artificial activation of PTP we added BSA to the incubation medium.

Fig. 1A shows that, under these conditions, the addition of 360 nmoles Ca^{2+}/mg induced cyclosporin-sensitive swelling, indicating triggering of PTP [26]. To follow the kinetics of the release of cytochrome *c* under these conditions, we followed the rate of mitochondrial respiration (Fig. 1B). Calcium stimulates the rate of respiration because the electrogenic uptake of Ca^{2+} reduces $\Delta\Psi m$; however, after about 2 min, when the mitochondria begin to swell (Fig. 1A), the outer membrane was apparently ruptured, cyt *c* was released, diluted in the suspending medium, and the respiration was greatly inhibited (Fig. 1B). The addition of saturating concentrations of cyt *c* restored the rate of respiration to the value obtained before the induction of the swelling, confirming that the outer membrane, which is normally impermeable to cyt *c*, was indeed ruptured. If we assume that the respiration rate depends linearly on cyt *c* concentration, we can estimate, approximately, the percentage of cyt *c* that was released from the ratio of the inhibited rate of respiration (R_2) to the rate of respiration after the addition of cyt *c* (R_3) (i.e. the fraction of released cyt $c = 1 - R_2/R_3$, Fig. 1B).

3.2. *The aliphatic amines spermine and spermidine cause release of cyt c and AK from mitochondria without inducing swelling or activating PTP*

Spermine, a highly charged aliphatic polyamine, was shown recently to induce the activation of caspase-3 in a cell free system, by inducing the release of cyt *c* from mitochondria [22]. However, spermine is known to inhibit PTP [23], and we have found that it does not induce mitochondrial swelling under various incubation conditions (cf. Fig. 2A). Spermidine, a natural precursor of spermine, mimics many of the biological effects of spermine, although it is usually less potent, apparently because it only has three amino groups compared to the four amino groups of spermine. We have found that similar to spermine it did not induce swelling under various incubation conditions (cf. Fig. 2A). Nevertheless, spermine and spermidine, at the mM concentration range, released cytochrome *c* from liver mitochondria. This was observed both from their effect on respiration (Fig. 2B), and from Western blot analysis of the mitochondrial pellet (Fig. 2C). Spermine and spermidine also released AK from mitochondria. When measured simultaneously the percentage of spermine-released AK was comparable to the percentage of the released cyt *c* (Fig. 2D). In all of these effects spermine was more potent than spermidine. Cyclosporin, a potent inhibitor of PTP, had no effect on the spermine- and

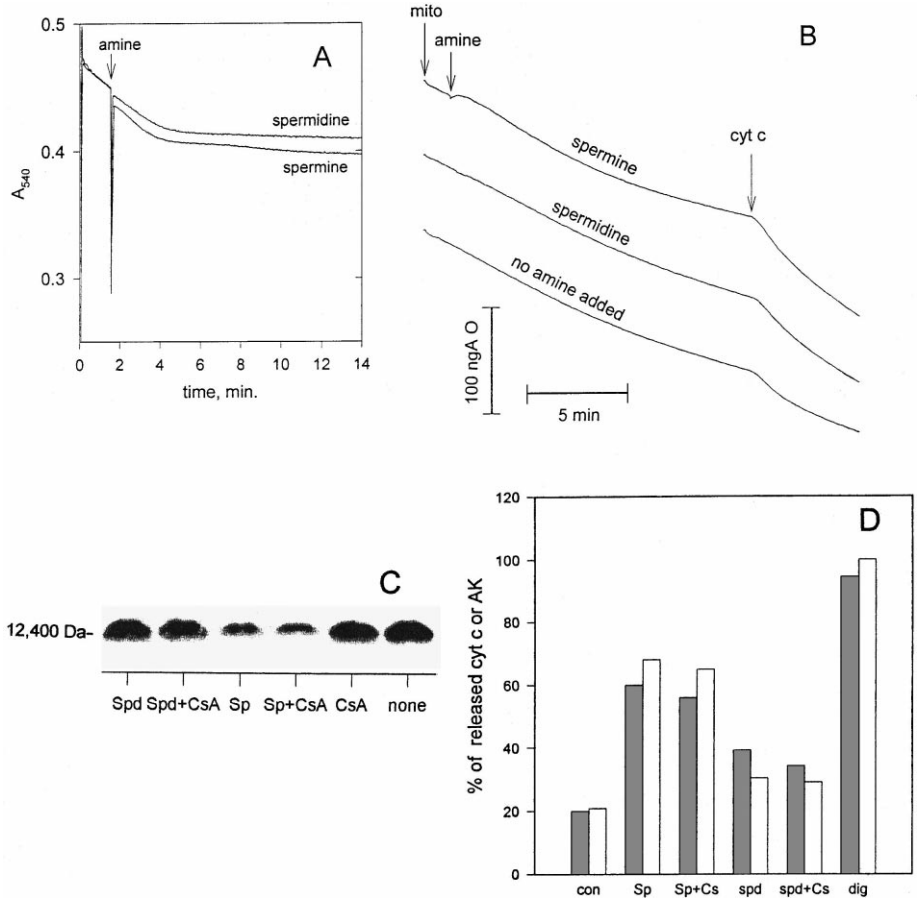

Fig. 2. Spermine and spermidine induce the release of cyt *c* and AK without inducing PTP. Assay conditions were as in Fig. 1, except that the mitochondrial concentration was 0.5 mg/ml. Added spermine and spermidine concentrations were 4 mM. Panel A shows that the addition of spermine or spermidine does not induce mitochondrial swelling. Panel B shows that spermine and spermidine released cyt *c*, as detected by the oxygen electrode assay. Panel C shows that spermine and spermidine released cyt *c* from the mitochondria, as detected from the Western blot analysis of the mitochondrial pellet. Cyclosporin did not inhibit the release. Panel D shows a comparison of the amount of spermine- and spermidine-induced release of cyt *c* (shaded) and AK (unfilled), with and without cyclosporin. Cyt *c* release was estimated from the OD of the Western blots and AK was determined enzymatically (Section 2).

spermidine-induced release of cyt *c* and AK (Fig. 2C,D). These results suggest that aliphatic polyamines release cytochrome *c* and AK by disruption of the outer membrane, and that this process does not depend on activation of PTP or on mitochondrial swelling. However, the amount of cyt *c* and AK that was released during a 15 min incubation with a polyamine varied from 30% to 60% of the maximal amount of cyt *c* and AK that was released by agents that induce large amplitude swelling, including digitonin. This observation suggests that swelling enhances the rate of release of these proteins from partially occluded sites in the mitochondrial cristae. Nevertheless, it is likely that, in vivo, a slow release of SIMP proteins, in the absence of mitochondrial swelling, is sufficient to trigger apoptosis.

3.3. *Aminoglycosides induce the release of cyt c and AK from mitochondria by both PTP-dependent and PTP-independent mechanisms*

Aminoglycoside antibiotics mimic many of the effects of aliphatic polyamines on several biological systems including mitochondria. Similar to aliphatic polyamines, aminoglycosides stimulate the electrogenic uptake of Ca^{2+} by mitochondria ([27]; Rottenberg, unpublished observation). However, unlike spermine, aminoglycosides do not inhibit PTP [28].

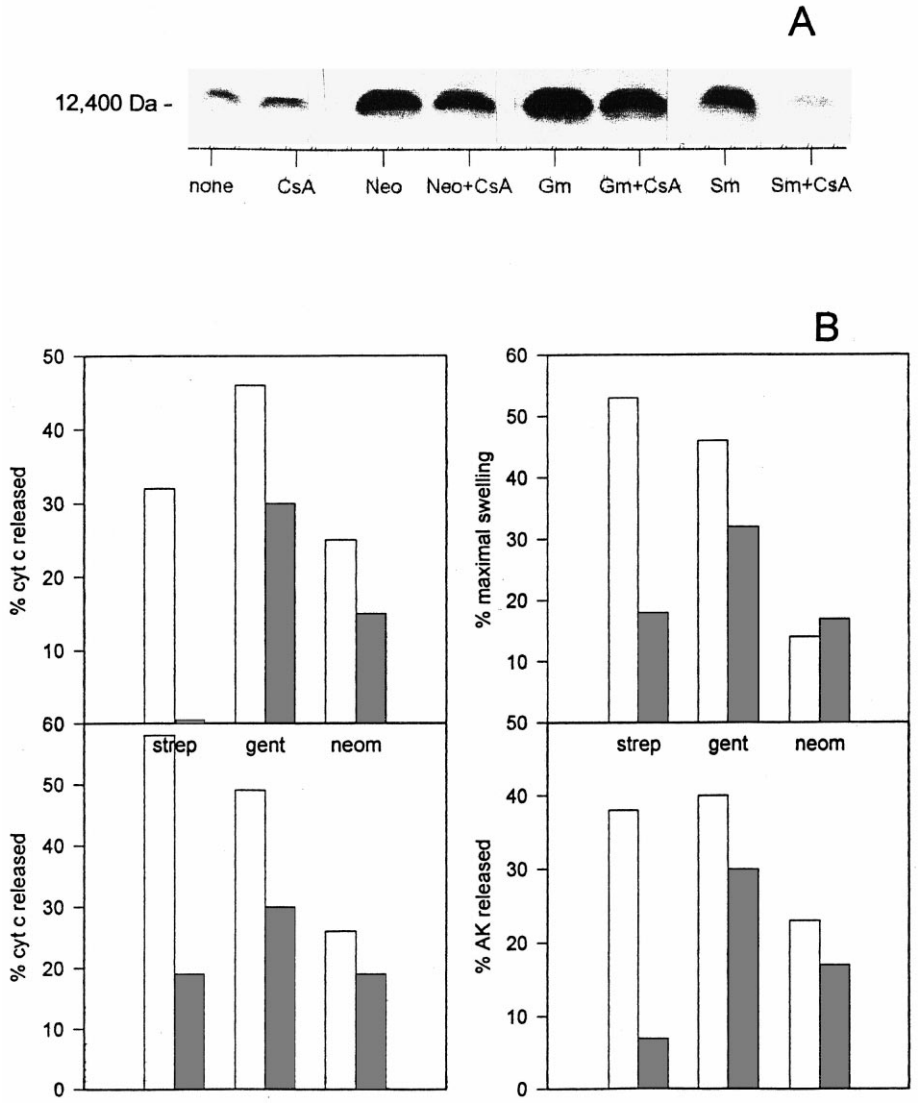

Fig. 3. Aminoglycoside-induced release of cyt *c* and AK is partially associated with the induction of PTP. Panel A shows the Western blots of cyt *c* in the supernatants of mitochondrial suspensions that were treated with streptomycin, gentamicin or neomycin (5 mM) and the inhibition of the release by cyclosporin (1 μM). Panel B shows the release of cytochrome *c* and AK and the swelling that was induced by the three aminoglycosides with cyclosporin (shaded) and without cyclosporin (unfilled). Top left: Western blot analysis of cyt *c* release; bottom left: oxygen electrode analysis of cyt *c* release; bottom right: enzymatic analysis of AK release; top right: swelling.

Fig. 3A shows that the aminoglycosides streptomycin, gentamicin and neomycin also induced the release of cyt *c* from isolated mitochondria. However, unlike aliphatic polyamines, the release of SIMP by aminoglycosides was inhibited by cyclosporin, to various extents, depending on the antibiotic. Streptomycin-induced SIMP release was almost completely inhibited by cyclosporin, gentamicin-induced SIMP release was partially inhibited, while neomycin-induced release was only slightly inhibited. Fig. 3B summarizes the effects of the three tested aminoglycosides without cyclosporin (unfilled columns) or with cyclosporin (hatched columns). The release of cyt *c* was measured by Western blot analysis (a, top right), and by cyt *c* stimulation of the respiration (b, bottom right); AK release was determined enzymati-

cally (c, bottom left), and the induction of swelling was measured by following the absorbance (d, top left). These polyamines differed in their ability to induce PTP-dependent and -independent release of SIMP. Neomycin, which is the most highly charged polycation in this group, released SIMP to a similar extent whether or not PTP is blocked by cyclosporin, and induced very little swelling, which was not cyclosporin sensitive. Streptomycin, which carries only two charges, released little SIMP when PTP was blocked, and induced cyclosporin-sensitive high amplitude swelling. Gentamicin with intermediate charge can release SIMP when PTP is blocked, but PTP greatly enhanced the release of SIMP.

These results suggest that the aminoglycosides can induce the release of SIMP both by directly destabilizing the outer membrane and by inducing PTP. For neomycin, which is not a potent inducer of PTP, destabilization of the outer membrane appears to dominate the release, while for streptomycin, which is a potent inducer of PTP, but is not potent in destabilizing the outer membrane, PTP dominates the release. Gentamicin appears to be equally potent in PTP induction and outer membrane destabilization.

3.4. Melittin causes release of cyt c and AK from mitochondria by activation of PTP

To find out if the positively charged cytotoxic peptides induce the release of SIMP by PTP activation or direct destabilization of the outer membrane, we

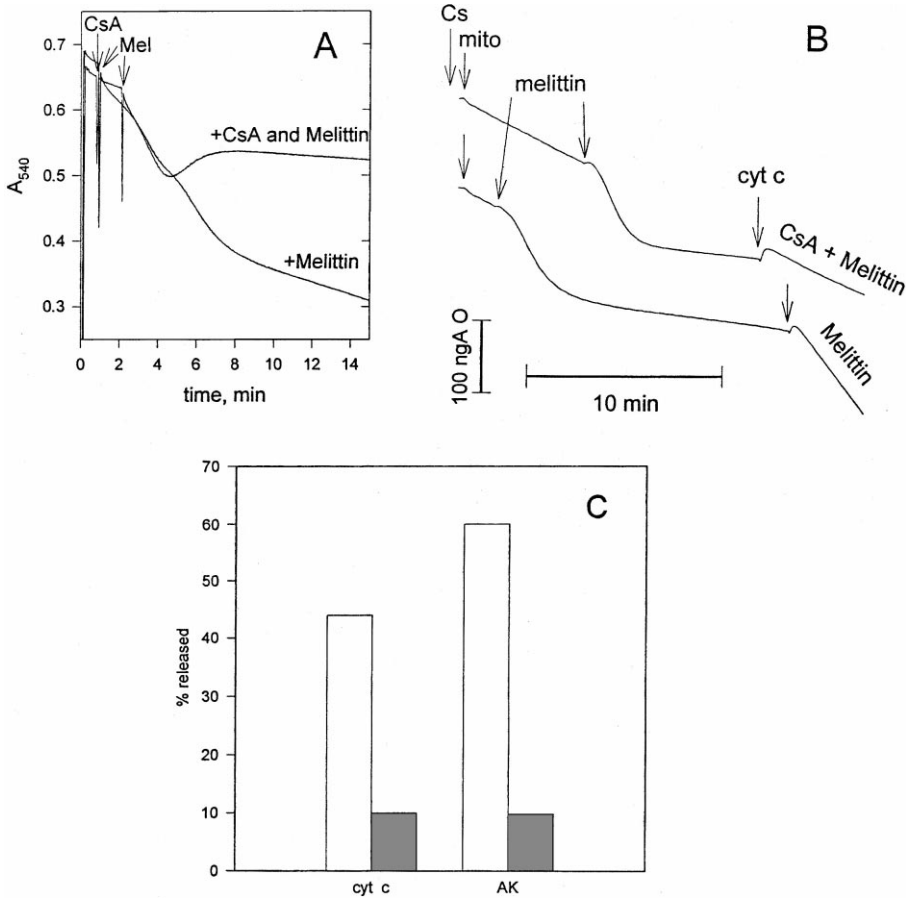

Fig. 4. Melittin induces the release of cyt *c* and AK by activating PTP. Panel A shows that melittin (2 nmol/mg) induces swelling that is inhibited by cyclosporin. Panel B shows that melittin caused inhibition of oxygen consumption that was restored by cyt *c*, and that this effect was inhibited by cyclosporin. Panel C shows a comparison of the melittin-induced release of cyt *c* (from Western blot analysis) and AK and the inhibition of the release by cyclosporin.

performed similar experiments with the cytotoxic peptide melittin. Fig. 4A shows that melittin, in low concentrations, induced mitochondrial swelling, which was inhibited by cyclosporin, indicating a strong potency in PTP activation. Under these conditions, melittin induces the release of cyt *c*, as demonstrated by the respiration assay (Fig. 4B). The release of cyt *c* together with AK was measured by Western blot analysis and enzymatic assay of the supernatant (Fig. 4C). The swelling, the release of cyt *c*, and the release of AK were all inhibited strongly by cyclosporin, indicating that the effect of melittin, at low concentrations, completely depends on the activation of PTP. However, when the melittin concentration was increased to 10 nmoles/mg protein from 2 nmoles/mg protein, these effects were not inhibited by cyclosporin (results not shown). Similar behavior was reported for mastoparan [20].

3.5. *The interaction of polyamines with phosphatidylinositol (PI) and cardiolipin (CL)*

In an effort to understand the origin of the different patterns of the effects of the different polyamines on mitochondria, we examined the interactions of polyamines with anionic phospholipids. Both the inner and outer mitochondrial membranes are rich in anionic phospholipids; however, the inner membrane major anionic phospholipid is CL, whereas the outer membrane major anionic phospholipid is PI [29]. To measure the interaction of polyamines with these anionic phospholipids, we reconstituted liposomes from mixtures of the neutral phospholipid phosphatidylcholine (PC) with either PI or CL and allowed the anionic phospholipids to bind Ca^{2+}. We then measured, with a Ca^{2+} electrode, the release of the tightly bound Ca^{2+} upon the addition of various concentrations of polyamines which competed with Ca^{2+} for the binding sites on the anionic phospholipids. The results of these titrations are shown in Fig. 5. There was a pronounced specificity for the interaction between polyamines and CL. Spermine and neomycin exhibited the highest affinity. Gentamicin has lower affinity, while streptomycin and spermidine exhibited low affinity. The interactions of polyamines with PI were similar, except that polyamines appear to replace more calcium from PI/PC

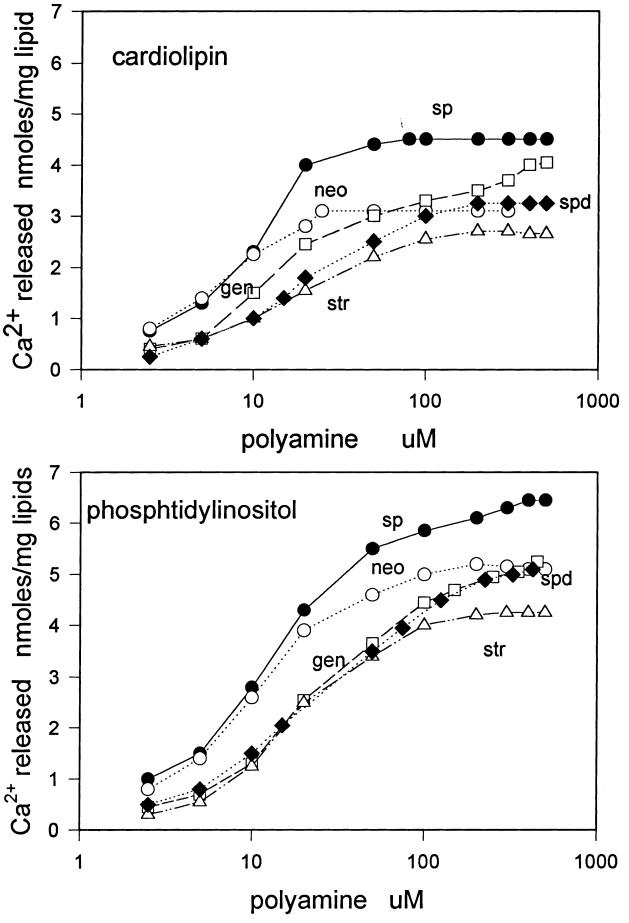

Fig. 5. Polyamine binding to the anionic phospholipids of mitochondria. Liposomes were prepare from a mixture of PC and either CL or PI in a ratio of 1CL/3PC (top) and 1PI/3PC (bottom). Calcium was added to the liposomal suspension (10 nmol Ca^{2+}/mg lipid). The binding of polyamines to the anionic phospholipids was measured, with a Ca^{2+} electrode, from the release of Ca^{2+} that was induced by polyamine binding. Sp, spermine; spd, spermidine; neo, neomycin; gen, gentamicin; str, streptomycin.

liposomes. Nevertheless, the affinity of the different polyamines was similar to that observed with CL, except that there was a smaller difference between gentamicin and streptomycin. Similar experiments with the anionic phospholipids phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylglycerol (PG) showed much lower affinity of polyamines to these phospholipids and almost no specificity (results not shown).

The patterns of the effects of the different polyamines on outer membrane destabilization and PTP activation may be related to their differential inter-

action with the anionic phospholipids of the inner and outer membranes (see Section 4).

4. Discussion

4.1. Membrane binding of molecules carrying a cluster of positive charges can induce the rupture of the outer mitochondrial membrane

Many biologically active polycations are known to disrupt the structure of biological membranes. Several families of peptides carrying clusters of positively charged residues have antibiotic activities that have been attributed to their ability to destabilize the membranes of microorganisms [18]. The mitochondrial membranes are similar in their phospholipid composition to bacterial membranes and may also be susceptible to the destabilizing effect of molecules carrying clusters of positive charges. Our observation that polyamines that interact strongly with the anionic phospholipids of the mitochondria appear to be more potent in direct destabilization of the outer membrane, is compatible with the suggestion that the rupture of the outer membrane results from the interaction between the positively charged polyamines and the negatively charged anionic phospholipids. The inner membrane has a very high content of integral membrane proteins, and cardiolipin, the anionic phospholipid of the inner membrane, is known to be mostly associated with the integral membrane proteins; this association may protect the inner membrane from direct destabilization by polycations.

It is not clear whether the effect of spermine on the stability of the mitochondrial outer membrane is relevant to its biological effects, in vivo. Spermine is known to inhibit PTP and stabilize rather than destabilize, mitochondria, in vitro [30]. However, the latter effect is most likely due not only to spermine inhibition of PTP [23], but also to inhibition of phospholipases [31], which produce free fatty acids that activate PTP, and appear to be the main reason for the rapid deterioration of isolated mitochondria. We have ameliorated this in vitro artifact by including BSA in the incubation medium, which allowed us to detect the destabilizing effect of spermine, in vitro. The concentration of spermine that causes the outer membrane rupture is higher than the physiological concentration of spermine in the cell. Therefore, under normal physiological conditions, it is unlikely that spermine induces significant rupture of the outer membrane. However, as has been shown recently, overloading cells with spermine or spermidine induces apoptosis [21,32], and this appears to be caused by the release of cytochrome *c* from the mitochondria [22]. The toxicity of aminoglycoside antibiotics is attributed to their interaction with RNA [33], but it is possible that their ability to induce the release of SIMP from mitochondria also contributes to their toxicity.

Bax was shown to destabilize planar phospholipid bilayers [24], and Bid and tBid were shown to release proteins encapsulated in liposomes [17]. In both studies the phospholipid membranes were prepared from a phospholipid mixture with a high content of negatively charged phospholipids, similar to the content of the mitochondrial membranes. A fragment of Bax, essentially composed of the critical cluster of positive charges, was reported to induce apoptosis in mammalian cells, and cell death in bacterial cells [34]. Most of the proapoptotic proteins of the Bcl-2 family carry clusters of positive charges, and some (e.g. harakiri [35], and Noxa [36]) are highly cationic. Moreover, in most of the proapoptotic proteins of the Bcl-2 family, the negative charges are concentrated near the carboxy terminus, which is often cleaved during apoptosis, and this cleavage activates (e.g. bid) or enhances their proapoptotic potency (e.g. bax) [16,37]. Nevertheless, because of the multiple modes of action of some of these proteins in apoptosis it is difficult to assess the importance of specific charged residues. Thus, removing some charged residues from bax abrogated the killing of yeast cells, but enhanced the apoptotic potency in mammalian cells, apparently because the binding to antiapoptotic Bcl-2 proteins was greatly enhanced [38].

It has been suggested that electrostatic interactions between a positively charged cluster on the SNARE complex with negatively charged phospholipids is critical to the induction of membrane fusion, which is preceded by transient breakdown of the apposed membranes ('fusion pore') [39]. We suggest that the proapoptotic proteins that induce membrane rupture do so by a similar mechanism. Indeed polyamines, which were shown here to induce rupture of the out-

er mitochondrial membrane, were also shown to be capable of triggering membrane fusion [40].

There are other cationic proteins that may contribute to the destabilization of the outer membrane in apoptosis. The adenine nucleotide translocator carries clusters of positive charges on its surface [41]. This abundant inner membrane protein is believed to interact with VDAC on the outer mitochondrial membrane to activate PTP [6]. However, there is a large excess of ANT over VDAC, and if the ANT is in a favorable conformation its charged surface may interact with and destabilize the phospholipid bilayer of the outer membrane. Similarly cytochrome *c* itself is a highly charged cationic protein that interacts with negatively charged phospholipids [42], and overexpression of cytochrome *c* resulted in the release of cyt *c* from mitochondria [43]. It is possible that in apoptosis lipid peroxidation induces the release of cyt *c* from its binding sites on the inner membrane [42], enhancing its binding to the outer membrane and contributes to its rupture. Indeed, it has been shown that overexpression of the mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx) prevents the release of cytochrome *c* in apoptosis [44].

4.2. Polycations can also induce PTP, swelling and the rupture of the outer membrane

It is evident that besides the ability of polycations to destabilize the outer membrane many are also capable of inducing PTP. It appears that when PTP is induced, the release of SIMP is faster and PTP becomes the predominate pathway of SIMP release, in vitro. This is most likely due to the fact that PTP, in vitro, is associated with large amplitude swelling, which facilitates the release of SIMP from partially occluded sites in the cristae. At present it is not clear why some polycations readily induce PTP, while others such as spermine [23], polylysine, and polyarginine [45] inhibit PTP. It is not unlikely that the induction or inhibition of PTP depends on the interaction of these polycations with cardiolipin in the inner membrane. It has been suggested recently that the triggering of PTP by calcium overloading results from the interaction between Ca^{2+} and cardiolipin, which enhances the production of ROS [46]. Since polycations compete with Ca^{2+} for cardiolipin binding sites (Fig. 5), it is possible that polycations either activate PTP (agonists) or inhibit PTP (antagonists) by binding to the Ca^{2+} binding sites on cardiolipin. Indeed, we have found that mitochondria that were preloaded with calcium are much more susceptible to PTP activation by aminoglycosides and melittin (results not shown).

In conclusion, our results suggest that electrostatic interactions between clusters of positively charged residues on peptides, glycosides and aliphatic compounds and the negatively charged phospholipids of both the inner and outer mitochondrial membranes contribute to the induction of SIMP release from mitochondria. The interaction with PI in the outer membrane could lead to direct destabilization and rupture of this membrane, while the interaction with CL in the inner membrane could lead to either inhibition or activation of PTP. These mechanisms may also contribute to the induction of SIMP release in apoptosis. The ability of some polycations to induce both direct destabilization of the outer membrane and PTP may be relevant to the mechanism of action of Bax, since evidence that supports both mechanisms has been accumulating.

Acknowledgements

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