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EFFECT OF POLYCATION PEPTIDES ON MITOCHONDRIAL PERMEABILITY TRANSITION

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Synthetic polycation peptides obtained with the basic aminoacids lysine, arginine and ornithine are able to inhibit the permeability transition induced in mitochondria by calcium ions and inorganic phosphate. At least three basic aminoacid residues must be present in the peptide in order to elicit the inhibitory effect.

In the presence of synthetic polycations and similarly to spermine, a lack of correlation between inhibition of swelling and glutathione release is apparent, since glutathione release occurs before the onset of a large amplitude swelling. The same lack of correlation is observed in the presence of cyclosporin. From the results obtained with the above reported polycations, different in both aminoacid composition and length, it appears that the effect is not to be referred to the individual properties of the molecules examined but rather to their cationic character; in addition, a critical number of positive charges is necessary to elicit the effect.

Polyamines are natural constituents of the animal and plant cells where they are present at millimolar concentration and are involved in several regulatory roles (1). According to an old observation, spermine and spermidine are able to inhibit the hypotonic swelling of rat liver mitochondria (2). More recent work (3-6) has demonstrated that spermine is able to inhibit the inner membrane permeability transition of isolated rat heart and liver mitochondria. The latter phenomenon is stimulated by an increase of matrix calcium in the presence of a variety of triggering agents chemically unrelated to each other (7, 8). The permeability transition induces the passage of small solutes (< 1500 Da) through a pore that is specifically inhibited by cyclosporin A (9). The mitochondrial pore appears to have the same molecular structure of the mitochondrial megachannnel identified with patch-clamp experiments by Szabò et al. (10).

In the present paper we have examined the effect of different synthetic polycations on mitochondrial swelling and release of glutathione. The results are discussed with reference to the possible role of membrane charge on mitochondrial permeability transition.

Materials and Methods

Rat liver mitochondria were isolated with differential centrifugation using a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4) and 0.5 mg/ml of bovine serum albumin. Mitochondrial swelling was measured spectrophotometrically by following the decrease of absorbance at 540 nm. Total glutathione was measured with the procedure of Tietze

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(11). Membrane potential ($\Delta\Psi$) was assessed by measuring the movements of tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a TPP^+ selective electrode (12). The tri- and tetrapeptides containing lysine (K_3, K_4), ornithine (Orn_3, Orn_4) and arginine (R_3, R_4) were synthesized by an automated synthesizer from Applied Biosystems (Model 431-A) using FastMoc™ chemistry according to the manufacturer. Other polycations and polyanions were purchased from Sigma (St Louis, MO, USA). Cyclosporin was a kind gift of Drs. G. Corbetta and G. Fiorella of Sandoz Prodotti Farmaceutici, Milano (Italy). Aminoacids are indicated with the one-letter abbreviations: D (aspartic acid); E (glutamic acid); K (lysine); R (arginine).

Results

In Table 1 the effect of various polycations of the basic aminoacids lysine, ornithine and arginine on mitochondrial swelling induced by Ca^{2+}/Pi is reported. The polycations utilized inhibit to a large extent mitochondrial swelling at variance with polyanions formed of aspartate and glutamate that slightly stimulate the swelling. Since the inhibitory effect appears mostly linked to the positive charges of polycations it is of interest to establish the minimum charge requirement to produce this effect. The results of Fig. 1A indicate that, under our experimental conditions, molecules with four residues of basic aminoacids almost completely prevent the Ca^{2+}/Pi-induced swelling, while with three residues a partial inhibitory effect is observed. In the latter case a complete inhibition is achieved if the concentration is raised from 25 μM to 80 μM (not shown). It is clear that, for inhibiting Ca^{2+}/Pi-induced swelling, at least three basic residues must be

Table 1. Effect of polycations and polyanions on mitochondrial swelling

POLYCATIONS	μM	swelling inhibition (%)
Lys	100	0
Poly **Lys** (M.W. 3 870)	5	70±10
Poly **Lys** (M.W. 43 700)	1	75±10
Arg	100	0
Poly **Arg** (M.W. 10 800)	0.8	90±5
Poly **Arg** (M.W. 24 000)	0.4	90±5
Orn	100	0
Poly **Orn** (M.W. 7 600)	1	85±5
Poly **Orn** (M.W. 38 000)	0.6	90±10
POLYANIONS		
Poly **Asp** (M.W. 90 000)	5	-10± 5
Poly **Glu** (M.W. 70 000)	5	-15± 5

Rat liver mitochondria (0.25 mg/ml) were incubated at 25 °C in 213 mM mannitol, 71 mM sucrose, 5mM Hepes/Tris (pH 7.4), 5 mM succinate, 3μg/mg protein rotenone, 4 μg/mg protein oligomycin, 13 μM EGTA. Single aminoacids and the corresponding polycations and polyanions were added at the indicated concentrations. Swelling was initiated by the addition of 40 μM $CaCl_2$ and 1 mM phosphate and its inhibition was reported as percentage with respect to the value reached by the control (no addition) after 15 minutes. Values in brackets indicate the average molecular weight of the compound given by the manufacturer (Sigma Chem. Comp.).

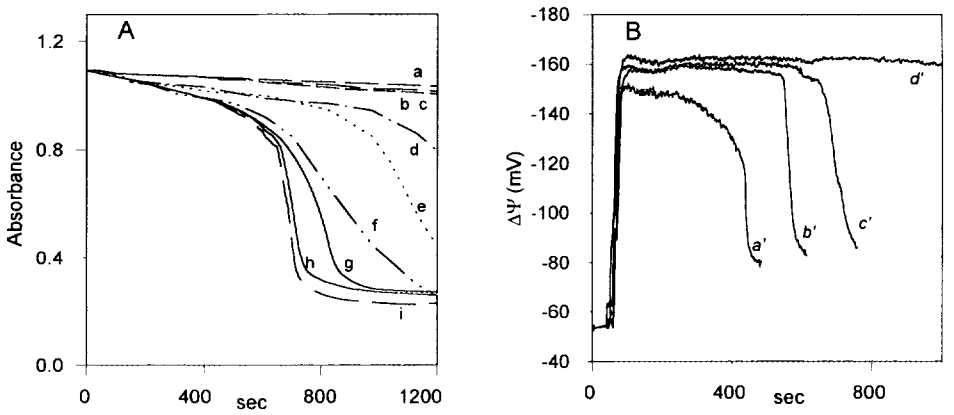

Fig. 1. Effect of basic tetrapeptides on mitochondrial permeability transition and membrane potential.

In A the experimental conditions are as in the legend of Table 1. Peptides were added at the following concentrations: Orn_4 (a), R_4 (b), K_4 (c) 5 μM; R_3 (d), Orn_3 (e), K_3 (f), D_4 (h) and E_6 (i): 25 μM. (g): no addition. Swelling was triggered by the addition of 1 mM phosphate followed, after 1 min, by 40 μM $CaCl_2$. In B rat liver mitochondria (1 mg/ml) were incubated at 25 °C in 213 mM mannitol, 71 mM sucrose, 5 mM Hepes/Tris (pH 7.4), 5 mM succinate, 1 μg/ml rotenone, 2 μM TPP^+, 3 mM phosphate and 80 μM $CaCl_2$. K_4 (*b'*), Orn_4 (*c'*), and R_4 (*d'*) were 100 μM. (*a'*): no addition. The data obtained with the TPP^+ selective electrode were acquired and analyzed by a PC software (Labview, National Instruments), converted to ASCII format and transferred to a graphic software. The ordinate $\Delta\Psi$ values of the curves were recalculated from the experimental values in order to obtain a linear scale and to correct for the TPP^+ non-specifically bound to mitochondria (27).

present in the molecule and therefore there is a correlation between the increase of the aminoacid residues and the inhibition of swelling. A strict correlation between the number of charges of different polyamines and their protective action was recently demonstrated by Tassani et al. (13). On the other hand, polypeptides with four or six residues of acidic aminoacids (aspartate and glutamate respectively) do not prevent Ca^{2+}/Pi-induced swelling but slightly stimulate it (Fig. 1A).

The effect of the tetrapeptides K_4, Orn_4 and R_4 on the mitochondrial membrane potential is reported (Fig. 1B). In the presence of Ca^{2+}/Pi mitochondria reaches a membrane potential that is rapidly lost. When polycations are present, the membrane potential is higher and maintained for longer times, particularly in the presence of R_4. Previously, it was shown that spermine strongly prevents the decrease of membrane potential induced by aging at room temperature or by adding palmitoylCoA and Ca^{2+}/Pi to rat heart mitochondria (14) or Ca^{2+}/Pi to rat liver mitochondria(5).

Since glutathione can be released through the mitochondrial pore opened during the permeability transition (15), swelling and glutathione release were followed simultaneously. As it can be seen in Fig. 2A, with all the tetrapeptides tested, swelling is far better protected than the release of glutathione. In fact, while at low concentrations of tetrapeptides (0.2 μM) there is a strict correspondence between swelling and glutathione release, when the concentration is increased up to 0.6 μM, swelling is almost completely inhibited, while glutathione release is still quite large and greater than 60% (Fig. 2A). The inset of Fig. 2A shows the time course of

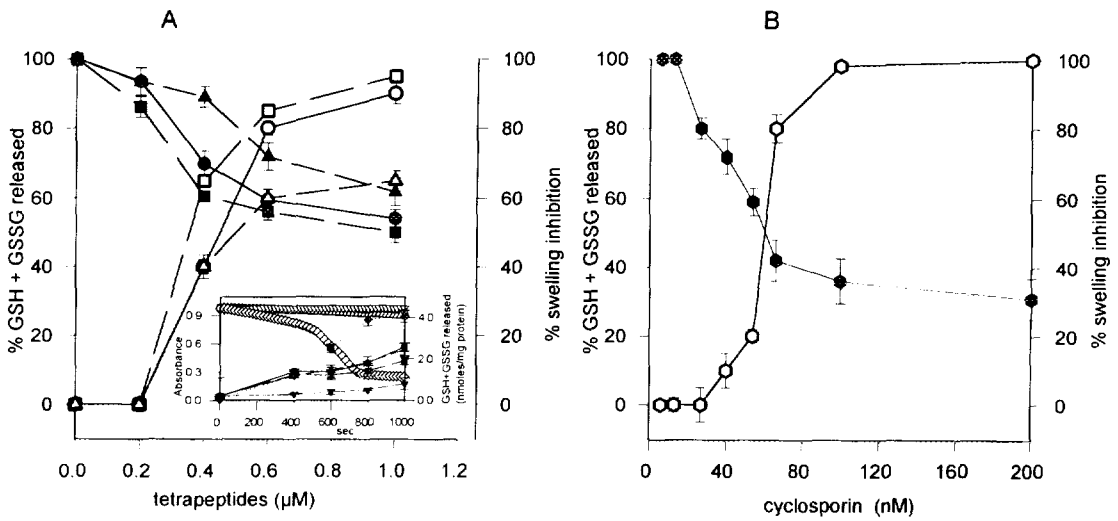

Fig. 2. Correlation between total glutathione release and mitochondrial swelling in the presence of the tetrapeptides Orn_4, K_4 and R_4 (A) and cyclosporin (B).
The experimental conditions are as in the legend of Table 1. Mitochondria were incubated in the presence of increasing concentrations of Orn_4 (○,●), K_4 (Δ, ▲) and R_4 (□,■) and cyclosporin (○ , ●). Swelling and glutathione release were initiated by the addition of 40 μM $CaCl_2$ and 1 mM phosphate. Glutathione released (filled symbols) is reported as percentage of the total mitochondrial content (4.8 ± 0.2 nanomoles/mg protein, before incubation) measured after 15 minutes of incubation. Inhibition of swelling (empty symbols) is reported as percentage with respect to the value reached by the control after 15 minutes. The inset of Fig. 2A shows the time course of swelling (empty symbols) and glutathione release (filled symbols) in the presence of 25 μM Orn_4 (○,●), K_4 (Δ, ▲) and R_4 (□,■). Control (no polycations) in the absence (▽,▼) and in the presence (◊,◆) of Ca^{2+} and phosphate is shown. The reported results are means ± SD of three to six experiments.

swelling and glutathione release in the presence of the tetrapeptides (25 μM): swelling is completely inhibited, while glutathione progressively leaks out. A similar behavior was found with cyclosporin, the most potent inhibitor of permeability transition (9, 16) that, at concentrations larger than 50 nM, is able to completely inhibit Ca^{2+}/Pi-induced swelling but not glutathione release (Fig. 2B).

Discussion

The reported results show that several different polycations formed from basic aminoacids are able to inhibit the mitochondrial permeability transition. This behavior is reminiscent of that of spermine (3-6) which is able to prevent pore opening, particularly in conditions of low ionic strength (4). The primary importance of the number of positive charges in the mechanism of prevention of mitochondrial permeability transition was recently reported for several polyamines for which any additional positive charge increases the protection of one order of magnitude (13).

Despite the impressive number of agents able to increase the probability of pore opening, Bernardi et al. (17) have recently recognized that the pore is regulated by a voltage sensor

sensible to $\Delta\Psi$, the oxidation of protein sulfhydryl groups and membrane surface potential. The latter, in particular, appears modified by changes in charges content, since polycations, by binding the negative charges, make the surface potential more positive (18). Mitochondrial surface potential can be altered by metabolites that render the surface potential relatively more negative (palmitic acid) and therefore facilitate pore opening, while long chain acyl cations (sterylamine, sphingosine and psychosine) act in the opposite way by inhibiting the permeability transition (17).

The effect of polycations in inhibiting mitochondrial swelling appears mostly dependent on their interactions with the negative charges of the membranes, essentially represented by phospholipids. In this respect, and similarly to Mg^{2+}, they can displace or compete with the Ca^{2+} ion that is the major responsible of the permeability transition (7). According to Åckermann (19) spermine inhibits Ca^{2+} binding to polar head groups of the phospholipids at the membrane surface. In addition, their capability to bridge negative charges (4), especially of cardiolipins, can have a marked influence on lipid arrangement. It is well known that cardiolipins undergo phase changes in the presence of cations, thereby probably changing the permeability properties of the membrane (20-22). It has recently been shown (23) that Ca^{2+} binding to protein-phospholipid domains of liver mitochondrial membranes containing cardiolipin can induce a bilayer-nonbylayer (hexagonal II) transition that might play a role in the fluorescence enhancement of the probe 1-anilino-8-naphthalene sulfonate (ANS) and in membrane permeability transition. Therefore, similarly to spermine, polycations could, in some way, "stabilize" the bilayer by reducing the effect of Ca^{2+}.

Conditions of oxidative stress such as radiations, chemical compounds, drugs, hyperoxia, ischemia/reperfusion determine a decrease of tissue glutathione (24) almost invariably associated with major alterations of mitochondria that, unable to synthesize glutathione, must import it from the cytosol (25). According to Savage et al. (15) the permeability transition of mitochondrial membrane induced by Ca^{2+}/Pi causes the release of glutathione. More recently, the same authors (26) have demonstrated that the prevention of Ca^{2+}/Pi-induced large amplitude swelling by oligomycin, antimycin and sulfide, did not prevent the release of mitochondrial glutathione, that, on the contrary, is prevented by cyclosporin A. The authors conclude that permeability transition can occur without large amplitude swelling. Previously, we have demonstrated that spermine at concentrations as low as 25 μM, fully prevent mitochondrial swelling, while for the complete inhibition of glutathione release the concentration of spermine should be no lower than 100 μM (5). A similar lack of correlation between large amplitude swelling inhibition and glutathione loss prevention was also observed in the presence of polycations formed of basic aminoacids. Cyclosporin A, completely inhibits both swelling and glutathione release at concentrations that are ineffective for polycations; nevertheless if cyclosporin is utilized in the range of 10-200 nM the same lack of correlation is again apparent; in fact, swelling is inhibited by 50 nM cyclosporin but glutathione leaks out. In conclusion, on occurrence of permeability transition, the release of glutathione appears to be an early event preceding the mitochondrial large amplitude swelling.

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