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Cation Permeability Induced by Spermine and Polybrene in Rat Liver Mitochondria

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Abstract

The effect of the polybasic substances Polybrene and spermine on the passive and active transport of monovalent cations in mitochondria was studied. These agents were found to stimulate the low amplitude swelling of mitochondria. Volume oscillations were induced by addition of substrate in the presence of spermine. In conditions where weak oscillations were obtained without these substances, oscillations were stimulated and their frequencies increased in the presence of Polybrene and spermine. Their effects were maximal with 100–300 μmoles spermine per litre and 3–5 mg Polybrene per litre. These results are discussed in relation to an interaction of the agents studied with membrane negative charges which may be important regulators of ion transport.

Introduction

In iso-osmotic sucrose or salt media, pH 7·4, the mitochondrial inner membranes are highly impermeable to monovalent cations.[1–3] Among the factors known to increase the permeability towards monovalent cations in mitochondria are increased pH of the medium and the presence of EDTA.[4–6] It has been suggested that the increased permeability is due to a higher affinity of EDTA for Mg^{2+} at a higher pH leading to depletion of membrane-bound Mg^{2+}.[4,6,7] The "ionophorous" antibiotics like gramicidin or valinomycin also greatly increase the permeability of mitochondria towards monovalent cations owing to the formation of lipophilic complexes.[8–11] The transport of monovalent cations and the accompanying anions can be studied by measuring the changes in mitochondrial volume occurring during ion uptake and release.[5,12–14] By recording the changes in light scattering or absorbance of mitochondrial suspensions information is obtained not only about structural alterations but also about coupled ion and water movements across the membranes.[13–16] In conventional ion transport media (see Materials and Methods) the energization of mitochondria results in reversible low amplitude swelling.[1,4,5,17] Contraction is achieved by deenergization. Swelling–shrinkage oscillating cycles can be obtained in suitable conditions.[15,18–21]

Substances with local anaesthetic properties such as phenthiazines and propranolol damp mitochondrial volume oscillations in the expanded swollen state, presumably by inhibiting the phase of contraction.[21,22] Fast shrinkage of mitochondria is obtained by stopping the input of energy by addition of uncoupling agents or respiratory inhibitors,[23] even in the presence of local anaesthetics.[21] Local anaesthetics also stimulate the energy-dependent transport of divalent cations in mitochondria.[24,25] Such stimulation has been

obtained also with another class of substances, the polyamines, which have a much higher charge density than the above-mentioned substances and lack aromatic ring structures.[26,27] In order to obtain information on the mode of interaction of these positively charged substances with biological membranes, we have investigated the effect of polyamines on the transport of monovalent cations in mitochondria using various controlled conditions.

Materials and Methods

Rat liver mitochondria were isolated as described by Wikström and Saris.[28] Protein concentration of the mitochondrial suspensions was estimated by the Lowry method.[29] Polybrene® (hexadimethrine bromide) was kindly donated by Dr. S. Nordling and FCCP (carbonylcyanide *p*-trifluoromethoxy phenylhydrazone) by Dr. P. G. Heytler. Spermine was obtained from Fluka AG, Bucks, Switzerland. All the other reagents were obtained commercially.

The assay medium for mitochondrial volume oscillations contained per litre 100 mmoles sucrose, 0·5 mmoles EDTA, 5 μmoles rotenone, 40 mmoles NaH_2PO_4 and 30 mmoles Tris, pH 8·0 (=medium 8 PT). Changes in the transmission of mitochondrial suspensions were measured with an Aminco-Chance dual wavelength spectrophotometer set at 520 nm and with the other wavelength excluded. The effect of spermine and Polybrene on the extent and rate of energy-dependent low amplitude swelling of mitochondria was studied in a conventional ion transport medium containing per litre 225 mmoles mannitol, 75 mmoles sucrose, 20 mmoles KCl, 13·3 mmoles KH_2PO_4, 5 μmoles rotenone and 10 mmoles Tris, pH 7·4 (=medium A).

Results

Rat liver mitochondria behave as osmometers, when suspended in solutions with varying osmolarity.[3,5,30,31] Thus they initially swell when they are suspended in the slightly hypotonic medium 8 PT (see Materials and Methods). After the initial osmolar volume equilibration a contraction is recorded in non-energized conditions as in the presence of rotenone to block the endogenous respiration. The contraction is due to passive energy-independent efflux of monovalent cations and water. The contraction is recorded in the left-hand traces in Fig. 1. It is seen that Polybrene stimulates this passive efflux of ions while propranolol inhibits. The right-hand traces show events following energization by addition of succinate. Following the initial energy-dependent swelling of mitochondria a contraction is recorded, which is inhibited by propranolol as shown also before.[21] It is also seen that Polybrene, by contrast, stimulates the rates of both contraction and swelling. As a result the period of oscillations is shortened and the oscillating behaviour becomes more clear-cut. The effect of spermine is qualitatively similar to that of Polybrene, Fig. 2. The oxygen is exhausted earlier with increasing concentrations of polyamine, suggesting that the mean respiratory rate is stimulated by the agents studied.

Table I shows the effects of different amounts of Polybrene and spermine on the rate of the passive efflux of ions, on the mean respiratory rate and on the mitochondrial volume oscillations. As seen in this table, the action of these agents is biphasic in nature. When the ratio of the concentration of spermine or Polybrene to the amount of protein present in

the suspension is high, the different parameters shown in Table I are again less than maximally stimulated. The same biphasic mode of action by the agents tested are also found in other conditions. The mean respiratory rates are seen to be increased in parallel due to stimulated ion transport. Mean respiratory rates were calculated because the respiratory rates oscillate along with the volume changes.[21,32] The presence of an uncoupling agent like FCCP did not influence the rate of passive efflux of ions, which indicates that the proton movements across the mitochondrial membranes are not affected by the agents tested to any large extent.

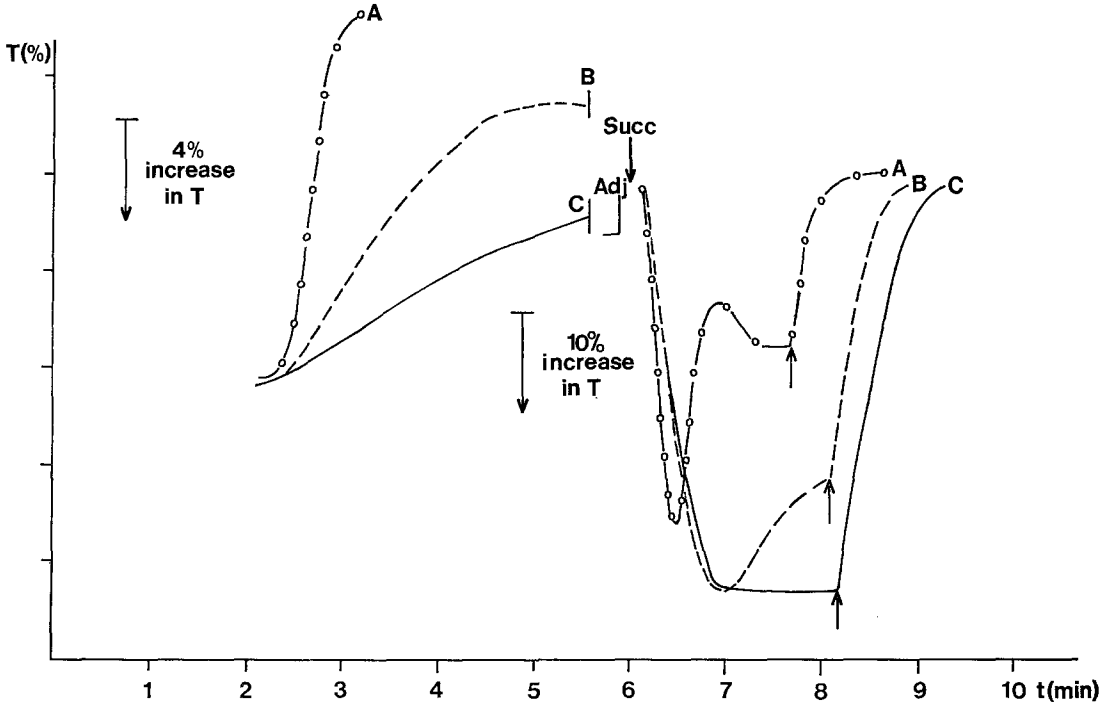

Figure 1. Effect of Polybrene® and propranolol on the passive contraction and energy-linked volume oscillations of rat liver mitochondria. In A 3·3 mg Polybrene® per litre was present and in B 167 μmoles propranolol per litre. The trace B is the control experiment. The final volume of medium 8 PT (see Materials and Methods) was 3·0 ml and the concentration of mitochondrial protein 1·0 mg/ml. Notice the change in scale before the addition of 6·7 mmoles succinate per litre by a stirring rod to start the oscillations. The oxygen was exhausted where indicated by the arrows immediately preceding the contraction. T = transmittance at 520 nm. For other conditions see Materials and Methods.

The effect of Polybrene and spermine on the reversible energy-dependent low amplitude swelling of mitochondria was tested also in a conventional incubation medium A. In the presence of phosphate ions the input of energy results in a slight but clear-cut swelling of mitochondria. The rate and extent of this swelling is stimulated by polyamines, as can be seen in Table II. The biphasic action of spermine is also clearly demonstrable in this medium. Though 167 μmoles spermine per litre effectively stimulated swelling, 0·33 μmoles gramicidin per litre was far more effective. Polybrene was also potent at the same concentrations as in stimulating volume oscillations.

Ionophorous antibiotics, by increasing the permeability towards monovalent cations,

can induce mitochondrial volume oscillations even in the conventional ion transport media.[32] Figure 3 clearly shows that this is also true of spermine which, when present at 167 μmolar concentration in medium A at the protein concentration of 1·2 mg/ml, induces an oscillatory cycle. Similar effects have been obtained with basic proteins.[33]

Fonyo and Bessman[34] have shown that *p*-mercuribenzoate inhibits the penetration of inorganic phosphate in rat liver mitochondria. In Fig. 4 it is seen that spermine stimulates the swelling–contraction cycle of mitochondria even when the transfer of phosphate ions across mitochondrial membranes is inhibited by Mersalyl (trace C). This stimulation again much resembles that caused by gramicidin (Fig. 4, trace B). The swelling of

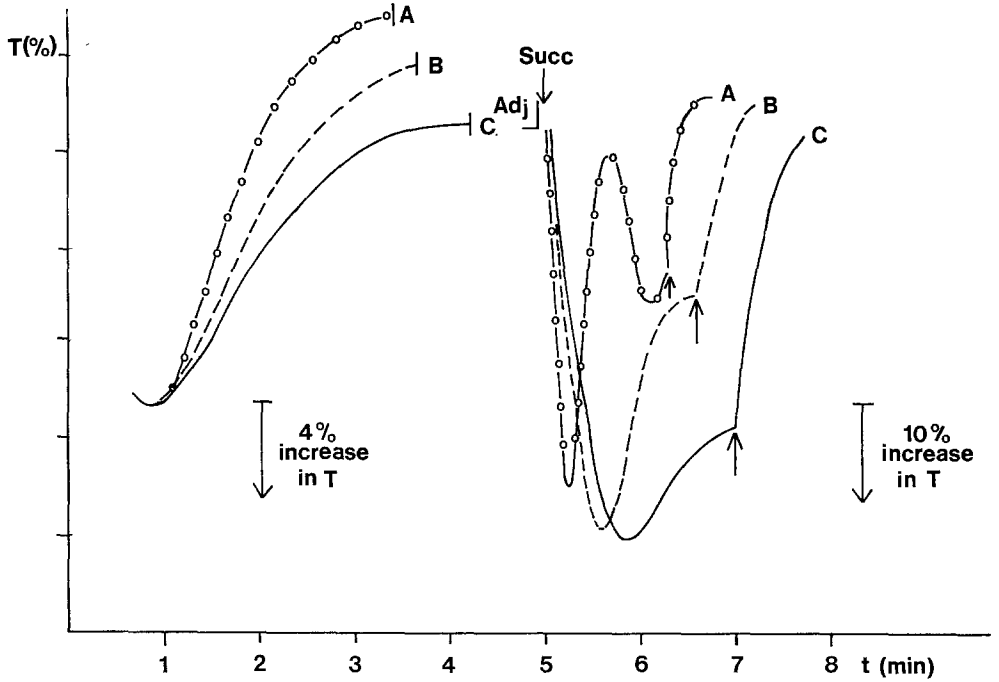

Figure 2. Effect of spermine on the passive contraction and energy-dependent volume oscillations of rat liver mitochondria. Trace C is the control, in B 33·3 μmoles spermine per litre was present and in A 167 μmoles spermine per litre. Mitochondrial protein was 1·0 mg/ml. Other conditions as in Fig. 1.

mitochondria in these experiments was started by adding ATP instead of succinate in order to minimize the transport of substrate anions along with the monovalent cations. In these conditions the addition of FCCP caused a further swelling of mitochondria, in contrast to the contraction obtained in the absence of Mersalyl (Fig. 4, trace D). This extra swelling is very probably due to the inorganic phosphate liberated by the highly stimulated activity of ATPase. The phosphate ions thus produced are not transported outside the mitochondria. This results in entry of water, which is seen in an increase in the intramitochondrial space.

When the ion transport is stimulated by gramicidin in the presence of Mersalyl the traces show some characteristics similar to the uncoupling effect of FCCP, Fig. 4, trace B. The mitochondria initially swell and then contract. The contraction phase,

TABLE I. Effects of Polybrene and spermine on the volume oscillations in rat liver mitochondria

Substance	Concentration	Rate of passive efflux T(%)/min	Mean respiratory rate μA O/min	Number of oscillatory cycles
No (=control)	0	2·6	280	1
Polybrene	0·84 mg/l	6·0	330	1+
Polybrene	3·4 mg/l	32·5	350	1½
Polybrene	33·4 mg/l	6·1	160	1
Spermine	16·7 μmoles/l	6·3	330	1
Spermine	33·4 μmoles/l	7·7	330	1
Spermine	66·8 μmoles/l	9·4	330	1+
Spermine	100·2 μmoles/l	11·3	350	1½
Spermine	167·0 μmoles/l	11·7	390	2
Spermine	334·0 μmoles/l	23·5	410	2+
Spermine	668·0 μmoles/l	13·7	370	2

Rotenone, 5 μmoles per litre, and Polybrene or spermine were present in the incubation medium before starting the volume oscillations by adding 6·7 mmoles succinate per litre. In these experiments 2·5 μg oligomycin was also present in the final volume of 3·0 ml of medium 8 PT. The concentration of mitochondrial protein was 1·0 mg/ml.

The initial (=maximal) rates of passive efflux of ions are expressed as percentual changes in transmittance (=ΔT).* The stimulation of oscillations is expressed as a number of oscillatory cycles recorded before reaching steady state or before exhaustion of oxygen. One cycle means that the mitochondria swell and contract once before reaching a steady state (see also controls in Figs. 1 and 2), 1+ indicates initiation of swelling following the first swelling contraction cycle. 1½ indicates that the mitochondria again swell following a complete cycle, and 2 that the mitochondria present two complete cycles of oscillation.

The time in minutes elapsed from the addition of succinate to the point of oxygen exhaustion was measured when the mean respiratory rates were calculated. The total amount of 520 μA oxygen (0) was divided by this time.

TABLE II. Effects of spermine, Polybrene and gramicidin on the reversible energy-dependent low amplitude swelling of mitochondria

Substance	Concentration	Amplitude of the swelling ΔT (%)	Half time (min)
No (= control)	0	4·9	1·45
Gramicidin	0·33 μmoles/l	54·0	seconds
Polybrene	3·4 mg/l	20·0	0·18
Spermine	100 μmoles/l	7·1	0·62
Spermine	167 μmoles/l	27·0	0·14
Spermine	334 μmoles/l	12·8	0·36
Spermine	668 μmoles/l	12·7	0·80
Spermine	1000 μmoles/l	10·9	1·16

3·0 mg mitochondrial protein was suspended to a final volume of 3·0 ml of medium A. The swelling was started by the addition of 6·7 mmoles succinate per litre in the presence of 5 μmoles rotenone per litre. Half times represent the rate of swelling. They are calculated by measuring the time in minutes elapsed until the change in transmittance (=ΔT) is 50% of the final level of transmittance, where the mitochondria are in a swollen steady state. In this state 1 μmole FCCP per litre was added to cause the contraction of mitochondria in order to ascertain the reversibility of the swelling. In some instances the rate of mitochondrial swelling was too high to obtain reliable measurements. This is expressed as seconds in the table above. The total amount of swelling is expressed as amplitude of the swelling obtained by measuring the total ΔT.

* The presence of 1 μmole FCCP per litre did not affect the rate of passive efflux (not shown).

however, is interrupted by a further swelling. This probably represents uncoupling by gramicidin, i.e., the permeability to protons in addition to potassium and sodium ions is increased and the activity of mitochondrial ATPase is stimulated.[35] This seems not to be the case with spermine, which only stimulates the ion transport in these conditions as does Polybrene. In medium A no noticeable swelling of mitochondria is obtained in the presence of Mersalyl, due to the impermeability of the membranes to monovalent cations in the absence of the substance studied.

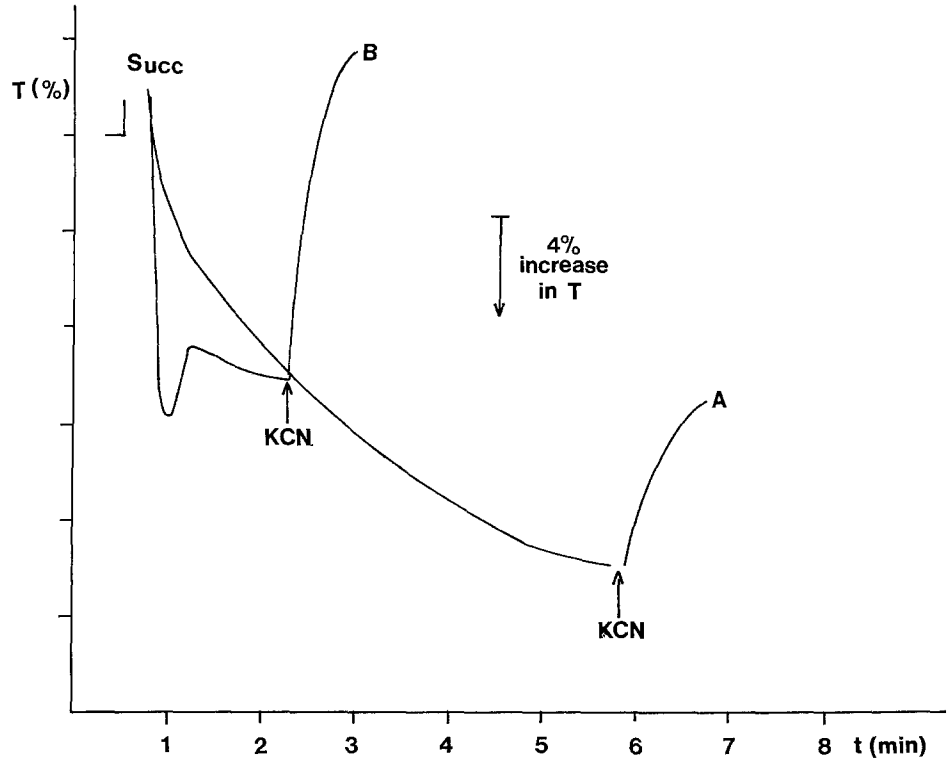

Figure 3. An oscillatory cycle of rat liver mitochondrial volume induced by spermine in medium A. Mitochondrial protein concentration was 1·2 mg/ml. The swelling of mitochondria was initiated by 6·7 mmoles succinate per litre. As in the control, in B 167 μmoles spermine per litre was present. The contraction is induced by 3·3 mmoles KCN per litre. Other conditions as in Fig. 1.

Discussion

The data in the present paper show that low concentrations of Polybrene and spermine increase the permeability of mitochondria towards monovalent cations. Thus the rate of contraction in the non-energized conditions of the preincubation was stimulated in the same way as in the presence of gramicidin. However, it seems to us improbable that polyamines act by the same mechanism as gramicidin in membranes. Negatively charged lipophilic gramicidin binds the monovalent cation and this complex probably shuttles in membrane transporting ions.[36] Strongly cationic Polybrene and spermine directly act on the membranes instead of complexing cations. These two agents also stimulate the energy-dependent swelling of mitochondria. The stimulation of volume

oscillations also demonstrates increased rates of both net ion influx and efflux. It is probable that these effects are due to changes in cation rather than anion permeabilities, since cation penetration seems to be the rate limiting factor in the experimental conditions used. This is especially true of the swelling experiments in the presence of Mersalyl with ATP hydrolysis as an intramitochondrial source of phosphate anions. In these conditions the transfer of inorganic phosphate is inhibited but the effects of Polybrene

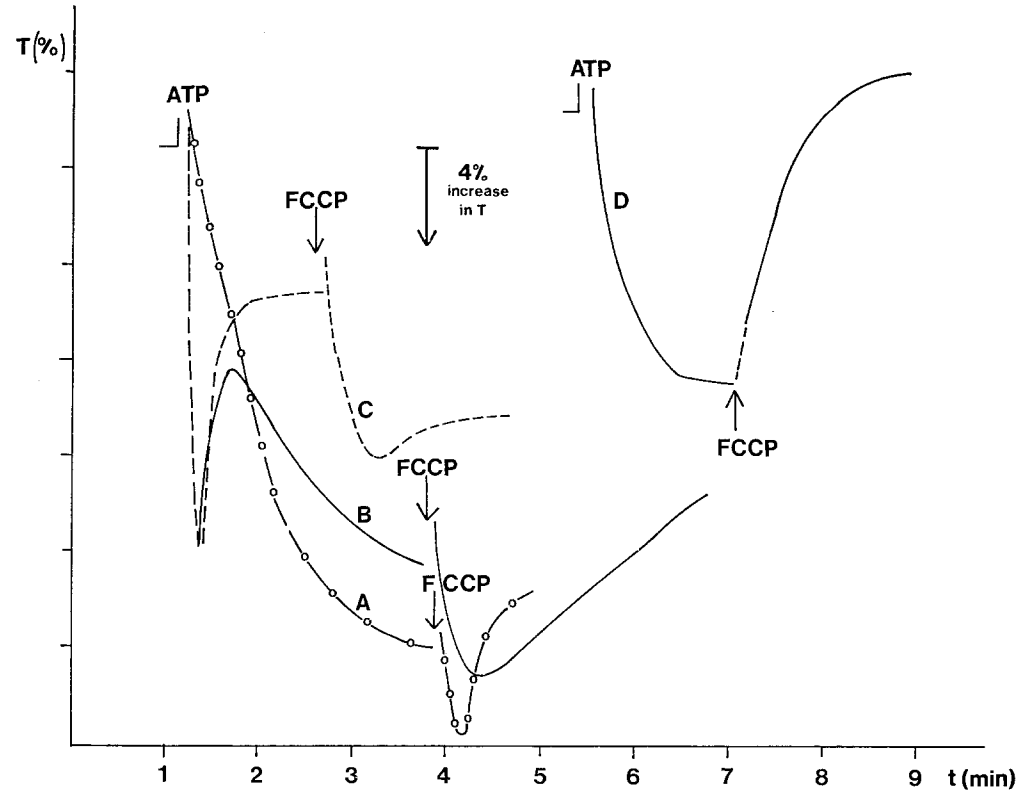

Figure 4. Effects of gramicidin and spermine on the rat liver mitochondrial volume oscillations in the presence of Mersalyl. The swelling was started by 1·3 mmoles ATP per litre in the presence of 320 μmoles Mersalyl per litre. The registration marked with D on the right of the figure represents the swelling in the absence of Mersalyl. Five times smaller amounts of Mersalyl gave identical results. The effect of 1 μmole FCCP per litre is shown. In the presence of Mersalyl the trace A represents the control experiment, in B 0·33 gramicidin is present, and in C 167 μmoles spermine per litre. 3·4 mg/l of Polybrene® acted in a similar way to spermine but the initial swelling was slower (not shown in the figure). The mitochondrial protein concentration was 3·4 mg in a final volume of 3·0 ml of medium 8 PT.

and spermine on ion transport in mitochondria are not essentially different to those obtained in the absence of Mersalyl. In iso-osmotic Medium A, pH 7·4, Mersalyl completely blocks the swelling of mitochondria. In medium 8 PT, pH 8·0, the accompanying anion in the presence of Mersalyl is very probably Cl^- instead of phosphate.[5] The extra swelling obtained in Fig. 4 by addition of FCCP proves that the transport of phosphate is indeed inhibited. These results suggest that the stimulation of ion transport in mitochondria by spermine, Polybrene, as also by gramicidin, is not substantially affected by the species of anion accompanying the cation transport. Furthermore, Table I suggests

that the proton movements are not primarily affected by the agents tested. The qualitative similarity between the effects of polyamines and gramicidin suggests that the stimulation of volume oscillations is indeed due to an increased permeability to cations.

In this connection it is of interest to compare the effects of Polybrene and spermine with those of propranolol and local anaesthetics. The last mentioned agents inhibit the passive efflux of ions in mitochondria under non-energized conditions. This is in accordance with the results of Azzi and Scarpa,[37] who used nupercaine to inhibit the transport of potassium ions in mitochondria. However, in oscillatory conditions the entire ion transport event is not inhibited. It rather seems that only the fluxes of ions and water directed outwards are inhibited by local anaesthetics.

The mechanism of mitochondrial volume oscillation is still rather poorly understood. However, there are known parameters found to be prerequisites for mitochondria to oscillate.[20, 21, 23] The data presented indicate that the decreased resistance of mitochondrial inner membranes to ion fluxes seems to be of paramount importance for the generation of volume oscillations. The presence of EDTA and high pH in slightly hypoosmolar conditions also increases the ion permeability and favours the oscillations.[15] Thus in conventional ion transport media, such as medium A, the mere presence of gramicidin or valinomycin can induce volume oscillations[32] when the cations to be transported are Na^+ or K^+. The species of cations and anions is also a factor known to affect the quality of oscillations, presumably due to their different abilities to permeate the membranes.

In beef heart mitochondria and digitonin particles of rat liver mitochondria, Polybrene and spermine stimulate while propranolol and chlorpromazine damp the volume oscillations (A. Huunan-Seppälä, unpublished). The effects of various agents studied on the volume oscillations in these particles were somewhat less pronounced. Nevertheless they suggest that the outer mitochondrial membrane is not of great importance in regulating the mitochondrial volume oscillations. This seems reasonable since only the inner membranes are known to be involved in the energy-linked alterations in mitochondrial volumes.

In considering the mechanism by which polyamines and Polybrene increase mitochondrial cation permeability, an interaction with membrane negatively charged sites seems highly probable.[21, 22] In mitochondria the phospholipids are presumably the principal binding sites for the positively charged substances studied[21, 22, 27] in view of the known *in vitro* binding of local anaesthetics and polyamines by various phospholipids.[38–40] Complexing with the negatively charged groups of membrane phospholipids and proteins would also result in changes in the conformation of mitochondrial membranes leading to altered physical and functional properties.

We have previously shown that local anaesthetics, Polybrene and spermine cause a change towards positive in the net surface charge of mitochondria.[22] This means that these agents cover the negative sites on mitochondrial surfaces. It is quite possible that, owing to their larger molecular masses and higher charge density, Polybrene and to a lesser degree spermine are not able to penetrate the hydrophobic membrane phase itself as efficiently as local anaesthetics, which in addition are more lipophilic in nature. Polyamines may thus be expected to interfere mainly with the charges in the outer boundary of hydrophobic phase. The resulting changes in charge distribution and associated molecular disorganization might well promote the passage of cations in this

region of the membrane. Local anaesthetics, on the other hand, might be expected to affect the properties of other regions in the membrane as well. Thus their action on mitochondria is of a more complex nature.

The profoundly changed properties of the mitochondrial membranes in the presence of local anaesthetics is also seen as an increased resistance towards lysis caused by added phospholipase A.[41,42] In spite of the inhibition of phospholipase A-induced swelling of mitochondria by spermine, it is not able to inhibit the formation of the reaction products to a large extent.[42] This may reflect the well-known properties of the polyamines to stabilize the membranes.[43] This suggestion is further strengthened by the biphasic action of spermine on the various parameters in Tables I and II, the inhibition by higher concentrations representing the phenomenon of membrane stabilization. Polybrene was found to be unable to inhibit the action of phospholipase A to any noticeable extent.[42] These results further suggest that local anaesthetics are bound in the hydrophobic membrane phase in addition to the binding on the outer and inner membrane surfaces. Their effect on the Donnan distribution arising from the fixed charges in the mitochondrial membranes and their diffusible paired ions[13] would thus be different from that of polyamines, which might affect this distribution by binding principally on the outer surfaces. In this case the inside of the membrane would become even more negative as compared to the outside, leading to increased potential difference between the two phases. Thus the mitochondria could also drive the monovalent cations inwards more efficiently, especially when energized.[44]

There is still the possibility that spermine might primarily act on the fluxes of water through mitochondrial membranes. The same would then be true of the other agents used. In fact it has been shown that chlorpromazine increases the permeability to water in erythrocytes.[45] The work of Tedeschi,[31] and Azzi and Azzone[5] indicates that the water movement in mitochondria is osmotic in nature. They further showed that the increase in mitochondrial volume dependent on the osmotic pressure of the permeating solutes is secondary to the uptake of solute particles and that this movement of water is due to increased permeability to cations and anions. In accordance with this idea, it is hardly possible that increasing the potential difference between the outer and inner mitochondrial membrane surfaces by polyamine binding would *per se* lead to an increased water flow.

By changing the external pH Wrigglesworth and Packer[16] have been able to show that along with changes in the state of membrane protonation the conformation of the mitochondrial membrane is altered. According to these authors, the change in pH affects the degree of ionization of fixed charges in the membranes, which leads to alterations in the electrostatic and repulsive forces. By this mechanism the proton gradient could change the membrane conformation.[16,44] In fact the lowering of pH in medium 8 PT inhibits the mitochondrial volume oscillations. This inhibition seems not to be due to the diminished capacity of EDTA to bind membrane Mg^{2+}, as previously thought,[4,6] because the inhibition is also observed in the presence of gramicidin or valinomycin (M. K. F. Wikström, unpublished). This renders the idea of Mg^{2+} regulating cation permeability in oscillatory conditions implausible, since the permeability to Na^+ or K^+ cannot be the rate limiting factor in the presence of the above-mentioned ionophorous antibiotics. It is possible that the action on membranes of the agents used in the present study, especially as regards local anaesthetics, is in some respect similar to the increased proton

concentration in the incubation medium. This would be true at least in covering partially the same negative groups and thus affecting the state of membrane protonation.

It may be concluded that the changes in the distribution and density of fixed charges in the membranes caused by Polybrene, spermine and local anaesthetics result in altered membrane properties. The idea of the importance of the fixed negative charges in regulating the conformational and functional membrane properties is further strengthened. The manipulation of these charges by changing the state of protonation or by binding with other positively charged agents capable of reacting with them offers a valuable method for studying the complex nature of ion transport coupled to energy-linked functions of membranes. Work is therefore in progress on the effects of local anaesthetics and related agents on the transport of monovalent and divalent cations in mitochondria and artificial membrane models consisting mainly of phospholipids. These results may be expected to be of use also in studies concerning the mode of action of local anaesthetics and related agents.

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