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Activation of the ADP/ATP carrier from mitochondria by cationic effectors

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The ADP/ATP carrier from the mitochondrial inner membrane was found to be influenced by cationic substances from the hydrophilic surroundings. Under low-ionic-strength conditions, addition of these cationic effectors fully activated the reconstituted adenine nucleotide translocator. The list of activators included divalent cations, polyamines, peptides and cationic proteins. The minimum requirement for an activator to be effective was the presence of at least two positive net charges, regardless of the size of the molecule. Cationic molecules were not activating when an intramolecular charge compensation was possible or when the two charges were too far apart from one another. The affinity of these activators varied from several hundred μM (diaminoalkanes, divalent cations) to 1 μM (cytochrome *c*, spermine) and even down to a few nM (polylysine). The activation by cations was fully reversible and was not due to fusion processes. It was not mediated by an interaction with the anionic substrates ADP and ATP, nor by interaction with the liposomes. The stimulation could directly and functionally be correlated to the reconstituted carrier protein. Activation was not observed in intact mitochondria, but could be demonstrated when the outer mitochondrial membrane had been removed by treatment with digitonin. These mitoplasts were stimulated by polycations similar to the ADP/ATP carrier in the reconstituted system.

Introduction

The transport activity of the adenine nucleotide translocator from the inner mitochondrial membrane is modulated by various factors pertaining to the surrounding hydrophobic and hydrophilic phases as well as by transmembrane parameters. The composition of the surrounding water phases with respect to monovalent and polyvalent anions and cations [1–3] and the composition of the surrounding lipid phase with respect to phospholipid headgroups and surface charge [2,4], cholesterol content [5] and fluidity of the membrane were all factors found to influence the transport activity of the adenine nucleotide translocator in the reconstituted system. The transmembrane parameters, i.e. substrate gradients and membrane potential, are the most important parameters which regulate the transport function in vivo [6,7].

Recently activation of the adenine nucleotide translocator by anions was described in detail for the reconstituted system [3]. When we tried to stimulate the adenine nucleotide translocator in intact mitochondria by anions we were not successful. The ADP-ATP exchange was nearly fully active also without added anions (not published). However, when we used mitoplasts, i.e. mitochondria from which the outer membrane has been removed, we suddenly found the same stimulation by anions as that observed in the recon-

Abbreviations: hexamethonium, hexane-1,6-bis(trimethylammonium bromide); Pipes, 1,4-piperazinediethanesulfonic acid; TEC^{3+}, tris(ethylenediamine)cobalt(III) cation (chloride salt).

stituted system. Thus some substance in the intermembane space must permanently activate the adenine nucleotide translocator in intact mitochondria. This cannot be done by simple anions, since the outer membrane is freely permeable to these molecules [8]. We thus had to look for a substance large enough to be restricted by the outer membrane and present in sufficiently high amounts. When testing cytochrome *c*, we found a definite stimulation of the reconstituted ADP-ATP exchange by this compound. However, in contrast to the anionic activators looked for in these experiments, cytochrome *c* is a strongly cationic molecule; the same holds true for a large number of activators which were subsequently found by experiments in the reconstituted system. These cationic activators share the minimum requirement of two positive net charges. They vary from small divalent cations to large polycationic proteins. The affinity of some of these cations is surprisingly high, so that an almost stoichiometric interaction with the carrier protein can be observed.

Materials and Methods

Chemicals. The chemicals used in these studies and their sources were as follows: carboxyatractylate, nucleotides, bovine serum albumin, lysozyme (Boehringer Mannheim); all other activator substances were obtained from Sigma; radioactive nucleotides (Amersham Buchler); Dowex 1-X8 (Fluka); Sephadex, SP-Sephadex, CM-Sephadex, DEAE-Sephacel (Pharmacia); Ecteola 23 (Serva); Triton X-100, polylysine agarose (Sigma). Bongkrekate was a gift from Professor Berends, Delft. All other chemicals were of analytical grade. Hydroxyapatite was prepared as described previously [9].

Lipids and liposomes. Egg yolk lipids were prepared according to Ref. 10. In all experiments, with the exception of those described in Fig. 3, purified egg-yolk phospholipids to which 5% cholesterol had been added were used in the reconstitution. For the preparation of liposomes with cetyltrimethylammonium bromide, the cationic lipid was added to the preformed liposomes of a composition as described above, which leads to incorporation into the outer leaflet of the bilayer [2].

Mitochondria. Bovine heart mitochondria [11] and rat liver mitochondria [12] were prepared as described previously. Removal of the outer membrane and purification of the inner membrane fraction (mitoplasts) were carried out by means of a controlled digitonin incubation of rat liver mitochondria [13]. In order to obtain mitoplasts active in ADP-ATP exchange, the amount of digitonin to be added to the rat liver mitochondria had to be tested separately before every experiment. Not more than 85% of the outer membrane could be removed without complete loss of exchange activity; therefore the amount of digitonin had to be reduced to about 0.12–0.14 g/g protein. Forward exchange in mitochondria and mitoplasts was carried out as described by Pfaff et al. [14].

Isolation, reconstitution and assay of the adenine nucleotide translocator. The adenine nucleotide carrier was isolated from bovine heart mitochondria by hydroxyapatite chromatography in a batch procedure using Triton X-100 as described previously [9]. The carrier protein was incorporated into performed liposomes and the ADP/ATP translocation activity was reconstituted by a freeze-thaw procedure [15] and a second sonication [16]. The sonication buffer included all ions and nucleotides which had to be present afterwards in the internal liposomal volume. For most experiments, the internal space contained 20 mM Na_4ATP/30 mM Na_2SO_4/10 mM Pipes (pH 7.0). The external medium was exchanged by chromatography on Sephadex G-75 columns in order to obtain the desired external ionic conditions. In most experiments the external buffer contained 3 mM Pipes (pH 6.5), 1 mM Na_2SO_4, 150 mM sucrose (low salt buffer). Measurement of reconstituted adenine nucleotide exchange at room temperature has been described previously [7]. After stopping the exchange reactions with carboxyatractylate and bongkrekate, radioactive external nucleotides were removed by ion-exchange chromatography on Dowex 1-X8 columns (Cl^- form). 100–200 μl of liposomal suspension were subjected to columns 5 × 30 mm. In order to minimize loss of lipid and protein, the columns were preequilibrated with egg-yolk phospholipid liposomes (3–5 mg phospholipid/column) and bovine serum albumin (2 mg/column). The liposomes of the exchange assay were eluted with a defined volume of 50 mM

NaCl. Aliquots of the eluate were analyzed by liquid scintillation counting of the amount of internal radioactively labeled nucleotides. When activators were tested in the reconstituted system, they were added to the assay system 1–2 min before addition of labeled substrate.

The true exchange rates were extrapolated according to Ref. 7. The $K_{0.5}$ values, i.e. the concentration of activator which causes half maximum stimulation, and the Hill-factor n were determined as described previously for the activation by anionic substances [3].

In one experiment, the reconstituted aspartate/glutamate carrier was used. Isolation and reconstitution of this mitochondrial carrier protein has been described elsewhere [17].

Results

Characterization of cationic activators

As already mentioned in the introduction, the first compound to be tested was cytochrome *c*. Fig. 1 shows the influence of cytochrome *c* or bovine serum albumin on the reconstituted adenine nucleotide translocator. Only the cationic protein cytochrome *c* was activating, the stimulation reaching the same level as that obtained by the addition of high salt concentrations, which led to full activation, as described previously [3]. Activation by cytochrome *c* showed sigmoidal dependence on the concentration of added activator. This could be analyzed in a way similar to the procedure developed for the activation by anions [3]. The analysis led to Hill coefficients of n = 1.4–2.3 for the various activators discussed below (cf. Table I).

Activation by cations was pH-dependent (Fig. 2). Obviously activation by spermine became less effective at higher pH values, whereas the diminished activation at lower pH (Fig. 2B) was mainly due to the fact that in this pH region the adenine nucleotide translocator was already substantially activated by the amount of sulfate present. Thus, at lower pH the baseline was shifted to higher activities. This pH profile was found to be qualitatively similar to that found with spermine when tested with cytochrome *c* and with di-

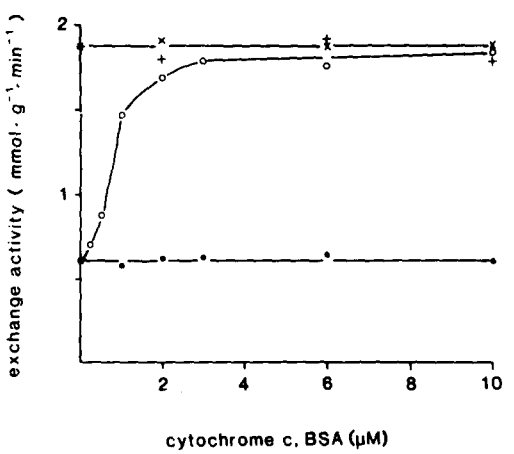

Fig. 1. Influence of cytochrome *c* and bovine serum albumin (BSA) on the reconstituted ATP-ATP exchange. Cytochrome *c* (○, +) and bovine serum albumin (●, ×) were added either in the presence of 3 mM Pipes (pH 6.5), 1 mM Na_2SO_4, 150 mM sucrose (○, ●) or in the presence of 20 mM Pipes (pH 6.5) 30 mM Na_2SO_4, 50 mM sucrose (+, ×).

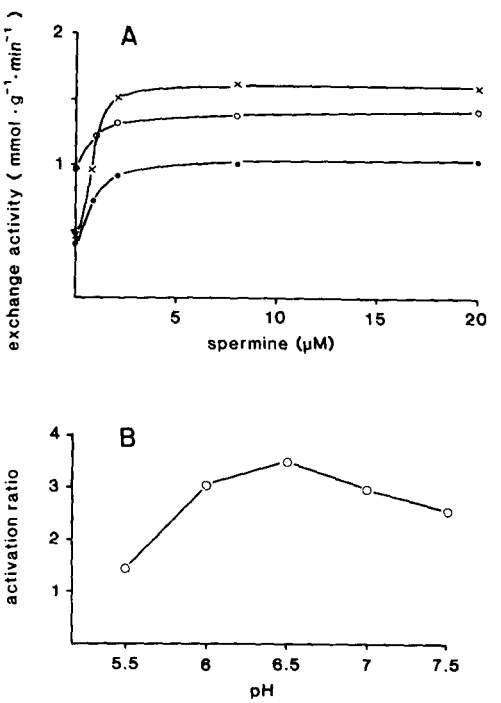

Fig. 2. Activation of reconstituted ATP-ATP exchange by addition of spermine at various pH valves. (A) Exchange activity in dependence of added spermine at pH 5.5 (○), 6.5 (×), and 7.5 (●). (B) Dependence of activation on the pH of the transport assay, activation ratio = (activity in the presence of 20 μM spermine)/(activity without spermine).

aminooctane. In the following, the activation by cations was investigated at pH 6.5.

Once the stimulating effect of cytochrome *c* on the adenine nucleotide translocator activity had been discovered, a large series of substances was tested for their activation properties. A list of these effectors is given in Table I. Several properties common to all activators can be derived from this table. (a) Activators had to be positively charged at pH 6.5 where activation was tested. (b) The molecular size of the activators was unimportant; this was made clear by the fact that e.g. both Ca^{2+} and polylysine were activating. (c) The minimum number of positive charges required was 2. Not only did monovalent cations fail to activate, also the strongly cationic amino acids lysine and arginine did not activate, presumably because one of the two positive charges was compensated by the adjacent carboxylic group. The dipeptide Lys-Lys had enough potency to stimulate the reconstituted adenine nucleotide translocator, although with low affinity. Lys-tripeptide was already a good activator. (d) The affinity for activation is expressed in $K_{0.5}$ values, representing that concentration of effector which led to half-maximum stimulation of transport. The $K_{0.5}$ values varied

TABLE I

ACTIVATION OF THE RECONSTITUTED ATP-ATP EXCHANGE BY CATIONS

The $K_{0.5}$ values represent the concentration of activator that causes half maximum stimulation of the adenine nucleotide translocator at pH 6.5.

Substance	Molecular weight (mass)	Charge at pH 6.5	Activating effect	$K_{0.5}$ (M)
(a) Proteins				
Cytochrome *c*	13400	+	+	$1 \cdot 10^{-6}$
Lactalbumin	17400	0	0	–
Myoglobin	16900	0	0	–
Lysozyme	13900	+	+	$1 \cdot 10^{-6}$
Serum albumin	66000	–	0	–
Histone	37000	+	+	$1 \cdot 10^{-7}$
Protamin	5000	+	+	$1 \cdot 10^{-6}$
Polylysine	(1–4 kDa)	+	+	$2 \cdot 10^{-6}$
Polylysine	(4–15 kDa)	+	+	$2 \cdot 10^{-8}$
Polylysine	(15–30 kDa)	+	+	$5 \cdot 10^{-9}$
Polylysine	(30–70 kDa)	+	+	$5 \cdot 10^{-9}$
(b) Amino acids, polyamines				
Lysine	146	+	0	–
Arginine	174	+	0	–
Lys-Lys	289	+	+	$1 \cdot 10^{-4}$
Lys-Lys-Lys	433	+	+	$5 \cdot 10^{-6}$
Putrescine	86	+	+	$3 \cdot 10^{-4}$
Spermidine	142	+	+	$5 \cdot 10^{-6}$
Spermine	198	+	+	$1 \cdot 10^{-6}$
(c) Diaminoalkanes, anorganic ions				
1,4-Diaminobutane	90	+	+	$3 \cdot 10^{-4}$
1,6-Diaminohexane	118	+	+	$5 \cdot 10^{-4}$
1,7-Diaminoheptane	132	+	+	$7 \cdot 10^{-4}$
1,8-Diaminooctane	146	+	+	$8 \cdot 10^{-4}$
1,10-Diaminodecane	174	+	+	–
Hexamethonium	202	+	0	–
Decamethonium	258	+	0	–
Mg^{2+}	24	+	+	$5 \cdot 10^{-4}$
Ca^{2+}	40	+	+	$1 \cdot 10^{-4}$
TEC^{3+}	239	+	+	$1 \cdot 10^{-5}$

over a wide concentration range from more than 0.5 mM (diaminooctane) to 5 nM (polylysine). (e) There must be a certain structural parameter which provides optimum activation. This is seen on the one hand in the case of the diaminoalkanes, which have a minimum structure for activation by organic cations and showed decreasing stimulation power with increasing alkyl length; on the other hand, when the two charges were separated more stringently, as in the case of hexamethonium, no activation at all could be observed. One may argue that this is due to a shielding of the positive charges. However, it could be shown that this was not the reason, since TEC^{3+}, a trivalent cation, the charge of which is even more effectively shielded, showed activation with good affinity. In contrast to Ca^{2+}, both methonium compounds and especially TEC^{3+} are known not to bind tightly to negative charges of the membrane surface [18]. Thus the reason for this stimulation could not simply be charge compensation.

What is the actual target of the activator?

The transport assay consisted of several components which, in principle, could all be targets for the cationic activators: phospholipids in general, negatively charged phospholipids in particular, the liposomes, the negatively charged substrate ATP, and the inserted carrier protein. In Table II, the actual concentration of all these possible targets in the reconstituted system is compared with the minimum amount of several activators necessary for full stimulation of transport. The concentration range of the various components in the transport assay could be calculated according to the data given in Materials and Methods. The activator concentration was expressed as $2 \times K_{0.5}$ on the basis of the following considerations. If we assume a direct interaction of activator and target and if the stimulating ligand is tightly bound, then all binding sites of the target would be saturated by two times that concentration of activator which led to stimulation of half of the carrier molecules, i.e. $2 \times K_{0.5}$. Since most of the activators are obviously not so tightly bound, the assumed $2 \times K_{0.5}$ value clearly overestimates the amount of binding sites. In other words, the concentration of the targets, stimulated by the cations shown in Table II, must be lower or at the best equal to the $2 \times K_{0.5}$ value given for the corresponding activators.

TABLE II

CORRELATION OF AVERAGE CONCENTRATIONS OF PHOSPHOLIPIDS, CARRIER PROTEIN AND NUCLEOTIDES IN THE TRANSPORT ASSAY WITH THE OBSERVED ACTIVATION CONSTANT $K_{0.5}$

For calculation of the concentrations see text.

Object	Concentration range in the assay system
Phospholipid	
External concentration	15–25 mM
External, negatively charged	0.1–0.4 mM
Liposomes (average diameter 400 Å)	3–6 μM
Carrier protein	
total concentration	0.6–1.2 μM
functional active	0.06–0.25 μM
active, right-side-out	0.03–0.12 μM
Substrate (ADP, ATP)	50–200 μM
Minimum amount of activator ($2 \times K_{0.5}$)	
Diaminobutane (putrescin)	600 μM
Diaminooctane	1600 μM
Ca^{2+}	200 μM
Lys-Lys-Lys	10 μM
Spermine	2 μM
Cytochrome *c*	2 μM
Protamine	2 μM
Polylysine (1–4 kDa)	4 μM
Polylysine (4–15 kDa)	0.04 μM
Polylysine (15–30 kDa)	0.01 μM

Phospholipids in general could be ruled out as targets for the activators on the basis of two main arguments. (a) Several polycations effectively activated the adenine nucleotide translocator at concentrations far below the concentration of phospholipids, below the concentration of negatively charged phospholipids in the outer leaflet of the membrane, and even below the concentration of liposomes in the transport assay (Table II). (b) If binding of the activators to negatively charged lipids at the surface of the liposomes triggered the stimulation event, this should be prevented when the negative surface charges are cancelled by addition of positively charged lipids, such as cetyltrimethyl ammonium bromide. This experiment is shown in Fig. 3. Addition of positively charged lipids to the liposomal membrane did not

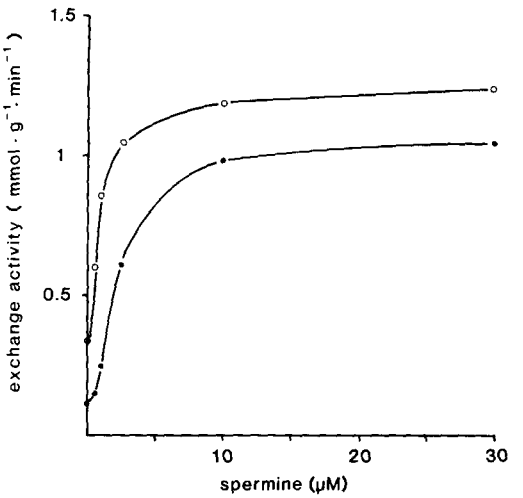

Fig. 3. Activation of ATP-ATP exchange by spermine. Exchange was measured in proteoliposomes prepared from purified egg-yolk phospholipids (○) or from purified egg-yolk phospholipids with addition of 2% (mol/mol) cetyltrimethylammonium bromide (●) in low salt buffer (see Materials and Methods). Addition of 2% cetyltrimethylammonium bromide (mol/mol) to egg-yolk phospholipid liposomes leads to virtually charge-balanced liposomes, as has been shown previously [2].

at all prevent the activation by spermine. The extent of activation remained the same, but the apparent affinity was somewhat lowered, presumably due to the altered (more cationic) surface potential of the liposomes.

The next candidate, the anionic substrate ATP, can easily be excluded for several reasons. (a) Firstly, the large discrepancy in the actual concentrations has to be considered (Table II). However, this argument could be questioned, because already a small portion of the ATP present in the assay would be sufficient to saturate the binding sites at the carrier protein. (b) The affinity of Mg^{2+} for ATP is about 10-times higher than that of Ca^{2+} [19], as has been shown also in the reconstituted system of the adenine nucleotide translocator [1]. If Mg^{2+} stimulates via substrate interaction, its activation effect, as compared to that of Ca^{2+}, should alsobe seen at lower concentrations. In fact, just the opposite is true (Table I). (c) The effect of spermine, cytochrome *c* and also other polycationic activators is not different, when ATP-ATP or ADP-ADP exchange is measured (experiments not shown). (d) If the activators did, in fact, interact with small amounts of the substrate, thereby changing some binding steps in the transport cycle – this was the objection raised in (a) – it should be revealed in a shift of the apparent K_m values for nucleotide transport [7]. Fig. 4 demonstrates that this is definitely not the case.

Besides the elimination of all other possible targets, we also have positive evidence that the protein is, in fact, the real target of these cationic activators. (a) The adenine nucleotide translocator was the only component in agreement with the concentration range of the activators with highest affinity. (b) If activation is an unspecific effect on the liposomes or phospholipids it should be seen also with other carrier proteins. We tested the reconstituted aspartate/glutamate carrier [18], which has a very similar transport mechanism, but found no stimulation at all by any of the substances listed in Table I (experiments not shown). (c) It is well known that the adenine nucleotide translocator is a very basic protein with an isoelectric point around 10 [20]. Thus, if one tries to bind this protein to ion exchangers, cation exchange resins have to be used. However, it turned out that neither cation exchangers (e.g. SP-Sephadex, CM-Sephadex), no –as expected– conventional anion exchangers (e.g. Ecteola 23, DEAE-Sephacel) were

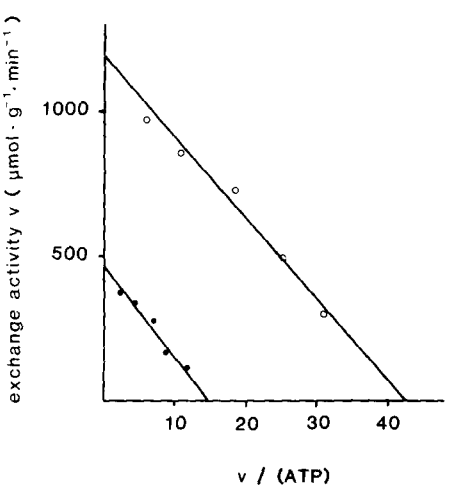

Fig. 4. Eadie-Hofstee plot for determination of K_m and V_{max} of the reconstituted ATP-ATP exchange. Transport was measured without (●) or with 10 μM spermine added (○) in low salt buffer (see Materials and Methods). ATP concentration in the external space was 10, 20, 40, 80 and 160 μM.

suitable for this purpose (Table III). Since polylysine was found to be a very efficient activator of the carrier protein and should thus bind tightly to the adenine nucleotide translocator, chromatography on polylysine agarose was tested. In fact, this anion exchange material could be used for reversible binding of the cationic adenine nucleotide translocator, as shown in (Table III). Obviously, this should be regarded more or less as an affinity chromatography.

Is activation mediated by fusion?

There are reports in the literature asserting that an increase in the size of liposomes significantly increases the apparent transport activity of reconstituted carrier proteins [21]. Thus one might explain the observed activation on the grounds of fusion events leading to enlargement of the liposomes. This was suggested especially by the fact that many of the effectors listed in Table I are well known fusogens. However, several arguments exclude this possibility. (a) Purified egg-yolk phospholipids, which include only a very small amount of negatively charged lipids [2] do not provide a suitable membrane for fusion events to occur. (b) In Fig. 3 an experiment with positively charged membrane is described in which activation can also be observed. Cations cannot mediate fusion of positively charged liposomes. (c) It has already been explained above that the aspartate/glutamate carrier, when reconstituted into the same liposomes, was not influenced by any of these activators. (d) The best argument against membrane fusion forming the basis for carrier activation was provided by reversibility studies. In Table IV experiments with three different activators are described. The stimulation event was shown to be reversible not only for activators with low affinity (Ca), but also for those with moderate (spermine) and high affinity (polylysine). Fusion cannot be considered to be reversed by passage over cation exchanger or by complexation of Ca^{2+} and polylysine.

Experiments with mitochondria

Activation of the reconstituted adenine nucleotide translocator by cationic effectors has been described here in detail. Does this phenomenon occur also in intact mitochondria?

When intact mitochondria were washed in low salt buffer, addition of spermine led to only a very small stimulation effect (Fig. 5). Mitochondria were found to be nearly fully active also without added cations. Therefore it is not surprising that this stimulation was not observed earlier. It has to be pointed out that the activation described here must be discriminated from the surface potential effects observed by Meisner [22], which mainly affect the apparent K_m values. This has been analyzed in detail in the reconstituted system [2].

Nevertheless, the polycation-induced activation of the ADP/ATP exchange is not an artefact of the reconstituted system. This was revealed when the outer membrane of mitochondria was removed

TABLE III

CHROMATOGRAPHY OF ISOLATED ADENINE NUCLEOTIDE TRANSLOCATOR ON VARIOUS ION-EXCHANGE COLUMNS

Elution of protein (% of material applied onto the columns) and elution of reconstitutively active adenine nucleotide translocator (% of activity applied onto the columns). Fract. 1: pass-through fraction in 15 mM Tris (pH 7.0), 150 mM sucrose (polylysine agarose, DEAE-Sephacel), respectively, 15 mM acetate (pH 7.0), 150 mM sucrose (SP-Sephadex); fract. 2: elution with 100 mM Na_2SO_4. The columns were preequilibrated to obtain the chloride form (polylysine agarose, DEAE-Sephacel) or the sodium form (SP-Sephadex). Column dimensions were 0.5×5 cm, 200–300 μg (1 ml) of adenine nucleotide translocator were applied, the pass-through fraction was 2.5 ml, the protein eluted by Na_2SO_4 was collected in 1.0 ml.

Chromatography on	Eluted protein (%)		Eluted adenine nucleotide translocator activity (%)	
	fract. 1	fract. 2	fract. 1	fract. 2
Polylysine agarose	9	82	3	56
DEAE-Sephacel	63	6	31	2
SP-Sephadex	46	1	26	1

TABLE IV

REVERSIBILITY OF ACTIVATION BY CATIONS IN THE RECONSTITUTED SYSTEM

In Expts. 1 and 2 the procedures (a), (b), and (c) refer to exchange measurements after the subsequent addition of the effectors, i.e. in experiment 1c e.g. the assay contains Ca and EDTA. In Expt. 3 spermine was first added (a → b), then removed by passage over Dowex 50W, Na^+ form (b → c), then added once more to the eluate of the Dowex column (c → d). The ATP-ATP exchange was measured in low salt medium (see Materials and Methods).

Expt.	Procedure	Exchange activity ($\mu mol \cdot g^{-1} \cdot min^{-1}$)
1	(a) before addition of effector	140
	(b) + $CaCl_2$ 0.5 mM	1075
	(c) + EDTA 1 mM	190
2	(a) before addition of effector	105
	(b) + polylysine (15–30 kDa) 15 nM	1045
	(c) + polyaspartic acid (20 kDa) 100 nM	175
3	(a) none	210
	(b) + spermine 10 μM	960
	(c) passage over Dowex 50W	230
	(d) + spermine 10 μM	905

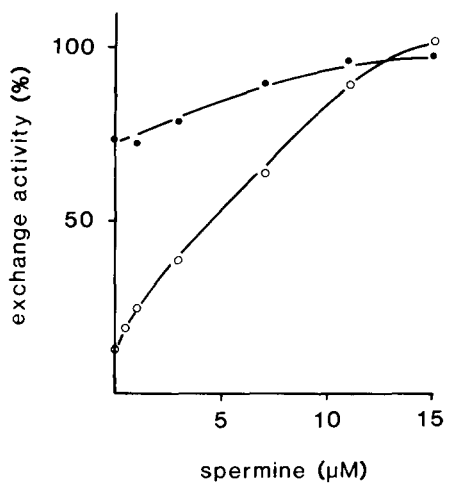

Fig. 5. Activation of adenine nucleotide exchange by spermine in mitochondria and mitoplasts. Mitochondria and mitoplasts were washed once in 150 mM KCl, 10 mM Tris (pH 7.5), then twice in 250 mm sucrose [^{14}C]ATP uptake in the presence of 0.5 μM FCCP was measured in the forward direction in dependence of added spermine, both in mitochondria (●) and mitoplasts (○). For better comparison, relative exchange activities (full stimulation = 100%) were shown, the absolute activities were 8 $\mu mol \cdot g^{-1} \cdot min^{-1}$ for mitochondria and 3.5 $\mu mol \cdot g^{-1} \cdot min^{-1}$ for mitoplasts (7°C).

by controlled digitonin treatment. Washed mitoplasts could effectively be stimulated by addition of spermine in concentrations similar to those determined in the reconstituted system (Fig. 5).

Discussion

Activation of the ADP/ATP transport by cationic substances has been characterized in the following respects.

(a) Activation is not an unspecific process. Although the list of activators includes very different substances, there are some important prerequisites which have to be fulfilled by all activators. At least two closely adjacent positive charges have to be present. If the distance between the charges is too large steric hindrance (hexamethonium) will preclude activation properties.

It is interesting to note that Møller et al. [23–25] found similar effects when studying the influence of surface charges on the rate-limiting steps in plant mitochondrial electron transport in extensive and careful investigations. They discriminated between the general influence of ions on the surface potential, which led to modulation of the K_m value, and specific effects on partial reactions, which modulate the V_{max} value. This analysis can be excellently correlated with investigations in the much simpler system of reconstituted adenine nucleotide translocator [2]. However, the results presented in this paper cannot be simply due to surface-charge screening. This is demonstrated mainly by the data given in Table I. (i) Monovalent cations fail to activate, even at concentrations above 10 mM. (ii) The divalent cations hexamethonium and decamethonium, which were successfully used by Møller et al. as screening agents for negative surface charges, did not activate the reconstituted adenine nucleotide translocator. (iii) There are significant differences with respect to the stimulation power between molecules with the same number of charges (compare Lys-Lys-Lys with spermidine or spermine) and inconsistencies in the order of stimulation power when only the number of charges is taken into account (compare polylysine (1–4 kDa) with spermine).

(b) Activation can be observed also in intact mitochondria. It is of basic interest to decide whether activation is observed only in the recon-

stituted system, or whether it is of physiological importance. So far, the 'physiological' importance could not be proved, but became obvious when it was shown that mitoplasts behaved in the same way as the reconstituted adenine nucleotide translocator. Several possible explanations for this observation can be considered. (i) There may be a component in the intermembrane space which functions as activator and which is lost after removal of the outer membrane. (ii) Protein-protein (carrier-carrier?) interactions may occur which are changed after removal of the outer membrane. (iii) There may be contact areas between the outer and the inner membrane [26] which lead to modulation of the carrier activity – an influence which may be mimicked by the polycations. Experiments to decide between these possibilities are in progress.

(c) Activation is fully reversible and no mediated by fusion. In our opinion, the proof for reversibility is very important since it elucidates that the activated protein is dependent on the permanent presence, i.e. binding, of the activator substance. Whenever the activator was removed or complexed, the adenine nucleotide translocator instantly fell back into a nonactivated state. Furthermore, the observation of reversibility provides the most convincing proof –besides other arguments (see Results)– against fusion being the explanation for the carrier activation. Nevertheless, it may not be purely by chance, that many of the stimulators are also classical fusogens. We have, therefore, to consider a mechanism, responsible for activation, which causes the activating substance to change the surface properties of some molecule (usually phospholipid) in the same manner as in the well-known case of fusogens.

(d) Functional correlation of activators to the adenine nucleotide translocator. There are unequivocal arguments against the substrate being the mediator for the observed stimulation. There are good arguments against phospholipids in general and against liposomes being the target of the activating substance. There are, furthermore, a couple of arguments in favor of a direct correlation of the effector molecules to the nucleotide carrier protein. However, if one assumes a molecule, tightly bound to the adenine nucleotide translocator, as the actual target of the activating effector, then we would have a situation which is not easily distinguishable from the interpretation given above, i.e. direct binding of the activator to the carrier protein. Such a hypothetical molecule, which, in fact, is not purely hypothetical anymore, has recently been discovered. Several molecules of cardiolipin have been found to be tightly bound to the adenine nucleotide translocator [27]. These tightly bound negatively charged phospholipid molecules might be the direct target of the polycationic activators. Experiments in the reconstituted system to substantiate this possibility are in progress.

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