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The cationically selective state of the mitochondrial outer membrane pore: a study with intact mitochondria and reconstituted mitochondrial porin

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The outer mitochondrial membrane pore at a voltage above 20 to 30 mV can adopt a state of low conductance which may restrict free permeability of mitochondrial substrates. In order to obtain insight into the physiological meaning of this property we took advantage of the fact that the low conductance pore state could be induced by a polyanion in lipid bilayer membranes as well as in intact mitochondria. Upon reconstitution in artificial bilayers the pore in this substate became exclusively cation selective when the polarity of the applied voltage was negative on the cis-side. This behaviour of the pore would explain why induction of the low conductance pore state in intact mitochondria led to a complete inhibition of mitochondrial intermembranous kinases, such as creatine kinase and adenylate kinase, but not of peripheral kinases, for example hexokinase, when utilizing external ATP. The possibility that the inner membrane potential might be transduced to the outer membrane in the contact sites, suggests the existence of cation selective pores in these sites. This aspect may be important in the regulation of peripheral kinases like creatine kinase, nucleoside diphosphate kinase and adenylate kinase which are located behind the outer mitochondrial membrane.

Introduction

The transport of adenine nucleotides and creatine phosphate across the outer mitochondrial membrane is restricted to a pore protein named 'VDAC' [1] or 'mitochondrial porin' [2] which subsequently was identified as a specific binding protein for glycerol kinase [3] and hexokinase [3,4]. This knowledge led us to suggest how mitochondrial ATP may be channelled to the peripheral bound hexokinase [5–7]. We gained further insight into the structural organization at the mitochondrial periphery by electron microscopy. It was found that hexokinase is located at the mitochondrial surface preferentially in the contact sites between the two mitochondrial boundary membranes [8–10]. By isolation of the contact sites from brain mitochondria we observed that the activity of the mitochondrial creatine kinase was also concentrated in these sites [10], which emphasized the involvement of contact sites in the organization of kinases. As the pore protein was found to be randomly distributed all over the outer membrane, we suggested that voltage-dependent regulation of the pore conductivity [1,2] may come into effect only in the contact sites leaving other pores unregulated. It appeared likely that the inner membrane potential might be transduced to the outer membrane in the contacts and by that may exert control over the transport through the outer membrane pore. As described earlier [1,2], at voltages above 30 mV the pore adopts a low conductance state in which ADP and ATP transport appears to be excluded [11]. In case of *Paramecium* mitochondria the closed pore exhibited cation selectivity [12]. Provided the inner membrane potential would regulate the pore transport, a dynamic compartmentation of adenine nucleotides at the mitochondrial periphery would result [7,13]. Such compartmentation would improve the exchange of mitochondrial energy [14] of the following reasons: firstly, the ATP/ADP transport system would be displaced from equilibration, which is necessary because the electrogenic ATP export, according to in vitro measurements [15] generates ATP/ADP ratios which are equivalent to the cytosolic steady-state concentrations in active, intact cells [16]. Secondly, the mitochondrial creatine kinase would not be exposed to the cytosolic level of creatine phosphate. The latter would explain the preferred utilization of creatine by the mitochondrial

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creatine kinase as postulated in the creatine phosphate shuttle [17,18,19].

In order to provide evidence of this considerations we studied the ion selectivity and single channel conductance of the outer membrane pore in the closed state by reconstituting rat liver porin in artificial lipid bilayer membranes and employing a synthetic polyanion which has been shown to be a potent inhibitor of the pore function [11,20,21]. Furthermore, we took advantage of this method to close the pore while investigating the activity of the mitochondrial creatine kinase, adenylate kinase and hexokinase in isolated brain and liver mitochondria.

Materials and Methods

Chemicals. All chemicals were purchased from Boehringer-Mannheim and Merck-Darmstadt, F.R.G.

Enzyme assays. Adenylate kinase (EC 2.7.4.3), hexokinase (EC 2.7.1.1) and creatine kinase (EC. 2.7.3.2) was measured in agreement with Bücher et al. [22] in the presence of 10 mM $MgCl_2$.

Preparation of mitochondria from rat liver and brain. Liver mitochondria from rats (250g body weight) were isolated by differential centrifugation in 0.25 M sucrose, 10 mM Hepes (pH 7.4). The mitochondrial sediment was washed two times using $6000 \times g$ and $3000 \times g$ (Sorvall, rotor SS-34) for sedimentation. Mitochondria from rat brain were prepared according to Rehncrona et al. [23] in a medium containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% fatty acid free bovine serum albumin. The mitochondria were further purified by 35 min centrifugation at 38000 rpm in a 60 Ti rotor (Beckman) on a 20% percoll gradient. Percoll was removed from the mitochondrial fraction by washing two times with the above medium.

Treatment of isolated mitochondria with the polyanion. The mitochondrial fraction, suspended in isolation medium, contained a protein concentration of approximately 20 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated in the presence of 5 mM $MgCl_2$ for 5 min at room temperature with concentrations of the polyanion ranging from 1.25 to 100 μg/ml. The polyanion is a copolymer of metacrylate, maleate, and styrene in a 1 : 2 : 3 proportion. The suspension was subsequently centrifuged for 1 min in a table-top centrifuge. The supernate was removed and the sediment was resuspended in the original volume of isolation medium. In case of hexokinase determination, polyanion pretreated liver mitochondria were incubated with 68 mU of isolated isozyme I, 10 mM $MgCl_2$, and 5 mM glucose and subsequently centrifuged. The activity of hexokinase in the sediment was determined in the presence of either 1 mM ATP or 0.5 mM ADP, 5 mM succinate and 5 mM phosphate and $MgCl_2$.

Treatment of mitochondria with digitonin. Mitochondria were treated with the polyanion as described above and resuspended in sucrose medium. The mitochondrial suspension contained a protein concentration between 20 and 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 30 sec at room temperature with concentrations of digitonin ranging from 10 to 500 μg/mg of protein. Aliquots of the suspension were directly used for determination of activity of creatine kinase and adenylate kinase. This resulted in a dilution of the digitonin in the enzyme assay by a factor of 100.

Assay of protein concentration. Protein was determined by the method of Lowry et al. [24].

Porin isolation. Mitochondrial porin was isolated from whole mitochondria essentially as described by De Pinto et al. [25].

Lipid bilayer experiments. The methods used for the 'black' lipid bilayer experiments have been described previously [26]. The membranes were formed from a 1% (w/v) solution of diphytanoylphosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane across circular holes (surface area about 0.1 mm^2 in the case of single-channel experiments, or 1 mm^2 for the macroscopic conductance and selectivity measurements) in the thin wall of a Teflon cell separating the two aqueous compartments. Porin was added from a concentrated stock solution (1 mg/ml porin, 0.1% Genapol X-80) to the aqueous phase with stirring to allow immediate equilibration prior to membrane formation, or after the membranes had turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart or a tape recorder. For macroscopic conductance measurements the current amplifier was replaced by a Keithley electrometer (model 610 C). The zero-current membrane potentials were the result of a 6.4-fold KCl gradient (50 mM versus 320 mM) across a membrane in which between 10^2 and 10^4 pores were reconstituted [27]. The membrane potential measured using a Keithley electrometer reached its steady state value within 3 to 5 min. The specific conductance of the aqueous salt solutions was measured using a conductometer (Metrom, Herisau Switzerland).

Results

Activity of peripheral kinases as analyzed by inhibition of the outer membrane transport

We observed recently that a polyanion (i.e., a 1 : 2 : 3 polymer of metacrylate, maleate and styrene) described by König [20] was able to inhibit the transport of ATP and ADP across the outer membrane [11]. We took advantage of this finding to study the organization of

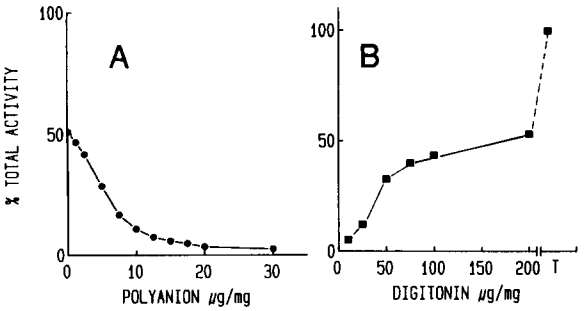

Fig. 1. Inhibition of mitochondrial creatine kinase by the polyanion and reactivation of the enzyme by digitonin. (A) Brain mitochondria were incubated with increasing concentrations of the polyanion as described in Methods. The mitochondria were subsequently sedimented, resuspended in the same volume of sucrose medium and subjected to determination of enzyme activity. (B) Aliquots of mitochondria pretreated with 30 µg/mg polyanion were incubated with different concentrations of digitonin and creatine kinase activity was determined immediately after 30 s incubation in sucrose medium within 20 µl of the digitonin treated samples. One sample was treated with 1% Triton X-100 (T) to determine the total activity. The data are mean values of four experiments.

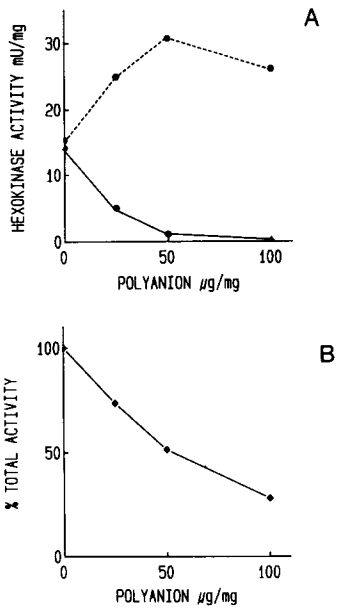

Fig. 2. Inhibition of mitochondrial hexokinase and adenylate kinase by the polyanion. Liver mitochondria were pretreated with different concentrations of polyanion as in Fig. 1. The mitochondria were washed in sucrose medium and adenylate kinase activity was determined (B). (A) Aliquots of the same mitochondria were incubated with isolated hexokinase I as described in Methods, centrifuged and activity of hexokinase was analyzed in the presence of 2 mM external ATP (dashed line) or endogenous ATP (solid line) provided by the oxidative phosphorylation in the presence of 5 mM succinate and 0.5 mM ADP.

the mitochondrial creatine kinase (Figs. 1A and B) and adenylate kinase (Fig. 2B) in comparison to the peripheral bound hexokinase (Fig. 2A). The activity of these kinases was determined in isolation medium to keep the structure of the freshly isolated brain and liver mitochondria intact. As reported earlier [11] the activity of adenylate kinase became inhibited by the polyanion. In contrast to adenylate kinase in liver mitochondria, 50% of the total (Triton-X-100 extractable) creatine kinase activity was latent in brain mitochondria without inhibition of the pore by the polyanion. However, as with adenylate kinase, the freely accessible activity of the latter enzyme became completely inhibited after preincubation of the mitochondria with 30 µg of polyanion (Fig. 1A) and the activity was regained after disruption of the outer membrane by addition of 200 µg of digitonin (Fig. 1B). The results suggested that the mitochondrial creatine kinase was excluded from its substrate ATP because it was located behind the outer membrane. This interpretation was completely consistent with the fact that glucose phosphorylation by the peripheral bound hexokinase utilising external ATP was not inhibited by the polyanion (Fig 2A). On the other hand, the polyanion was effective when the ATP was internally provided by the oxidative phosphorylation. In this case it could not be distinguished whether the polyanion inhibited the oxidative phosphorylation, as postulated by König [20], or the export of ATP. Supposing that the polyanion switches the pore into its low

TABLE I

Comparison of three different methods to liberate creatine kinase activity from polyanion treated mitochondria

Brain mitochondria were incubated with increasing concentrations of the polyanion as described in Methods. The mitochondria were subsequently sedimented, resuspended in the same volume of sucrose medium and subjected to determination of enzyme activity. Aliquots of polyanion-treated mitochondria were incubated with 700 µg/mg digitonin or 1% Triton X-100 or were sonicated four times for 30 s. Double determinations of creatine kinase activity were made in the mitochondrial suspensions immediately after treatment.

Polyanion	Creative kinase activity liberated							
	untreated		digitonin		triton		sonifier	
(µg/mg)	mU/mg	%	mU/mg	%	mU/mg	%	mU/mg	%
0	145	47	312	101	295	96	307	100
5	125	40	290	94	308	100	296	96
20	65	21	279	90	324	105	280	91
50	75	24	263	85	314	102	272	88

conductance state [11,21], we concluded from these experiments that this pore state excludes transport of adenine nucleotides across the outer membrane. In addition, these experiments showed that no transport system other than the pore can contribute significantly to the adenine nucleotide exchange. This conclusion is valid because of several reasons: firstly, the free kinases were not effected by the addition of the applied polyanion concentrations, provided the Mg^{2+} concentration was 10 mM. Secondly, digitonin or Triton did not specifically interact with the polyanion. This was proved by an experiment summarized in Table I, where it did not matter how the barrier of the outer membrane was disrupted either by digitonin treatment, solubilization by Triton X-100, or sonification in all cases we regained at least 86% of the kinase activity.

An interesting side-effect of the polyanion was that upon preincubation with increasing concentrations of this compound increasingly more hexokinase I became bound to the mitochondria (Fig. 2A). This observation may also point to the structural change of the pore protein caused by the polyanion which would not only apply to changes in conductivity but also to increased binding of hexokinase.

Regulation of the outer membrane pore by the membrane potential.

Porins from eucaryotic cells are voltage-dependent [28]. When polyanion is added to one side (the cis-side) of the membrane this voltage dependence is shifted to smaller voltages provided the sign of the voltage is negative at the side of the addition of the polyanion. The effect of the polyanion on the voltage-dependence was studied in more detail. Increasing concentrations of the polyanion were added to one side of the membrane containing reconstituted rat liver porin and the voltage-dependence of the channels was determined as a function of the polyanion concentration. Table II shows that increasing polyanion concentrations on one side led to a dramatic decrease of V_0 (the potential where 50% of the channels are in the closed state [28]) while the number of gating charges n moving through the entire electric field remained constant. It has to be noted that these effects were only observed when the applied membrane potential was negative at the cis-side. For opposite polarity (positive at the cis-side) we found no influence of the applied membrane potential on the channel, i.e., the channel was no longer voltage-dependent up to 100 mV, starting with a polyanion concentration of 0.1 μg/ml. The asymmetric effect of the polyanion on rat liver porin was stable during the whole lifetime of the membranes (up to several hours) indicating that the polyanion could not penetrate the channel. The addition of the polyanion to both sides of the membrane resulted in a bell shaped voltage-dependence with the same values for V_0 and n given in Table II for the asymmetrical case.

TABLE II

Influence of increasing concentrations (C_p) of the polyanion on the voltage-dependence of rat liver porin

The polyanion was added only to one side of the membrane. The aqueous phase contained 1 M KCl, pH 6 and 20 ng/ml rat liver porin. V_0 is the voltage where 50% of the channels were in the closed state. n is the number of gating charges that are assumed to move through the entire transmembrane potential gradient for channel gating. V_0 and n are given as the mean (± S.D.) of at least three individual experiments. Note that the pores only closed when the cis-side was negative. For opposite polarity (positive at the cis-side) the pores were always open in the presence of the polyanion up to voltages of at least 100 mV.

C_p (μg/ml)	V_0 (mV)	n
0	55 ±5	2.3±0.2
0.01	32 ±3	1.9±0.3
0.03	10 ±2	2.1±0.2
0.1	6.5±0.8	2.0±0.2
0.3	5.0±0.7	2.2±0.3

Effects of the polyanion on single-channel conductance of the mitochondrial porin

The experiments described above can be explained by assuming that the pore is switched into the closed or low conductance state as a consequence of the action of the polyanion at a much smaller trans-membrane potential. So far it was not known if the closed state of rat liver porin had a different ion selectivity than the open state. To study this in detail we performed single channel experiments in the absence and in the presence of the polyanion. Fig. 3 shows the influence of the polyanion on the conductance steps observed with rat liver porin in 1 M KCl. In Fig. 3A polyanion was added in a concentration of 0.1 μg/ml to the side of the positive polarity only and the conductance did not change compared with the pores in the absence of polyanion in the control experiments. However, when the same con-

TABLE III

Average single-channel conductance of the open and the polyanion-induced closed state of rat liver porin in different 0.5 M salt solutions

The pH of the aqueous salt solutions was adjusted to 7.2. The membrane voltage was 10 mV; $T = 25°C$. To induce the closed state, the aqueous phase contained 0.1 μg/ml polyanion. The data correspond to the mean of at least 100 single events.

Salt	Single-channel conductance (nS)	
	open state	closed state
KCl	2.2	1.2
LiCl	1.8	0.40
KCH_3COO	1.1	0.85
K-Mes	0.89	0.74
Tris-Cl	1.5	0.25

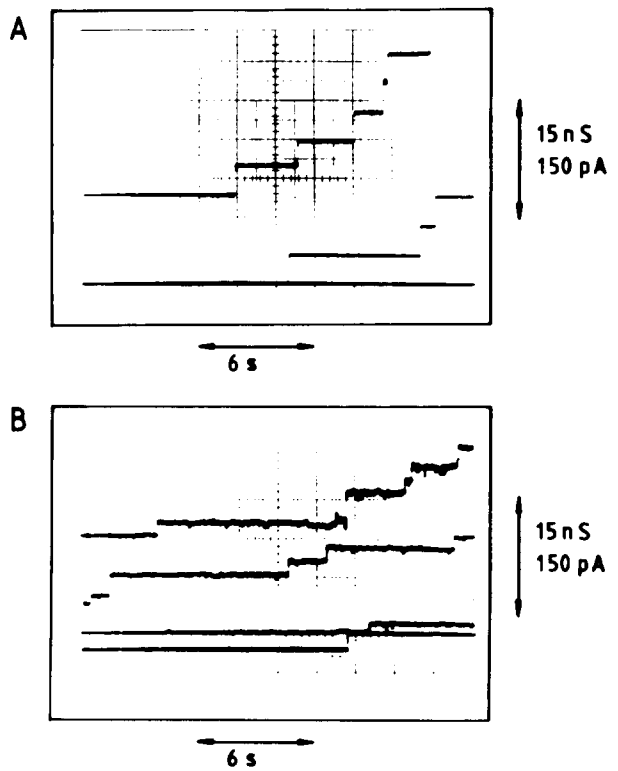

Fig. 3. Effect of polyanion on single-channel conductance of rat liver porin. 10 mV were applied to membranes formed of diphytanoylphosphatidylcholine/*n*-decane in 1 M KCl solution containing 2 ng/ml rat liver porin. (A) the aqueous phase contained 0.1 μg/ml polyanion on the side of positive polarity. (B) The aqueous phase contained the same concentration of polyanion on the side with negative polarity of another membrane; $T = 25\,^{\circ}C$.

centration of the polyanion was added to the opposite side (with negative polarity, Fig. 3B) the single channel conductance was about half of that observed in Fig. 3A and in the absence of polyanion.

Similar measurements were performed with a variety of salts. The results are summarized in Table III. The reduction of the single-channel conductance caused by the polyanion is especially large when a mobile anion (for example chloride) was combined with a less mobile cation (for example Tris). In this case the single-channel conductance was reduced to less than 10% of the open state value. The effect of the polyanion on a combination of a mobile cation with a less mobile anion (for instance K-Mes) was very small. These results would be consistent with the assumption that the slightly anion selective channel in the open state becomes cation selective in the closed state.

Influence of Polyanion on the ion selectivity of mitochondrial porin

After reconstitution of rat liver porin into diphytanoylphosphatidylcholine membranes in the presence of 50 mM KCl (pH 6.0) the concentration of KCl on one side of the membrane was increased by a factor

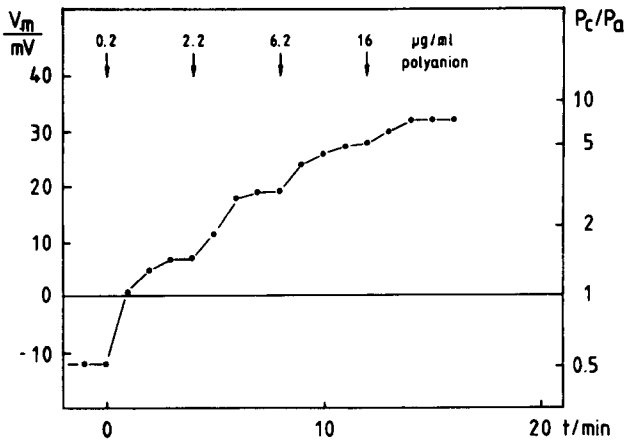

Fig. 4. Influence of the polyanion on the ion selectivity of rat liver porin. A membrane was formed of diphytanoylphosphatidylcholine/*n*-decane in a solution containing 50 mM KCl and 20 ng/ml rat liver porin. After reconstitution of about 300 pores the KCl concentration on one side of the membrane was increased to 320 mM and the instrumentation was switched to the measurement of zero-current potentials. The 6.4-fold increase of the KCl gradient resulted initially in a negative potential on the more dilute side. Increasing the concentration of the polyanion (as indicated at the top of the the figure) shifted the potential to positive values. Simultaneously, the permeability ratio (P_c/P_a) calculated from the Goldman-Hodgkin-Katz equation changed to favour the cation.

of 6.4. The zero-current membrane potential (V_m) caused by the balancing of the concentration gradient across the membrane according to the specific properties of rat liver porin was measured. This potential was negative by 13 mV on the more dilute side of the membrane, which means that the channel was anion selective (i.e., anion moved preferentially through the pore) [1,2]. Then, we added increasing concentrations of the polyanion to both sides of the membrane and studied its influence on the zero-current membrane potential, and thereby on

TABLE IV

Zero-current membrane potentials (V_m) of rat liver porin containing membranes as a function of the polyanion concentration (C_p)

The membranes were made from diphytanoylphosphatidylcholine/*n*-decane in the presence of 20 ng/ml rat liver porin. The membrane potential was measured for a 6.4-fold KCl gradient as a function of the polyanion concentration in the aqueous phase; $T = 25°C$. V_m defined as the difference between the potential on the dilute side (50 mM) the potential at the concentrated side (320 mM). The pH of the aqueous salt solutions was approximately 6. P_c/P_a was calculated from the Goldman-Hodgkin-Katz equation [29]. The results are given as the mean (± S.D.) of at least three individual experiments.

C_p (μg/ml)	V_m (mV)	P_c/P_a
0	−13.0±1.5	0.48±0.06
0.03	3.4±0.5	1.2 ±0.03
0.3	8.7±1.1	1.6 ±0.15
3.0	21.0±2.8	3.3 ±0.50
10.0	29.0±3.1	5.8 ±1.4
20.0	36.0±3.7	11.0 ±3.1

the ratio of the permeabilities P_c/P_a of the pore (c for cation, a for anion) as calculated from the Goldman-Hodgkin-Katz equation [29]. Fig. 4 illustrates the influence of increasing concentrations of polyanion on the zero-current membrane potential in such an experiment: the membrane was first anion selective (permeability ratio $P_c/P_a = 0.52$) but switched then to cation selectivity in a dose-dependent fashion on the polyanion concentration. Table IV summarizes the effects of the polyanion on the ion selectivity of rat liver porin. It is evident that the presence of increasing concentrations of the polyanion shifts the selectivity of the mitochondrial pore from slightly anion selective to a much stronger cation selectivity with a ratio of P_c/P_a larger than 10 at high polyanion concentrations.

Discussion

Properties of the low conductance pores in planar bilayers.

The polyanion interacts with rat liver porin reconstituted into lipid bilayer membranes. The most obvious effect is the shift of the voltage-dependence of the channel. The closed or low conductance state is obtained at very low voltages in the presence of the polyanion [21,11]. Asymmetric addition of the polyanion changes channel gating only when the polarity of the trans-membrane voltage is negative on the cis-side, the side of the addition of the polyanion. For opposite polarity (positive at the cis-side) single channel conductance and selectivity of the pore cannot be distinguished from those in the absence of the polyanion. The voltage-dependence is changed under the latter conditions (positive at the cis-side) in such a way that the channel remains always in the open state for voltages up to at least 100 mV. This means that the polyanion is probably not directly bound to the channel interior but interacts with positively charged groups located in some distance away from the mouth of the channel. In fact, electron microscopic analysis of two-dimensional crystals of mitochondrial porin of *Neurospora crassa* (formed by phospholipase A_2 treatment of mitochondrial outer membranes) have shown that the polyanion binds at the protein/lipid boundary along the channel exterior (Mannella, C.A., personal communication).

The polyanion-induced shift of the channel gating allowed the study of the pore properties in the low conductance state in intact organelles and in vitro. The single-channel data obtained from the reconstitution experiments suggested that the closed state could be cation selective. This was deduced from the observation that the polyanion had little effect on the single channel conductance of a salt composed of a mobile cation and a less mobile anion (K-Mes) while the polyanion caused a dramatic reduction of the conductance when the salt was of the opposite combination (mobile anion and less mobile cation, i.e. for Tris-Cl). In this case the single-channel conductance was reduced at least by a factor of 5. This result was confirmed by studying the selectivity of the channel in the presence of increasing concentrations of the polyanion. In fact, the ratio of the permeabilities P_c/P_a for a polyanion concentration of 20 μg/ml was larger than 10 (Table IV) which means that the anion used in our experimental conditions had a very small permeability through the closed channel. ATP and ADP with a much larger molecular mass and with a larger charge density would become most probably excluded from the channel in the closed state. Thus, the in vitro data serve to explain the polyanion-mediated inhibition of kinases in the intermembrane space of intact mitochondria (Figs. 1 and 2).

Effects of the low conductance pores in intact mitochondria

As described recently [11] the transport of ATP and ADP through the low conductance pores was inhibited presumably in both directions (Figs. 1, 2). This was deduced from the fact that the activity with external ATP of the surface bound hexokinase was not inhibited by the polyanion while the activity of the intermembranous kinases (creatine kinase and adenylate kinase) behind the outer membrane barrier was completely suppressed. Thus, the effect of the polyanion on the activity appeared to depend on the position of the various kinases in relation to the pore protein and whether or not the substrates and products of the kinase had to permeate the pore. The polyanion which was used in our experiments was not able to pass the pore because of the average M_r of 10000 according to König [30]. This was demonstrated by the fact that the asymmetric polyanion effect persisted for hours: the conductance of the pore remained unchanged when the side of the bilayer where the polyanion was added was positive, while the closed state of the pore was observed with the opposite polarity.

How the inner membrane potential may regulate the outer membrane pore

Considering that the effects of the polyanion on the pore reflect the physiologically occurring voltage-sensitivity, we arrived at the question whether a membrane potential exists at the outer membrane and of which polarity it may be. Taking into account the high ion conductivity of the pore, the existence of an electrochemical potential across the outer membrane can't be expected. However, in the contact sites it appears possible that the potential at the inner membrane influences the outer membrane, because, as analyzed by freeze fracture, a distance between the two membranes of 1–2 nm can be assumed [31]. We can therefore think of a capacitative coupling between the two membranes similar to that between two condensators. The transfer of positive charges through the inner membrane would

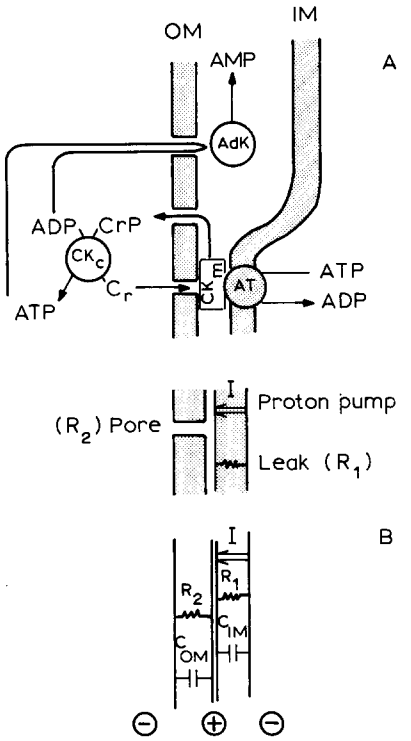

Fig. 5. Scheme showing the difference in transport properties of the pore inside and beyond the contact sites as regulated by the inner membrane potential. Panel A summarizes how the difference in ion selectivity of the pores creates the asymmetry of the phosphocreatine shuttle. Abbreviations are: AT, adenylate translocator; IM, inner membrane; Om, outer membrane; Cr, creatine; CrP, phosphocreatine, AdK adenylate kinase; CK_m, creatine kinase; CK_c, cytosolic isozyme. Panel B: equivalent circuit showing how the pore inside the contact sites is influenced by the charge transfer (I) across the inner membrane. We assume a capacitative coupling between the two membranes like between two condensators (C_{OM} and C_{IM}). This results in a polarity as depicted in the scheme and changes the pore structure to the low conductance, cationically selective state, while the pores beyond the contacts have a high conductance and are anionically selective.

than result in a field across the outer membrane with a polarity outside negative as proposed in Fig. 5B.

In view of these facts it appeared possible that two states of the pores might be co-existent in the outer membrane: those which would be influenced inside the contacts by the inner membrane potential and consequently would be of low conductance, and other pores beyond the contacts which would be unregulated and therefore of high conductance.

Topology of peripheral mitochondrial kinases

Recent ultrastructural [32] and biochemical [9,10,33] localization studies agreed in that the mitochondrial creatine kinase was located inside the contact zones while adenylate kinase resided outside these zones. The meaning of this organization is understood if one considers the possibility discussed above, that two states of the pore protein may be co-existent in the outer membrane. Thus, adenylate kinase beyond the contacts may readily equilibrate with the extramitochondrial compartment through anionically selective pores, whereas, the metabolite exchange of creatine kinase inside the contacts may be restricted because of cation-selective pores in these sites. Such regulatory implications of the specific kinase topology at the mitochondrial surface have been recently postulated [14,33] and are schematically presented in Fig. 5A. It was assumed that exclusively creatine passes the cation-selective pores, while phosphocreatine leaves the outer compartment through the anion selective pores.

In agreement with this idea, we observed that the enzyme reaction (from creatine and ATP) was less effectively inhibited by external phosphocreatine as long as the outer membrane or at least the contact sites were intact [14].

Furthermore, Gellerich et al. [34] reported that extramitochondrial pyruvate kinase could not compete with mitochondrial creatine kinase for the ADP in the outer mitochondrial compartment, and recent kinetic work of Brooks and Suelter [35] suggested the importance of the outer membrane for the regulation of the mitochondrial creatine kinase.

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