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Transport properties and inhibitor sensitivity of isolated and reconstituted porin differ from those of intact mitochondria

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The pore-forming protein porin has been isolated from rat heart mitochondria and reconstituted in phospholipid vesicles of different composition. Rapid release of anions, cations and non-charged molecules has been demonstrated from the proteoliposomes but not from the protein-free liposomes. In spite of its higher molecular mass and charges, the movement of ATP was almost as fast as that of inorganic phosphate. Polyanion (1:2:3 copolymer of methacrylate/maleate/styrene), a potent inhibitor of porin residing in the mitochondrial contact sites decreased the solute movements but did not completely block any of the investigated transport processes (phosphate, chloride, ATP). Alterations of the lipid environment had significant effect: an increase in the proportion of soybean phospholipids to egg yolk phospholipids resulted in a decrease in the amount of transported substance but did not fully inhibit the ion movements. It is concluded that the transport properties of porin reconstituted in artificial phospholipid membranes are different from the characteristics of porin prevailing in the mitochondrial contact sites and additional regulatory factors are suggested to be effective in the intact mitochondria.

Introduction

Mitochondrial porin (also referred to as voltage-dependent anion channel (VDAC)) is a 32 kDa protein that forms wide channels ($r = 2$ nm) of high conductivity and low ionic selectivity [1,2]. In intact mitochondria it is situated in the outer membrane and it was shown to be a typical constituent of the 'contact points' [3–5]. These specialized sites where the inner and outer membranes seem to fuse are also highly enriched in different kinases [5–9]. Hexokinase – bound to porin – faces the extramitochondrial space whereas creatine kinase and adenylate kinase are located between the two membranes [5,8]. The kinetic properties of these enzymes suggest restricted transport of their substrates and products through the outer membrane. Specifically, hexokinase was shown to utilize preferentially intramitochondrial ATP [10,11] and the rate of creatinephosphate production (in the intermembrane space from extramitochondrial creatine and intramitochondrial ATP) proved to be independent from the cytoplasmic creatine phosphate concentration [12]. The apparently strict organization of these transport processes is not compatible with the high conductance of isolated porin and raised the idea about possible regulatory mechanisms.

Porin reconstituted into planar membranes proved to be voltage-dependent: application of a voltage difference of 20 to 30 mV (either positive or negative) decreased the conductance by 50% [13–15]. These findings provided the experimental basis for the hypothesis about 'capacitive coupling' in the contact sites where the high potential difference across the inner membrane could decrease conductance through porin residing in the outer membrane. The porin pores were reported to be also 'closed' by a polyanion (1:2:3 copolymer of methacrylate/maleate/styrene) that in intact mitochondria inhibited the transport of various small molecular weight compounds (ADP, phosphate, succinate, etc.) [16] and almost completely blocked the activity of both adenylate kinase and creatine kinase [17,18]. The effect of the polyanion could be demonstrated also in planar lipid membranes: the voltage-sensitivity of porin was increased and the 'closed' state could be achieved at a significantly lower potential difference [17,19]. Although in this 'closed' state electric conductance was decreased by a factor of 2, the diameter of the channel was estimated to be reduced only to 0.9 nm [19] and the calculated residual solute flux was still higher than the transport rate of any

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mitochondrial carrier [2]. It is thus improbable that the 'closed' state achieved under these conditions would block the movement of the adenine nucleotides as completely as it was observed in intact mitochondria in the presence of the polyanion [17,18].

A clear discrepancy appears in the behaviour of porin residing in the mitochondrial contact sites or inserted into artificial phospholipid membranes. This fact prompted us to study the transport properties of isolated porin in reconstituted proteoliposomes where the movements of various solutes can be followed directly. The inhibitory action of the polyanion and the modulatory effect of the lipid environment have been investigated. Polyanion was not able to inhibit completely the movements of either phosphate or ATP in porin-containing lipid vesicles and under the least conductive conditions ion flux was estimated to exceed 100 000 ions per minute per porin molecule.

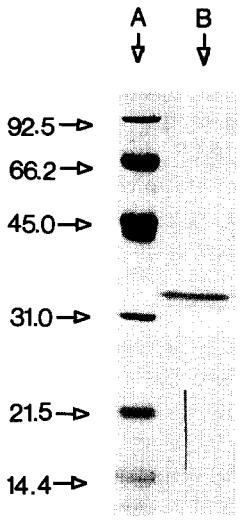

Fig. 1. Control of purity of isolated porin. SDS-PAGE with silver staining. Lane A: molecular weight standards (the corresponding molecular weights are indicated on the left); lane B: eluate from the hydroxylapatite-celite column.

Materials and Methods

Materials

Hydroxylapatite was obtained from Bio-Rad, celite from BDH, Serdolit AS-6 (0.3–0.8 mm) anion exchanger and Serdolit GS-2 (0.3–0.8 mm) cation exchanger from Serva, radioactive substances from Izinta (Hungary) and soybean type II-2 phospholipids from Sigma. Egg-yolk phospholipid has been prepared according to Refs. 20 and 21. Polyanion (1:2:3 copolymer of methacrylate/maleate/styrene) was a generous gift of Dr. Tamás König. All other reagents were of analytical grade.

Isolation of porin

Porin was isolated from rat heart mitochondria according to Ref. 22. Mitochondria (25 mg protein) were lysed by osmotic shock in 5 ml buffer A (10 mM Tris-HCl, 1 mM EDTA, pH = 7.0) at 4°C for 5 min. Membrane fragments were separated by centrifugation (5 min, 20 000 × *g*) and the sediment was solubilized at 4°C for 30 min in 4 ml of buffer A supplemented with 3% Triton X-100. After sedimentation of the insoluble material (45 min, 37 000 × *g*), 0.6 ml of the supernatant was placed on a column containing 0.6 g of the 2:1 mixture of dry hydroxylapatite and celite powder. The protein was eluted from the column by the same buffer that was used for solubilization. The first fraction (0.6 ml) was employed for reconstitution. The purity of the porin preparation was controlled by SDS-PAGE and silver staining (Fig. 1).

Preparation of proteoliposomes preloaded with labelled substances

The sonication-freeze-thaw method was used. 500 mg of the respective lipid or lipid mixture was dispersed in 10 ml buffer B (10 mM K-phosphate, 50 mM KCl, 20 mM Hepes, 1 mM EDTA, pH = 7.0) and after vigorous vortexing the suspension was sonicated (Branson sonifier 250, 1 min 40% power + 2 × 2 min 20% power, the tube kept in icebath). The radiolabelled compound was then added to the sonicated liposome suspension at a final concentration of approx. 500 000 cpm per ml and the total volume was divided in two parts: 2 ml served as control (protein-free liposomes) whereas the remaining 8 ml was supplemented with 400 μl isolated porin (eluate from the HTP-celite column). Following this, both portions of the liposome suspension were treated identically: immersed in liquid nitrogen for 2 min followed by thawing at room temperature (approx. 30 min). In order to obtain effective reconstitution and to reach large intraliposomal volume the freeze-thaw cycle was repeated two more times [23]. In accordance with previous reports [24,25] this method allowed the preparation of unusually large liposomes with high entrapped volumes. In our hands, the internal volume of liposomes prepared from 100% egg-yolk phospholipid varied – depending on the applied batch and on the age of the preparation – between 7 and 18% whereas liposomes from 100% soybean phospholipid exhibited a significantly larger (30–35%) internal volume. These data are in good agreement with earlier findings [23,25,26]. In our experiments, the mixture of 80% egg-yolk plus 20% soybean phospholipid resulted in the largest liposomes (50–55% internal volume) but this value is still lower than the highest volumes obtained with various other lipid mixtures [27,28].

During the freeze-thaw cycles liposomes open and reclose allowing thereby insertion of the protein and equilibration of extra- and intraliposomal solute con-

centrations (including the radioactive material). Before starting the transport assay, the liposome suspension was kept at room temperature for at least 20 min to attain appropriate sealing of the liposomes [23]. When the transport of ATP or sucrose was investigated, 2 mM, respectively, 10 mM unlabelled substance was also added to the sonicated liposome suspension before the freeze-thaw cycles.

Transport assay

Solute transport through the liposomal membrane was assessed on the basis of release of radiolabelled substances from the preloaded liposomes, similarly to the approach of Roos et al. [14]. Separation of the extra- and intraliposomal radioactivity proceeded in most experiments by ion-exchange chromatography whereas in one experiment, when a non-charged compound has also been investigated, dialysis was carried out. This procedure had to be applied as the extremely high transport rate and the lack of suitable inhibitor precluded any traditional transport measurement under equilibrium exchange conditions.

In the typical experiments, where transport of phosphate was followed, an aliquot (200 μl) of the preloaded liposome or proteoliposome suspension was layered on the top of the anion exchange column (the bed volume varied between 1.9 and 5 ml) followed by washing of the column by 10 ml of 160 mM sucrose. 10 ml of the effluent (containing the opalescent fraction) was collected in scintillation vials and the radioactivity was counted in a Beckman LS-200 liquid scintillation spectrometer on the basis of the Cerenkov effect. Radioactivity detected in the effluent liposomes was expressed as the percent of the total activity applied on the column.

In case of protein-free liposomes only ions of extraliposomal localization are bound by the ion exchange column, thus the effluent radioactivity represents the intraliposomal amount of the investigated substance (later on called 'entrapped activity'). (In case of constant solute concentration, variations of the entrapped activity are proportional and due to alterations of the intraliposomal volume.) Liposomes containing active porin molecules allow the release of the preloaded ions resulting in a decrease of the radioactivity eluting with the liposomes. The difference in the effluent radioactivity of non-reconstituted and reconstituted liposomes corresponds to the amount of intraliposomal content released during the time of passage through the ion exchange column (typically less than 10 s). Any inhibition of the transport results in an increase of the radioactivity of the effluent proteoliposomes.

In the experiment summarized on Fig. 4, 4.5 ml of the preloaded protein-free or porin-containing liposomes was dialysed three times against 1 liter of buffer

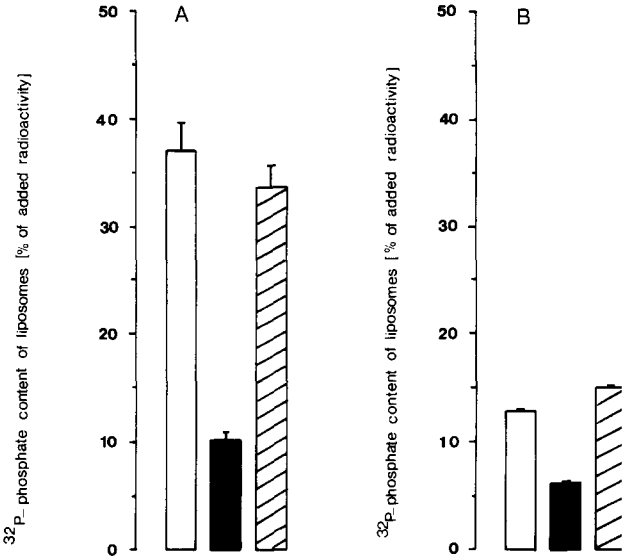

Fig. 2. Permeabilizing effect of porin in reconstituted liposomes Reconstitution was carried out with native (black columns) or heat denaturated (hatched columns) HTP-celite eluate. Non-reconstituted liposomes served as control (white columns). Release of [^{32}P]phosphate from preloaded liposomes was followed and the radioactivity retained after passage through the anion exchange column is expressed as the percentage of the total radioactivity applied to the column. Liposomes were prepared: in (A) from a mixture of 80% egg-yolk + 20% soybean phospholipids and in (B) from 100% egg-yolk phospholipids. The columns show the average of five parallel measurements (± S.E.) carried out on the same liposome batch. The figure demonstrates the results of one representative experiment out of 15 similar ones.

B. Radioactivity remaining after the last, overnight dialysis in the liposomal fraction was counted (200 μ aliquot in 10 ml scintillation cocktail) and expressed as the percentage of the total (extra- plus intraliposomal radioactivity. Further calculations were carried out as detailed for the effluents of the ion exchange columns

Miscellanous

SDS-PAGE was performed as described by Laemmli [29] and silver staining was carried out according to [30]. Protein was determined by the method of Lowry et al. [31]. Cytochrome *c* has been measured at 550 nm wavelength in a Perkin-Elmer Lambda-3 spectrophotometer.

Results

Permeability properties of porin-containing proteoliposomes

As transport of different solutes has hitherto not been measured directly in proteoliposomes containing porin, first we had to test some basic properties of the isolated porin reconstituted into phospholipid vesicles. Our approach consisted of preloading the liposomes with radioactively labelled substances and following the release of radioactivity while liposomes passed through an ion-exchange column. Fig. 2 shows the results of a typical experiment where the transport of ^{32}P-labelled

phosphate was investigated in liposomes of different lipid composition. Although the basic phenomena are identical, the numerical values showed big differences. The entrapped activity (see Methods) of liposomes prepared from pure egg-yolk phospholipid was 13% (Fig. 2B) whereas that of liposomes consisting of a mixture of egg-yolk and soybean phospholipid amounted to 37% (Fig. 2A), indicating a significantly larger intraliposomal volume of the latter vesicles. In both types of liposomes reconstitution with isolated porin resulted in a drastic decrease of the radioactivity in the effluent suspension (to 5%, respectively, 10% of the total activity). The anion-exchanger was chosen to allow rapid flow of the liposomes. In this way the time of passage through the column could be kept around 10 s. As an increase of the passage time had no influence on the residual radioactivity in the effluent proteoliposomes, we suggest that the entire releasable phosphate-pool was emptied within 10 s. The residual radioactivity probably represented the phosphate-content of liposomes containing no transport-active porin molecules. The data of Fig. 2 show clearly that the amount of transported phosphate varies in parallel to the initial entrapped activity: it was about 8% (13% minus 5%) of the total added activity in proteoliposomes from pure egg-yolk phospholipid (Fig. 2B) but about 27% (37% minus 10%) in proteoliposomes prepared from a mixture of egg-yolk and soybean phospholipid (Fig. 2A).

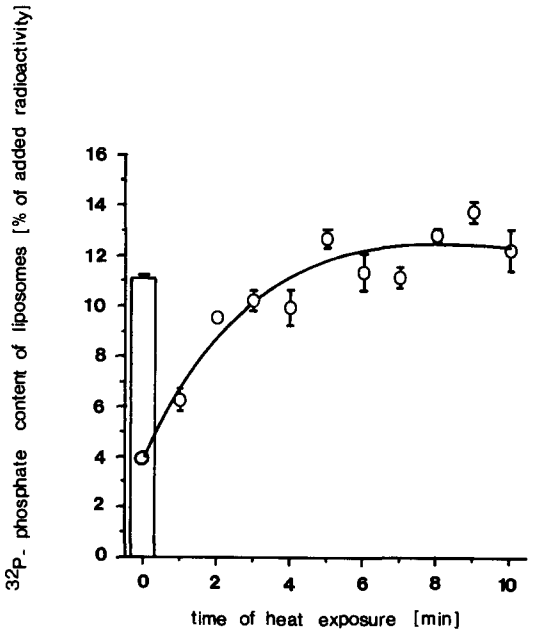

Fig. 3. Effect of heat treatment on the permeability of porin-containing proteoliposomes. Proteoliposomes were prepared from 100% egg-yolk phospholipids and porin as described in Materials and Methods followed by immersion into 100°C water-bath for the indicated period. After cooling down the samples to room temperature, release of preloaded [^{32}P]phosphate was followed and the radioactivity remaining in the liposomes after the anion exchange chromatography is shown by circles. The column represents the entrapped activity of protein-free liposomes (for further details see Methods). The results of five parallel measurements ±S.E. are shown in the case of one typical experiment out of three similar ones.

Two kinds of control measurements were carried out in order to prove that the decrease of radioactivity observed in the effluent reconstituted proteoliposomes can be attributed to movement through active porin molecules. One approach consisted of reconstitution of liposomes with previously heat-denatured porin. As shown in the hatched columns of Fig. 2, phosphate content of these proteoliposomes was very near to that of non-reconstituted liposomes. In the second procedure liposomes were reconstituted with active porin and the transport-active proteoliposomes were exposed in Eppendorf tubes to 100°C for precisely controlled time. As shown in Fig. 3, radioactivity detected in the effluent proteoliposomes increased gradually until (after approx. 5 min boiling) the original value of non-reconstituted liposomes was attained. The radioactivity of protein-free liposomes was not changed by the heat-treatment.

In order to provide a negative control, cytochrome *c*, a protein of 13 kDa molecular mass has been enclosed into porin-containing or control liposomes following the same procedure as described for [^{32}P]phosphate. After ample washing of the different vesicles the cytochrome *c* content has been compared. In two experiments, 51 and 51.8% of the total amount of added cytochrome *c* was retained in reconstituted proteoliposomes whereas the control liposomes contained 53 and 55.3% of the entrapped protein. Under similar conditions the [^{32}P]phosphate or [^{32}P]ATP content of porin-containing liposomes decreased from 52–55% to 7–11% (see Fig. 6).

For testing the selectivity of reconstituted porin we needed a method of separation of extra-and intraliposomal radioactivity that could be equally applied for negatively or positively charged and non-charged molecules. After several preliminary experiments, overnight dialysis of pure egg-yolk proteoliposomes proved to be the most suitable method. Fig. 4B shows the results obtained when movements of labelled phosphate, chloride, rubidium and sucrose were followed. In order to verify that the radioactivity detected in the control liposomes after the overnight dialysis has been indeed retained in the vesicles and can be mobilized under appropriate conditions, we repeated the experiment with $^{86}Rb^+$ in the presence and absence of valinomycin (Fig. 4C). Fig. 4A summarizes the data obtained with the same type of liposomes on which the previously detailed anion exchange column separation was applied. The amount of the released radioactivity proved to be rather similar irrespectively of the species of solute studied and the method of separation used.

This finding is compatible with previous suggestions of poorly selective, rapid transport of different solutes through porin in intact mitochondria.

Effect of polyanion on ion movements in reconstituted proteoliposomes

When porin was reconstituted in liposomes of pure egg-yolk phospholipid, no effect of the polyanion could be demonstrated at all. As shown in Fig. 2, liposomes prepared of a mixture of egg-yolk and soybean phospholipid (80:20) have a larger entrapped volume and are expected to be a more sensitive indicator for partial inhibitory effects. This presumption proved to be correct and in the presence of 50 μg/ml polyanion the radioactive phosphate content of the effluent proteoliposomes was significantly higher than in the absence of the polyanion (16% versus 11%) pointing to a decrease of the amount of phosphate released during the passage through the anion exchange column (Fig. 5A). Further increase of the polyanion concentration had no additional effect. On the other hand, the effect of polyanion disappeared completely, when the time of passage through the ion exchange column was prolonged by increasing the length of the column (data not shown). This finding is in good accordance with the results obtained in planar membranes and in liposomes by an indirect technique: in both preparations polyanion induced a narrowing of the porin channel but did not fully block the conductance. Part B of Fig. 5 demonstrates the effect of the polyanion in a narrow concentration range: above 20 μg/ml the inhibition of transport became maximal. In six different proteoliposome preparations, measuring either phosphate or chloride transport, the half-maximal effect was achieved between 3 and 7 μg polyanion per ml. This value is in good agreement with earlier results obtained on proteoliposomes by an indirect – shrinking/reswelling – technique [19].

The effect of the polyanion on isolated reconstituted porin consisted of a slowing down of the transport of small molecular weight anions whereas in intact mitochondria the complete blocking of the adenylate kinase and creatine kinase reaction (in a Triton-sensitive manner) suggested full inhibition of the transport of the substrates and/or products of these enzyme reactions. This discrepancy has been resolved by the suggestion that highly charged larger molecules (like ATP^{4-}) behaved basically differently from the previously studied small anions. With this possibility in mind we investigated the transport of ATP in reconstituted porin vesicles.

Fig. 6A compares the kinetics of the outflow of [^{32}P]ATP and [^{32}P]phosphate from porin-containing vesicles. When measured on the same liposome batch, a modest but clear difference could be detected in the transport rate of the two substances at the early time-points. Nevertheless 75% of the total releasable pool of [^{32}P]ATP has left the liposomes within the first 30 s

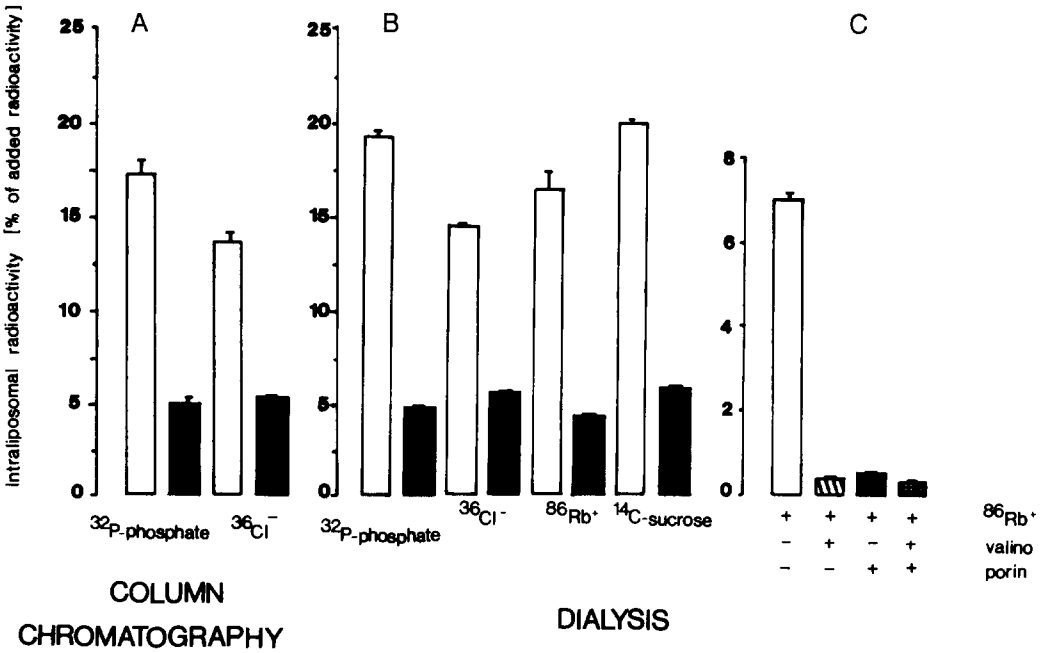

Fig. 4. Demonstration of the non-selective nature of the permeability induced by porin in liposomes. Proteoliposomes (black columns) and protein-free liposomes (white columns) were prepared from 100% egg-yolk phospholipids and preloaded with the indicated radioactive substance as described in Materials and Methods. Separation of extra-and intraliposomal radioactivity was carried out by anion exchange chromatography (A) or overnight dialysis (B, C). Radioactivity remaining in the liposomes after separation is shown as the percentage of the total applied activity. Where indicated, 5 μg/ml valinomycin has been added to the liposome or proteoliposome suspension before starting the dialysis. The average of five parallel measurements ±S.E. is demonstrated. The experiment shown in panel C has been carried out with a different batch of egg-yolk phospholipid than the measurements summarized in panels A and B.

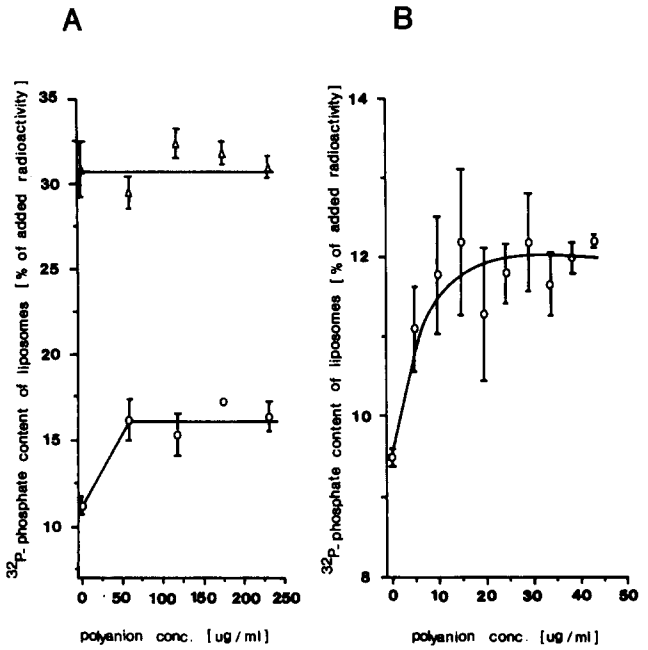

Fig. 5. Effect of polyanion on [^{32}P]phosphate release from porin-reconstituted proteoliposomes. Liposomes were prepared from 80% egg-yolk and 20% soybean phospholipids. Polyanion was added to the reconstituted, preloaded proteoliposomes (○) or to the protein-free preloaded liposomes (△) in the indicated concentrations 10 min before beginning of the transport assay. Part A and B show the same effect in two different concentration range. The average ±S.E. of four parallel measurements carried out on the same liposome batch is shown.

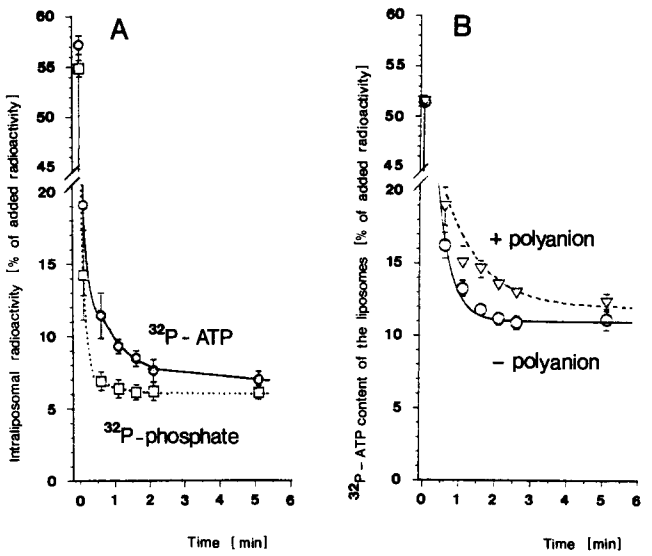

Fig. 6. Release of [^{32}P]ATP and [^{32}P]phosphate from porin-containing proteoliposomes (A) and effect of polyanion on [^{32}P]ATP movement (B). Liposomes were prepared from 80% egg-yolk and 20% soybean phospholipids, then reconstituted and preloaded as described in Materials and Methods. Radioactivity remaining in the liposomes after anion exchange chromatography is expressed as the percentage of the total applied activity. The time of passage was varied by stopping the flow of the column. (A) Comparison of the release of [^{32}P]phosphate (□) and [^{32}P]ATP (○) from proteoliposomes. (B) Release of [^{32}P]ATP was followed in the presence of 150 μg/ml polianion (△) or in its absence (○). The average ±S.E. of three parallel measurements is shown.

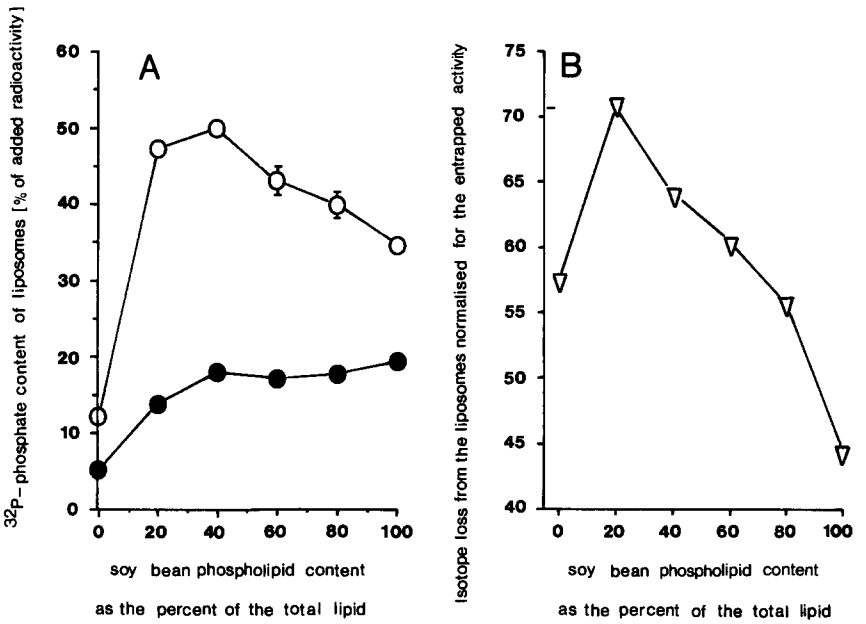

Fig. 7. Effect of the lipid composition on the permeabilizing property of porin. Liposomes of differring composition were prepared by adding soybean phospholipids in the indicated proportion to egg yolk phospholipids. Total lipid concentration was kept in each mixture at 50 mg/ml. Reconstitution and preloading of the proteoliposomes proceeded as described in the Methods. (A) Radioactivity remaining in the protein-free liposomes (entrapped activity, ○) and in the reconstituted proteoliposomes (●) after anion exchange chromatography. The average ±S.E. of five parallel measurements carried out on the same liposome batch is demonstrated. (B) Release of [^{32}P]phosphate during anion exchange chromatography. The represented data were calculated from the values of part A in the following way: entrapped activity (○) minus the remaining activity (●) divided by the respective entrapped activity.

(the same value for [^{32}P]phosphate is 83%) and the ion movement ceased after about 2 min. The influence of a maximally effective polyanion concentration (150 μg/ml) on the time-course of [^{32}P]ATP movement is demonstrated in Fig. 6B. The presence of polyanion visibly slowed down but did not totally prevent ATP movements. At the early timepoints the difference in the labelled nucleotide content of proteoliposomes in the presence and absence of polyanion was 3% and corresponded to the value obtained when the release of phosphate was measured (Fig. 5). Thus, in phospholipid vesicles reconstituted with porin the movements of inorganic phosphate and ATP showed the same basic characteristics and both were only partially inhibited by the maximally effective concentration of the polyanion. Consequently, the virtual ATP-impermeability of the outer membrane reported in intact mitochondria under certain conditions, must depend on additional factors.

Effect of the lipid composition on the transport properties of porin-containing proteoliposomes

The differences of the entrapped volume and consequently the total releasable solute pool of proteoliposomes of altering lipid composition showed in Fig. 2 prompted us to carry out a more detailed study of the effect of the lipid environment. In the experiment of Fig. 7A the outflow of [^{32}P]phosphate was followed from liposomes and proteoliposomes prepared from a mixture of egg-yolk and soybean phospholipid, varying the proportion of the soybean lipid from 0 to 100%. Incorporation of 20% soybean phospholipid resulted in a 4–5-fold increase in the entrapped activity (proportionally to the increase of the entrapped volume) of the protein-free liposomes but further increase of the soybean component caused a slight decrease. The labelled phosphate content remaining in the proteoliposomes after passage through the anion exchange column rose from 5 to about 19% as the proportion of soybean phospholipid was augmented. Part B of Fig. 7 demonstrates the quantity of solute release (difference of the entrapped and remaining activities) as the percentage of the respective entrapped activity. The phosphate content of 70% of the total intraliposomal pool was released within about 10 s from proteoliposomes composed of 80% egg-yolk and 20% soybean phospholipid whereas phosphate outflow from porin-containing vesicles prepared of pure soybean phospholipid amounted only to 42% of the internal pool.

In order to prove that porin has been incorporated also into liposomes prepared of pure soybean phospholipid, the following control experiment has been carried out (Fig. 8). In the first step porin was either added to the liposomes (composed of 100% soybean phospholipid) in the usual way before the freeze-thaw

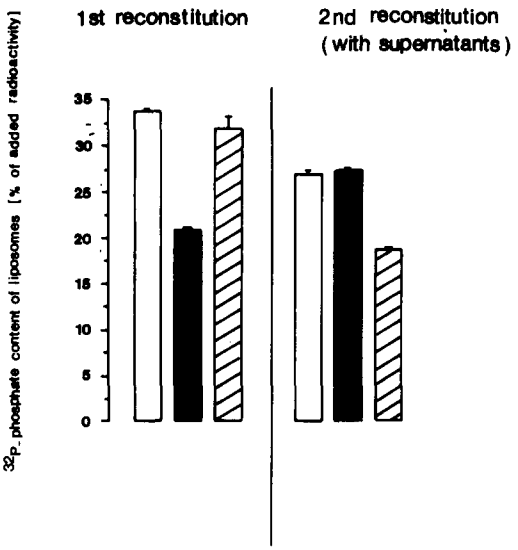

Fig. 8. Control of the effectivity of the insertion of porin into liposomes. A two-step reconstitution assay was applied. For the first reconstitution (Fig. 8A) liposomes were prepared from 100% soybean phospholipid, preloaded with [^{32}P]phosphate and either reconstituted with porin as described in the Methods or simply mixed with porin by vortexing. Radioactivity remaining after anion exchange chromatography in protein-free (white columns), porin-reconstituted (black columns) or porin-mixed (hatched columns) liposomes is expressed as the percentage of the total applied activity. An aliquot of the three different liposome suspensions were ultracentrifuged and the supernatants were used for the second reconstitution (Fig. 8B). In this step liposomes were prepared from 80% egg-yolk and 20% soybean phospholipids and sonicated only for 2×10 s resulting in a lower entrapped volume. The symbols are the same as for part A The average ± S.E. of five parallel measurements is shown.

cycles (results are shown in the black columns) or it was mixed to the prepared vesicles after the freeze-thaw phase (hatched columns). Proteoliposomes were then separated by ultracentrifugation and the porin content of the supernatant was tested by means of a second reconstitution step. Incorporation of porin into the liposomes in the first or second reconstitution step was assessed on the basis of release of the labelled phosphate. When porin was added to the liposomes before the freeze-thaw cycles (i.e., in case of normal reconstitution) [^{32}P]phosphate content of the effluent liposomes was decreased after the first reconstitution step, however, not after the second one (black columns). In contrast, when porin was only mixed with prepared liposomes after the freeze-thaw cycles, transport activity was hardly detectable in the first step but significant decrease of the liposomal [^{32}P]phosphate content was found after the second reconstitution (hatched columns). Thus, reconstitution carried out by repeated freeze-thaw cycles results in the complete disappearance of functional porin molecules from the water phase even if the applied liposomes are composed of pure soybean phospholipid.

Discussion

In the experiments summarized in Figs. 2–4 some basic transport properties of porin could be reproduced in reconstituted proteoliposomes: rapid net movement of small anions, cations and uncharged molecules that disappeared upon heat-denaturation of the protein. This experimental approach offers the possibility of direct measurement and comparison of the movement of various molecular species through the porin channel and the obtained results can be considered relevant to the situation in the intact mitochondrial membrane.

In our reconstituted proteoliposomes the release of the investigated substances (phosphate, chloride, ATP) was only partially inhibited by polyanion, the known inhibitor of porin channels. This finding is consistent with the results of previous investigations carried out in planar membranes or in proteoliposomes by indirect (shrinking/reswelling) measurements. All these previous studies reported the narrowing of the channel upon polyanion treatment, the achieved 'closed' state having an estimated radius of approx. 0.9 nm [19]. Our direct transport measurements provide as new information that ATP – in spite of its higher molecular mass and charges – behaves basically similarly to the small anions studied previously. The movements of ATP are also slowed down but are not fully inhibited by the polyanion in our reconstituted system. It has to be remarked that no alterations of the transmembrane voltage have been applied in our proteoliposomes. However, the voltage-dependency of porin molecules prevailing in the contact sites became questionable by the results of recent patch-clamp studies. The ion channels detected in the contact sites proved not to be voltage-sensitive whereas those present in other parts of the outer or inner mitochondrial membrane demonstrated clear voltage-dependency [32]. All these data suggest that in intact mitochondria additional factors should be responsible for the low permeability of the contact sites for ATP under basal conditions and the complete inhibition of the kinase reactions by the polyanion. A similar conclusion has been reached very recently by Moran et al. [33] on the basis of patch-clamp measurements.

The results shown in Figs. 7 and 8 call the attention to another factor that might play a role in the modulation of the transport properties of porin situated at different locations in the outer mitochondrial membrane. Variation of the lipid composition was found to have considerable influence on the amount of transported substance. The control experiments summarized in Fig. 8. prove that association of the transport-active porin molecules to the liposomes was complete even in the case of the least favourable lipid composition (100% soybean phospholipid). However, significant differences were found in the size of the releasable pool (from 70% to 42% of the intraliposomal pool) as the proportion of the soya component was increased. This change might be due to the enrichment of a vesicle population not containing transport-active porin or due to a drastic change in the transport properties of a fraction of porin molecules. In either case some components of the soybean phospholipids can be suggested to create unfavourable conditions for porin in the high conductance state. As the lipid composition of the contact sites was shown to be significantly different from the rest of the outer membrane, regulatory functions of some specific components should be considered. It is interesting to note that both the isolated contact points [34] and the soybean phospholipid applied in our experiments were rich in cardiolipin and other negatively charged lipids. However, it has to be emphasized that in our experiments even under the least conductive conditions, 42% of the total solute content was released within approx. 10 s, corresponding to a 'turnover number' of 120 000 per minute (provided that all the added porin molecules are functionally active). This value should be compared to the turnover number of the adenine nucleotide carrier (500 per min) [35]. Thus the investigated changes of the lipid environment do not account entirely for the limited transport properties of porin localized in the contact sites. The effect of further factors (more efficient lipid species, role of the bound kinases, etc.) has to be studied in future experiments.

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