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INCORPORATION OF Na^+-Ca^{2+} ANTIPORTER AND OF ($Na^+ + K^+$)-ATPase INTO LIPOSOMES AND DEMONSTRATION OF THEIR NON-IDENTITY

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($Na^+ + K^+$)-ATPase was isolated from the grey matter of brain and incorporated into liposomes. Most of the reconstituted enzyme was oriented 'inside-out' with respect to its in vivo orientation and externally added ATP promoted Na^+ uptake that was inhibitable by internally trapped ouabain. Using the same proteoliposomes, an Na^+-Ca^{2+} exchange system was observed as indicated by the following pieces of evidence. (1) The Na^+ gradient provided the only readily apparent driving force for acceleration of Ca^{2+} accumulation into proteoliposomes. (2) The antiporter was specific for Ca^{2+}, high Mg^{2+} excess did not inhibit Ca^{2+} antiport. (3) The Na^+ efflux was dependent on the extravesicular Ca^{2+} concentration. (4) The Na^+ efflux was not inhibited by tetrodotoxin. The demonstrated Na^+-Ca^{2+} exchange could not be related to ($Na^+ + K^+$)-ATPase protein, since it was not purified with ($Na^+ + K^+$)-ATPase, as followed from transport studies with liposomes containing ($Na^+ + K^+$)-ATPase of different specific activity. The results strongly indicate that plasma membranes isolated from the grey matter of brain contain an Na^+-Ca^{2+} exchange system and that the proteoliposomes are suitable for further purification of the carrier molecule.

Introduction

In cardiac muscle, synaptosomal and kidney preparations, and also in dialyzed squid axons, two mechanisms for the extrusion of Ca^{2+} from cytoplasm, an Na^+-Ca^{2+} exchange and an ATP-driven Ca^{2+} transport have been reported to reside in the plasma membrane [1–7]. In these studies, either intact tissues or plasma membrane vesicles were used so that the nature of the protein involved in Ca^{2+} extrusion remained open.

Various pieces of circumstantial evidence pointed to the eventual involvement of ($Na^+ + K^+$)-ATPase. First, both systems are found to coexist or to copurify with (Na^+,K^+)-ATPase [3,7–10]. Second, both the Na^+-Ca^{2+} exchange and the ($Na^+ + K^+$)-ATPase show an absolute specificity for sodium [11,12]. Third, ($Na^+ + K^+$)-ATPase, after incorporation into liposomes, appears to act as an ionophore for Na^+ [13] or K^+ [14]. Fourth, Ca^{2+} in concentrations lower than 10 μM can substitute for Na^+ in eliciting transphosphorylation from ATP to enzyme protein [15]. Fifth, Ca^{2+} may also substitute for Na^+ in inducing the phosphoenzyme conformation necessary for ouabain binding [16]. These observations suggested that ($Na^+ + K^+$)-ATPase might be involved in Na^+-Ca^{2+} exchange by serving as an ionophore not requiring ATP interaction or in

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Abbreviations: PC, phosphatidylcholine from egg yolk; DOPC, dioleoylphosphatidylcholine; PS, phosphatidyl-L-serine from bovine brain; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetate.

ATP-driven Ca^{2+} transport by sharing the transport pathway of Na^+ [15].

In the present paper, we used the reconstitution technique to answer the question of whether ($Na^+ + K^+$)-ATPase is a molecular machine which, besides effecting Na^+/K^+-antiport, is also involved in promoting Ca^{2+} movements across the membrane of proteoliposomes. Na^+-Ca^{2+} exchange activity has been found in cardiac plasma membrane after cholate dilution procedure in the presence of phospholipids. However, neither the protein nature of the Na^+-Ca^{2+} antiporter nor a possible involvement of ($Na^+ + K^+$)-ATPase have been clarified. To answer the above-formulated question, we compared liposomes in which we had incorporated brain or kidney ($Na^+ + K^+$)-ATPase preparations by means of the cholate dialysis method [18] or the octylglucoside dilution technique [19], respectively.

Materials and Methods

Materials

$^{45}CaCl_2$ (17 mCi/mg) was from the Radiochemical Centre, Amersham, U.K.; $^{22}NaCl$ (54 mCi/mg) from Rotop, Dresden, G.D.R.; ouabain as well as NaCl, KCl and $CaCl_2$ (all suprapure grade) were from Merck, Darmstadt, F.R.G.; disodium ATP from Reanal, Budapest, Hungary; bovine brain phosphatidyl-L-serine, mercaptoethanol and cholic acid came from Ferak, Berlin, West Germany; 1-*O*-*n*-octyl-β-D-glucopyranoside from Sigma; Sephadex G-50 medium grade from Pharmacia, Uppsala, Sweden; tetrodotoxin from Sankyo, Tokyo, Japan; A 23187 from Eli Lilly, Indianapolis, U.S.A.; NADH from Calbiochem – Behring Corp., U.S.A.; pyruvate kinase and lactate dehydrogenase from Boehringer, Mannheim, F.R.G.

Preparations

Phosphatidylcholine from egg yolk (PC) and dioleoylphosphatidylcholine (DOPC) were prepared as described by Singelton et al. [20] and Cuberobles and Van den Berg [21], respectively. Aliquots dissolved in ethanol were stored at −70°C under N_2. 0.5–2 nmol oxidation products per μmol phosphatidylcholine were present according to the thiobarbituric lipoxidation assay [22]. The commercially available phosphatidylserine was purified as described [23]. ATP was freed from sodium by passage through CM-Sephadex G-25 columns, and was neutralized with imidazole base.

($Na^+ + K^+$)-ATPase from the grey matter of pig brain was prepared according to Samaha [24]. 1–2 ml aliquots of the membrane suspension in 150 mM Tris-HCl buffer solution, pH 7.4 (15 mg protein/ml) were frozen in liquid nitrogen and stored at −70°C. Before use, the suspension was centrifuged for 30 min at $100000 \times g$ and 2°C, and resuspended as described below. The properties of the preparation are described under Results.

($Na^+ + K^+$)-ATPase from pig kidney red outer medulla was prepared according to the isopycnic zonal centrifugation procedure described by Jørgensen [25]. The membranes were suspended in 250 mM sucrose, 1 mM EDTA and 30 mM histidine-HCl buffer solution (pH 7.3) at a protein concentration of 2–3 mg/ml, and stored at −20°C. ($Na^+ + K^+$)-ATPase activity was 1300–1600 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$; ouabain-insensitive ATPase activity was not detectable.

Proteoliposomes from the brain ($Na^+ + K^+$)-ATPase preparation and phosphatidylcholine or dioleoylphosphatidylcholine

The cholate dialysis procedure described by Goldin [26] was modified as follows. 1 ml aliquots of stock phospholipid solution containing 30 mg PC or DOPC were freed from ethanol in an N_2 stream at room temperature. The lipid was suspended in 1 ml of 30 mM imidazole-HCl buffer solution (pH 7.3) containing 250 mM sucrose, 10 mM mercaptoethanol and ouabain, NaCl, KCl, $MgCl_2$ or $CaCl_2$, respectively, in the concentrations given in the legends to the figures and tables (referred to in the following as reconstitution buffer A). After thorough vortexing at room temperature under nitrogen the lipid suspension was chilled in a 10 ml tube. A 2.5 mg protein aliquot of the ($Na^+ + K^+$)-ATPase preparation suspended in 0.25 ml of the reconstitution buffer A was vortexed under nitrogen into the lipid suspension for 5 min at 28°C. To 1.25 ml of this mixture, 20 mg of sodium cholate dissolved in 1 ml of buffer A solution were added. After vortexing under nitrogen

for 20 min at 28°C, the mixture was centrifuged for 20 min at 100000 × *g* and 2°C. The clear supernatant was dialyzed under nitrogen for 36–48 h at 4–8°C against a total volume of 5 liters of buffer A solution. The dialyzed suspension contained the proteoliposomes with the required intravesicular contents. For comparison, liposomes without protein were occasionally formed by omitting the ($Na^+ + K^+$)-ATPase preparation from the procedure.

Proteoliposomes from the kidney ($Na^+ + K^+$)-ATPase preparation and a mixture of phosphatidylcholine and phosphatidylserine

In principle, the octylglucoside dilution procedure described by Racker et al. [19] was followed. Reconstitution was performed with 0.6 mg highly purified ($Na^+ + K^+$)-ATPase in the presence of 9 mg of a sonicated mixture of PC and PS (6:1, w/w) in 30 mM imidazole-HCl buffer solution (pH 7.3) containing 160 mM NaCl, 1 mM mercaptoethanol and 2% octylglycoside. The final volume was 0.44 ml. The suspension was vortexted for 15 s at 0°C. After addition of 9 ml of the same cold buffer solution without octylglucoside (named reconstitution buffer B) the mixture was immediately centrifuged with a Beckman Ti-75 rotor for 30 min at 48000 rpm at 2°C. The pellet was resuspended in 0.32 ml of the above-mentioned buffer solution.

Determination of radioactive cation contents of proteoliposomes

The movements of sodium and calcium between the extra- and intravesicular space were followed by determination of the changes in the $^{22}Na^+$ or $^{45}Ca^{2+}$ contents of the proteoliposomes. They were separated from the incubation medium by chromatography on Sephadex G-50 columns (11 cm in height and 1 cm inner diameter). The columns were equilibrated and eluted at 5°C with the corresponding reconstitution buffer which, in case of the $^{45}Ca^{2+}$ studies, also contained 2 mM EGTA for complete Ca^{2+} removal from the extravesicular space. 80–200 µl aliquots of the incubated proteoliposome suspension were passed through a column at a flow rate of 1–1.5 ml/min; 0.5–0.8-ml fractions were collected and counted for radioactivity by means of an LKB or Philips liquid scintillation spectrophotometer. The separation of the proteoliposomes, which were in the void volume, was fast (3–5 min) and the cation permeation through the proteoliposome membrane even at elevated temperature (28°C) was comparatively slow (cf. Results) so that a significant cation leakage during chromatography appeared to be unlikely.

Other methods

The protein concentrations were determined as described by Bensadoun and Weinstein [27]. The ATPase activities were followed by the determination of either P_i [28] or ADP by means of the coupled enzyme test system [29].

The electrophoretic separation of proteins was carried out in principle as described in Ref. 30 by using sodium dodecyl sulfate-acrylamide gels with 7.5% acrylamide and 0.1% bisacrylamide. The gels were stained with Coomassie blue. The coloured proteins were traced by means of a Shimadzu Dual Wavelength Scanner CS 910. The gels were calibrated with cytochrome *c*, myoglobin, chymotrypsinogen, albumin from chicken eggs, catalase, bovin serum albumin and ferritin as molecular weight markers.

The freeze-fracturing analysis was done after suspending the proteoliposomes in 30% glycerol for 30 min at room temperature and after freezing the suspension in liquid freon 12 at about −150°C. The specimens obtained were fractured at −100°C in a Balzers BA 360 M, and immediately shadowed with platinum at an angle of 45° and replicated with carbon. The replicas were analyzed in a TESLA BS 500 electron microscope.

Results

1.0. Proteoliposomes from brain ($Na^+ + K^+$)-ATPase preparation and egg yolk phosphatidylcholine or dioleoylphosphatidylcholine

1.1. General characteristics of the proteoliposomes

The process of co-solubilization of the membrane fragments and PC with cholate did not result in further purification of ($Na^+ + K^+$)-ATPase. The scans of the electrophoretograms of the original preparation (not demonstrated) and the proteoliposomes (Fig. 1) showed virtually no

differences. In each case, the catalytic protein and glycoprotein subunit of $(Na^+ + K^+)$-ATPase accounted for about 50% of the total protein, the other 50% was distributed mainly among four proteins with molecular weights of 110000, 74000, 43000 and 20000.

The freeze-fracture electron micrographs of the proteoliposomes (Fig. 2) showed a uniform population of fractured membrane faces which appeared either concave or convex. The diameters of most vesicles lay in the range of 100–130 nm. The liposomes from PC alone were devoid of intramembranal particles. However, the proteoliposomes showed particles which were distributed between concave and convex fracture faces, which represent the outer or inner leaflet of the vesicle bilayer. The distribution of protein particles in the vesiculized preparation is randon; some vesicles contain 1–2 protein particles, others up to 15. The diameter of most particles fell in the range of 5 to 10 nm (Fig. 2) and was thus similar to that of the particles in membrane fragments of highly purified $(Na^+ + K^+)$-ATPase preparations [31,32].

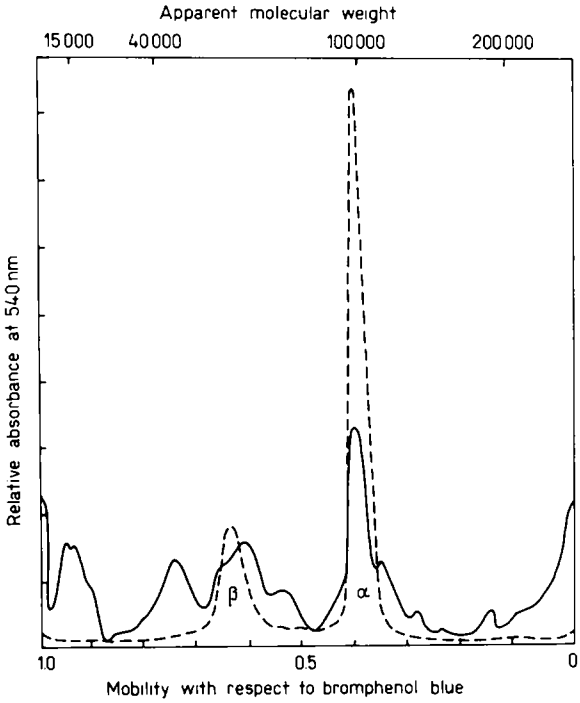


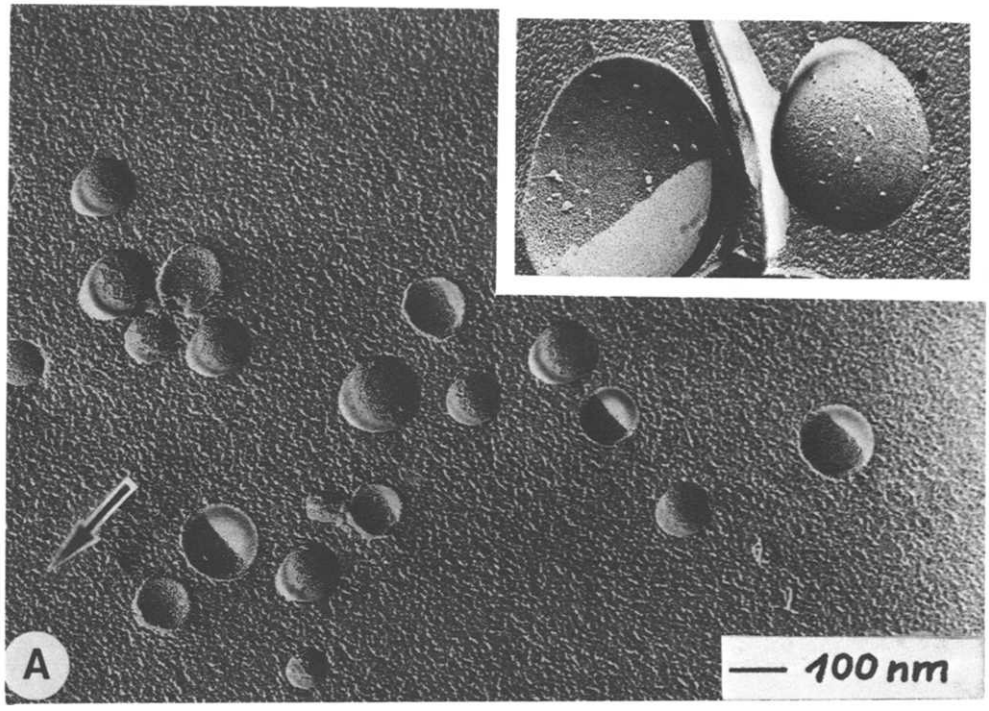
Fig. 1. Protein composition of the proteoliposomes formed either from brain $(Na^+ + K^+)$ATPase preparation and PC (———) or from kidney $(Na^+ + K^+)$-ATPase preparation and a 6 : 1 PC/PS mixture (- - - - -). The curves represent the 540 nm absorption scans of Coomassie blue-stained proteins after SDS-polyacrylamide gel electrophoresis. Whereas the kidney preparation consists almost exclusively of the catalytic protein α and the glycoprotein β of $(Na^+ + K^+)$-ATPase, accounting for greater than 90% of the absorption, the brain preparation contains in addition unidentified proteins of higher and lower molecular weight.

The comparison of mean specific activities of $(Na^+ + K^+)$-ATPase before and after incorporation into PC or DOPC liposomes (Table I) showed that the former phospholipid is more suitable for effective reconstitution with respect to the recovery of specific ATPase activity. As shown in Table I the presence of 0.25 mM ouabain, sufficient to suppress the $(Na^+ + K^+)$-ATPase activity of nonvesicular preparations reduced the total ATPase activity by 15% (PC vesicles) or 35% (DOPC vesicles) showing that at least the majority of the proteoliposomes are not permeable to ATP and ouabain. However, after disruption of the continuity of vesicle membrane formed either from PC or DOPC by 2-fold freeze-thawing, ouabain reduced the total ATPase activity by about 70%. This value corresponds to the inhibition obtained after loading the vesicles with ouabain or after addition of 0.3 mM digitoxigenin to the vesicles (not shown). The results indicate that the reconstituted transport molecules are oriented predominantly with their formed cytoplasmic site to the extravesicular space.

1.2. Cation permeabilities and transport activities of the proteoliposomes

The cation permeability of the PC liposomes was much increased after incorporation of the $(Na^+ + K^+)$-ATPase preparation (Fig. 3). The permeation curves of Na^+ and Ca^{2+} were monophasic. After plotting the logarithms of the increments of radioisotope equilibration against time we obtained linear slopes (inset to Fig. 3). These observations suggest that the individual proteoliposomes in the population had similar Na^+ and Ca^{2+} permeabilities.

The presence of ATP in the extravesicular space accelerated Na^+ transport into the intravesicular space of both PC and DOPC proteoliposomes (Fig. 4). Ouabain is known as a specific inhibitor of $(Na^+ + K^+)$-ATPase, but suppressed the Na^+



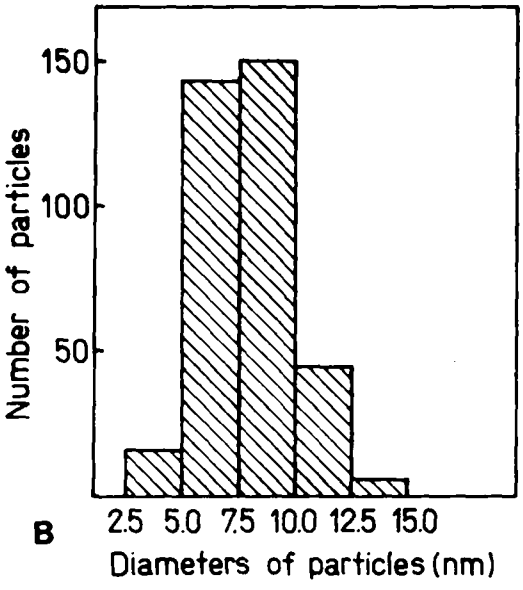


Fig. 2. (A) Presence of intramembranal particles in proteoliposomes from brain ($Na^+ + K^+$)-ATPase preparations and phosphatidylcholine visualized by freeze-fracture electron microscopy. The proteoliposomes consist of unilamellar vesicles with a globular protein substructure. — Inset: ×100000. Both the concave and convex fracture face representing the outer or inner leaflet of the vesicle bilayer show globular particles which, however, are absent in liposomes from PC alone (not demonstrated). (B) Diameter frequency of intramembranal protein particles in proteoliposomes from brain ($Na^+ + K^+$)-ATPase preparation and PC determined in freeze-fracture electron micrographs as shown in (A). The total number of particles evaluated was 360.

transport into PC proteoliposomes only when intravesicularly applied. These findings evidenced the inside-out orientation of ($Na^+ + K^+$)-ATPase with respect to in vivo conditions and the reconstitution of Na^+/K^+-antiport function in the proteoliposomes.

Actually, the Na^+ permeability of the DOPC proteoliposomes was lower than that of the PC

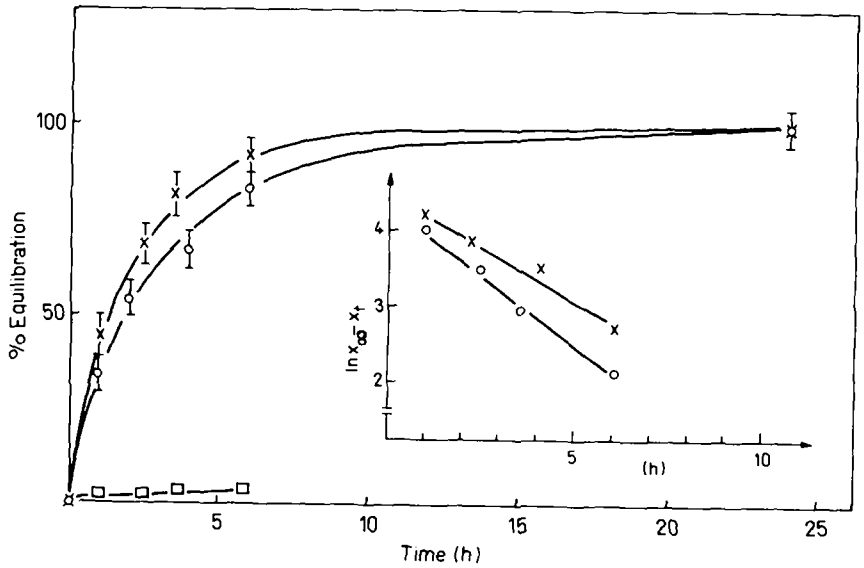


Fig. 3. Permeability at 23°C of liposomes, formed from PC alone, for Na^+ (□——□) or of proteoliposomes, formed from brain ($Na^+ + K^+$)-ATPase preparation and PC, for Ca^{2+} (○——○) and for Na^+, respectively (×——×). Aliquots of 2–4 μCi of carrier-free $^{22}NaCl$ or 1–4 μCi $^{45}CaCl_2$ were added to conical tubes and dried at 80°C. 700 μl of the liposome or proteoliposome suspension in a solution of 250 mM sucrose, 30 mM NaCl, 30 mM KCl, and 0.5 mM $CaCl_2$ (the latter in case of Ca^{2+} studies only) were added and immediately vortexed. At the times indicated, 80-μl aliquots were taken to determine the $^{45}Ca^{2+}$ or $^{22}Na^+$ contents. The data are the averages with standard errors for three preparations. Inset: The logarithms of the increments of intravesicular $^{45}Ca^{2+}$ or $^{22}Na^+$ content show linear slopes.

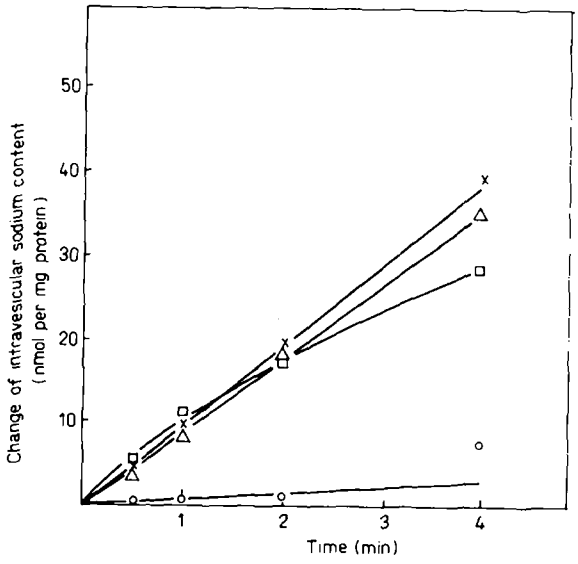


Fig. 4. ATP-promoted Na^+ transport into proteoliposomes formed from the brain ($Na^+ + K^+$)-ATPase preparation and DOPC (□——□) or PC (×——×). The transport into the PC proteoliposomes remained uninfluenced (△——△) or became suppressed (○——○) by 0.5 mM ouabain when extravesicularly applied or intravesicularly trapped, respectively. 400 μl of the proteoliposomes, prepared and suspended in 250 mM sucrose, 30 mM NaCl and 30 mM KCl were added and immediately vortexed to dissolve $^{45}NaCl$. After addition of 24 μl buffer medium or 24 μl 10 mM ouabain (final ouabain concentration 0.5 mM), 15 μl $MgCl_2$ solution (final concentration 1.3 mM) were added, and after preincubation for 1 min at 28°C, the reaction was started by adding ATP (final concentration 0.9 mM). The ouabain-containing proteoliposomes were prepared by including 0.5 mM ouabain in the dialysate. The data are corrected for the blank values obtained by omitting ATP from the incubation medium. The data are the averages of double determinations.

proteoliposomes (not demonstrated). Since this property appeared to be an advantage for measuring Na^+-Ca^{2+} exchange the DOPC proteoliposomes were preferred in the Na^+-Ca^{2+} exchange studies described below.

The Ca^{2+} influx into the proteoliposomes occurred slowly when high NaCl, LiCl or KCl concentrations were present on both sides of the membrane (Figs. 5 and 6), and was not changed when a K^+ or Li^+ concentration gradient was present (Fig. 6). Contrary to the K^+ and Li^+ gradient, the Na^+ gradient strongly enhanced the Ca^{2+} influx

TABLE I

COMPARISON BETWEEN MEMBRANE FRAGMENTS AND PROTEOLIPOSOMES FORMED FROM BRAIN ($Na^+ + K^+$)-ATPase PREPARATION AND EGG YOLK PHOSPHATIDYLCHOLINE OR DIOLEOYLPHOSPHATIDYLCHOLINE WITH RESPECT TO PROTEIN RECOVERY, ATPase AND OUABAIN INHIBITORY EFFECT

The ATPase activities were determined at 37°C in a final volume of 1.0 ml and a reaction medium containing 250 mM sucrose, 50 mM imidazole/HCl buffer (pH 7.2) and additionally either 60 mM KCl and 5 mM $MgCl_2$ (Mg^{2+}-dependent ATPase) or 170 mM sucrose, 120 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$ (total ATPase). ($Na^+ + K^+$)-ATPase activity was the difference between these activities. The data are the averages with standard errors of four preparations.

Preparation	Protein recovery (%)	Specific activities (μmol $P_i \cdot mg^{-1} \cdot h^{-1}$)		Inhibition by 0.25 mM ouabain (%)		
				Externally added		Intravesicularly trapped
		Mg^{2+}-ATPase	($Na^+ + K^+$)-ATPase	Native	Freeze-thawed	
Membrane fragments	100	12 ±2	115 ±15	90 ± 5	80 ±5	-
PC vesicles	50±10	2 ±1	52 ± 9	15 ±10	70 ±5	85 ±5
DOPC vesicles	50±10	2 ±1	26 ± 3	35 ± 5	75 ±5	85 ±5

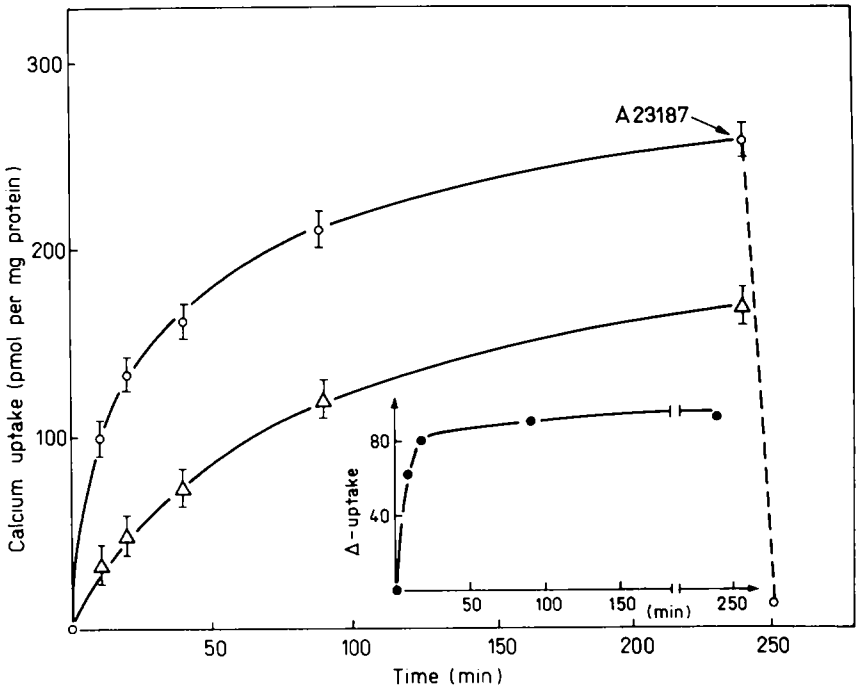


Fig. 5. Ca^{2+} influx at 28°C into proteoliposomes from brain ($Na^+ + K^+$)-ATPase preparation and DOPC in presence (○——○) or absence (△——△) of an Na^+ concentration gradient over the liposome membrane directed from the intra- to the extravesicular space. The proteoliposomes were loaded with 160 mM NaCl during the vesiculation procedure. 130 μl aliquots of the proteoliposome suspension were added to 1170 μl of buffer solutions containing besides 25 μM $^{45}CaCl_2$ (0.2–0.4 mCi/ml) either 160 mM LiCl (Na^+ gradient) or 160 mM NaCl (no Na^+ gradient). At the times indicated the extravesicular $^{45}Ca^{2+}$ was rapidly removed by passing aliquots through the Sephadex column with the buffered solution containing additionally 2 mM EGTA. 20 μM A23187 was included at $t = 240$ min (arrow). Inset: Na^+-gradient-dependent Ca^{2+} accumulation in the vesicles. The data are the averages and standard errors of triple determinations.

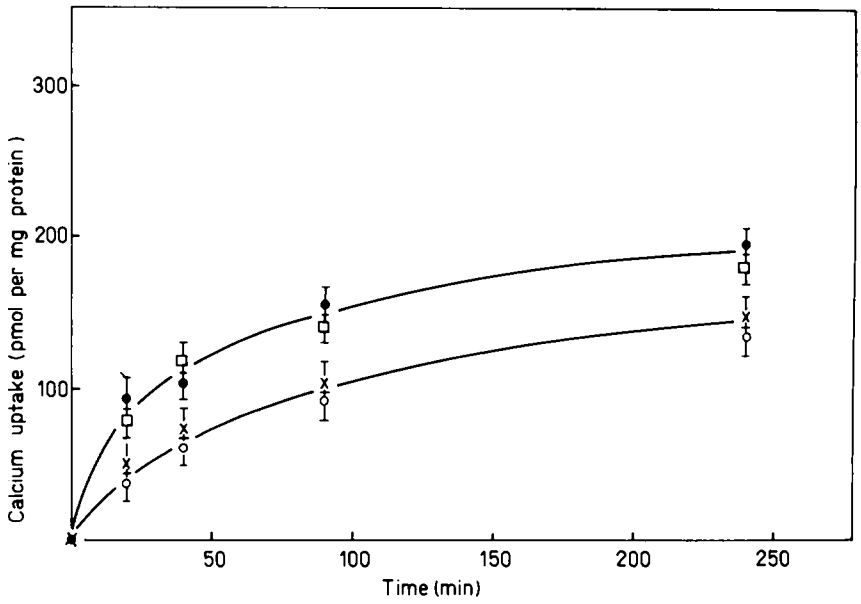


Fig. 6. Ca^{2+} influx at 28°C into proteoliposomes from brain ($Na^+ + K^+$)-ATPase preparation and DOPC in the presence (□ ——— □) or absence (● ——— ●) of a K^+ concentration gradient, and in the presence (○ ——— ○) or absence (× ——— ×) of an Li^+ concentration gradient, in both cases directed from the intra- to the extravesicular space. The proteoliposomes were loaded with 160 mM KCl or LiCl during the vesiculation procedure. 130 μl of the proteoliposome suspension were added to 1170 μl of buffer solutions containing besides 25 μM $^{45}CaCl_2$ (0.2–0.4 mCi/ml) either 160 mM NaCl (K^+ gradient) or 160 mM KCl (no K^+ gradient), and 160 mM NaCl (Li^+ gradient) or 160 mM LiCl (no Li^+ gradient), respectively. The analytical steps were carried out as described in the legend to Fig. 5. The data are the averages and standard errors of triple determinations.

(Fig. 5). The Na^+-gradient-driven Ca^{2+} accumulation essentially ceased within 15 min, apparently due to the exhaustion of the Na^+ gradient (inset to Fig. 5). In the presence of the Ca^{2+} ionophore A23187, all Ca^{2+} was removed from the proteoliposomes during their separation from the incubation medium (Fig. 5). This suggests that Ca^{2+} binding to the vesicle constituents was not involved in the Ca^{2+} uptake data. In the presence of an extravesicular Ca^{2+} concentration far below the

TABLE II

INFLUENCE OF Mg^{2+} AND OUABAIN ON THE Na^+-GRADIENT-DEPENDENT Ca^{2+} UPTAKE INTO PROTEOLIPOSOMES FORMED FROM BRAIN ($Na^+ + K^+$)-ATPase PREPARATION AND PC

The Na^+-gradient-dependent Ca^{2+} uptake was obtained by correcting the measured values for the Na^+-gradient-independent Ca^{2+} influx as shown in Fig. 5. To get complete inhibition of ($Na^+ + K^+$)-ATPase by ouabain, the proteoliposome suspension before dilution was incubated for 2 min in presence of 0.1 mM ATP and 1 mM $MgCl_2$ to form the phosphoenzyme known for optimum interaction with ouabain. The data are the averages and standard errors of triple determinations.

Original effector concentrations (mM)		Na^+-gradient-dependent Ca^{2+} uptake (pmol·(mg protein)$^{-1}$·(40 min)$^{-1}$)
Intravesicular space	Extravesicular space	
160 NaCl	160 LiCl, 16 NaCl	75 ± 10
160 NaCl	160 LiCl, 16 NaCl 5 $MgCl_2$	60 ± 9
160 NaCl, 0.6 ouabain	160 LiCl, 0.06 ouabain, 1 $MgCl_2$	78 ± 9

$K_{0.5}$ (Ca) value of near 40 μM [33], the Na^+ efflux along its concentration gradient occurred slowly. At a Ca^{2+} concentration surpassing the $K_{0.5}$ (Ca) value the Na^+ efflux increased (Fig. 7). The Ca^{2+}-dependent fraction of Na^+ efflux essentially ceased within 15 min, apparently due to the dissipation of the gradient (inset to Fig. 7). A similar time parameter was found for the Na^+ gradient-dependent Ca^{2+} uptake (inset to Fig. 5). These observations led to the conclusion that an Na^+-Ca^{2+} antiporter sensitive to sodium and calcium ions has been incorporated into the liposomes. 0.2 μM tetrodotoxin, intravesicularly trapped during vesiculation, did not inhibit Na^+ efflux (not shown).

The Na^+-gradient-dependent Ca^{2+} uptake into PC proteoliposomes was not depressed by an Mg^{2+} concentration 200-times higher than the Ca^{2+} concentration applied, and also not by a high ouabain concentration (Table II). These findings mean that the exchange mechanism easily distinguishes between Ca^{2+} and Mg^{2+}, and remains fully operative after complete inhibition of ($Na^+ + K^+$)-ATPase by high ouabain concentration. However, the latter observation does not disprove an involvement of ($Na^+ + K^+$)-ATPase in Na^+-Ca^{2+} exchange because ouabain binding to the enzyme does not need to inhibit a ionophoric function in Na^+-Ca^{2+} antiport across vesicle membranes. Hence, we searched for unequivocal proof or disproof of this possibility.

2.0. Proteoliposomes from kidney ($Na^+ + K^+$)-ATPase preparation and a mixture of egg yolk phosphatidylcholine (PC) and phosphatidylserine (PS)

The scan of the electrophoretogram showed the catalytic protein and the glycoprotein subunits of ($Na^+ + K^+$)-ATPase (Fig. 1). None of the four additional proteins found in the ($Na^+ + K^+$)-ATPase preparation from brain (Fig. 1) was

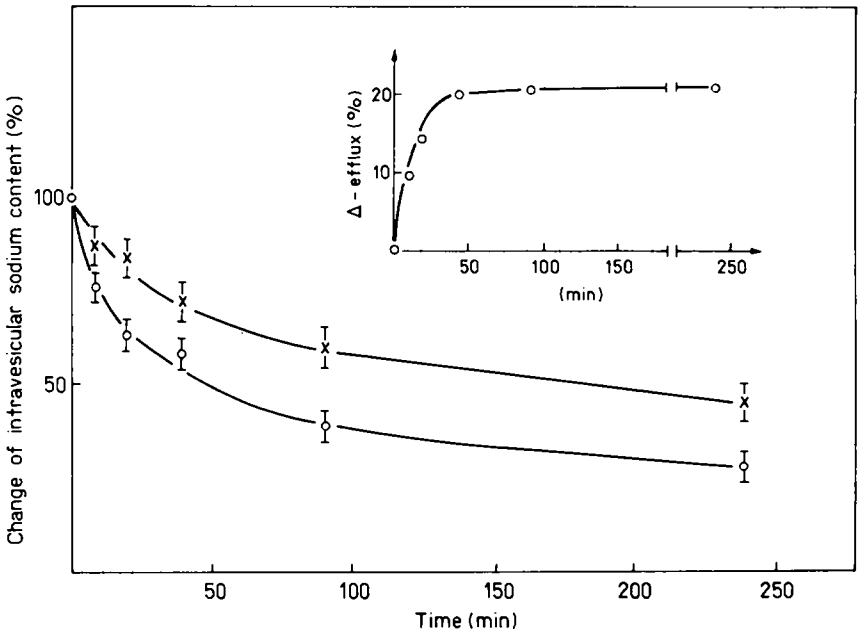


Fig. 7. Na^+ efflux at 28°C from proteoliposomes, formed from brain ($Na^+ + K^+$)-ATPase preparations and DOPC, in the absence (×——×) or presence (○——○) of a Ca^{2+} gradient directed from the extravesicular to the intravesicular space. The proteoliposomes were loaded with 160 mM NaCl containing 0.3 mCi/l ^{22}NaCl and 0.9 mM EGTA during the vesiculation procedure. Aliquots of the proteoliposome suspension were diluted 10-fold with buffer solutions containing 160 mM LiCl, 1.0 mM $CaCl_2$ and EGTA concentrations to obtain 0.23 μM Ca^{2+} (virtually no Ca^{2+} gradient) or 160 μM Ca^{2+} (steep Ca^{2+} gradient). The Ca^{2+} concentrations were calculated from $K_{(Ca\text{-}EGTA)} = 0.15$ μM [38]. The analytical steps were carried out as described in the legend to Fig. 5. The data are the averages and standard errors of triple determinations. Inset: Ca^{2+}-dependent fraction of Na^+ efflux from the vesicles.

traceable in that from kidney. The following experiments have been carried out to answer the question of whether the $(Na^+ + K^+)$-ATPase can operate as the Na^+-Ca^{2+} antiporter. To this end we measured in parallel the ATP-promoted Na^+ transport and the Na^+-gradient-dependent Ca^{2+} uptake with liposomes containing $(Na^+ + K^+)$-ATPase preparations of different specific activities. As expected the proteoliposomes from the kidney $(Na^+ + K^+)$-ATPase preparation with high specific activity (300 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$) accumulated in the presence of ATP by about one order of magnitude more Na^+ than the less active (42 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$) liposomes containing $(Na^+ + K^+)$-ATPase from brain (Fig. 8). It is interesting to note that under the same conditions but omitting Na^+ from the incubation medium no ATP-dependent Ca^{2+} uptake could be found (not shown).

In contrast to the co-purification of $(Na^+ + K^+)$-ATPase and ATP-promoted Na^+ transport demonstrated in Fig. 8, the proteoliposomes from kidney did not accumulate significantly higher amounts of Ca^{2+} in the presence of an outwardly

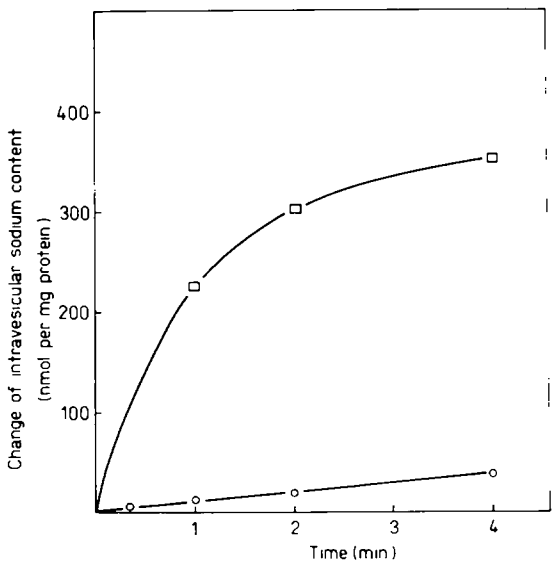


Fig. 8. ATP-promoted Na^+ transport into proteoliposomes from kidney $(Na^+ + K^+)$-ATPase (□——□) or into proteoliposomes from brain $(Na^+ + K^+)$-ATPase preparations (○——○). Conditions for transport studies as described in the legend to Fig. 4. The data are the averages of double determinations.

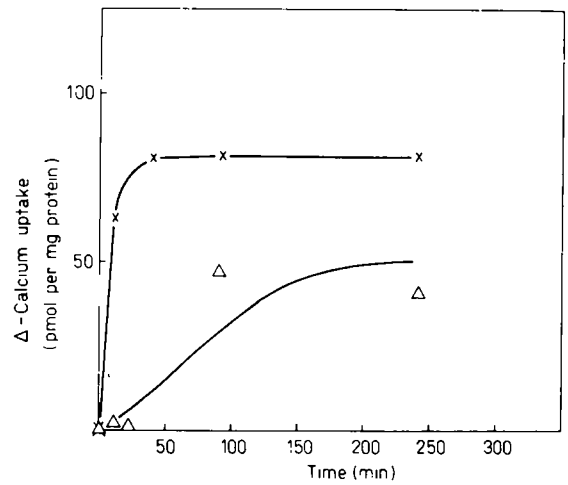


Fig. 9. Na^+-gradient-dependent Ca^{2+} accumulation into proteoliposomes from kidney $(Na^+ + K^+)$-ATPase preparations (△——△) or into proteoliposomes from brain $(Na^+ + K^+)$-ATPase preparation (×——×). Conditions were the same as described for Fig. 5 except that the extravesicular Ca^{2+}-concentration amounted to 55 μM.

directed Na^+ gradient (Fig. 9). This finding cannot be explained by differences in the passive permeabilities of the two types of proteoliposomes to Na^+ or Ca^{2+}. The passive cation permeabilities were similar in all preparations. To replicate the result shown in Fig. 7 we tried to detect a Ca^{2+}-sensitive fraction of Na^+ efflux in kidney proteoliposomes (not shown). However, the liposomes containing highly purified kidney $(Na^+ + K^+)$-ATPase did not catalyze Na^+ efflux sensitive to extravesicular calcium ions.

Discussion

The analysis of an Na^+-Ca^{2+} exchange mechanism in cells of excitable tissues has been attempted hitherto by the use of purified membrane fractions from heart muscle [3,5,8,9] or brain synaptosomes [2,10,35]. The results suggested that vesicular membranes derived from heart muscle or from synaptosomes contain both reversible Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake processes although contaminations from mitochondria or endoplasmic reticulum could not be excluded [3,10]. No serious attempts are known to have been made to identify the supposed carrier,

which presumably is an integral plasma membrane protein. This seems to be extremely difficult using sarcolemmal vesicles, as even the best-characterized preparations consist of at least 20 proteins of different molecular weights [36,37].

We decided to purify plasma membranes from brain showing higher purity with respect to ($Na^+ + K^+$)-ATPase activity and to the protein composition of the plasma membrane. The ($Na^+ + K^+$)-ATPase preparations purified by the use of a deoxycholate-KCl treatment of the brain microsomes showed no vesicular structures according to electron microscopic examination (unpublished results) thus being unsuitable for transport studies. For demonstration of an Na^+-Ca^{2+} exchange activity in ($Na^+ + K^+$)-ATPase preparations from brain it was therefore necessary to prepare proteoliposomes. The cholate dialysis-reconstitution procedure described by Goldin [26] has been improved in this work, resulting in increased ($Na^+ + K^+$)-ATPase activities and transport efficiency of the ($Na^+ + K^+$)-ATPase vesicles. In contrast to Sweadner and Goldin [37], who after reconstitution of ($Na^+ + K^+$)-ATPase from brain found recoveries of 7–13% of the ($Na^+ + K^+$)-ATPase activity and close to 100% of the Mg^{2+}-ATPase activity, we obtained in the vesiculized ($Na^+ + K^+$)-ATPase preparation formed from phosphatidylcholine recoveries of about 50% of ($Na^+ + K^+$)-ATPases and only 16% of Mg^{2+}-ATPase activities. This means that the majority of Mg^{2+}-ATPase activity could be removed during the incorporation procedure consisting finally of no more than 5% of the ($Na^+ + K^+$)-ATPase activity. Therefore, the present data strongly suggest that the plasma membrane is the source of the measured cation transports. This was further proved by showing that the proteoliposomes catalyze the ATP-dependent and ouabain-inhibitable Na^+ transport.

Miyamoto and Racker [17] described a procedure for preparation of proteoliposomes from plasma membrane of bovine heart muscle catalyzing Na^+-gradient-dependent Ca^{2+} accumulation. The authors did not give any data which would characterize the reconstituted and native vesicles with regard to protein composition. Thus, no information is available to allow the conclusion that the proposed Na^+-Ca^{2+} antiporter has been purified by the reconstitution procedure. However, the following pieces of evidence support our conclusion that an Na^+-Ca^{2+} antiporter of brain plasma membranes has been incorporated into proteoliposomes.

First, an Na^+ concentration gradient was required to accelerate Ca^{2+} movement across vesicle membrane (Fig. 5). The gradient-promoted Ca^{2+} transport was specific for Na^+, K^+ or Li^+ gradients were ineffective (Fig. 6). Second, the antiporter was specific for Ca^{2+}; a high Mg^{2+} excess did not inhibit Ca^{2+} antiport (Table II). Third, the Na^+ efflux depended on the Ca^{2+} concentration at the other side of the membrane, as shown by Blaustein and Russel [11] for Na^+-Ca^{2+} exchange in squid giant axons. Fourth, the Na^+ efflux was not inhibited by tetrodotoxin, known as an inhibitor of Na^+ conductance channels [39].

However, the Na^+-Ca^{2+} antiporter activity demonstrated could not be related to ($Na^+ + K^+$)-ATPase protein, since it was absent from proteoliposomes with incorporated kidney ($Na^+ + K^+$)-ATPase devoid of contaminating proteins (Fig. 1).

The exclusion of ($Na^+ + K^+$)-ATPase involvement in Na^+-gradient-promoted Ca^{2+} antiport (Fig. 9) or in ATP-driven Ca^{2+} transport coincides with the demonstration that proteoliposomes with incorporated kidney ($Na^+ + K^+$)-ATPase showed neither Ca^{2+}-activated K^+ transport nor bumetanide-inhibited N^+/K^+ cotransport [34].

The antiporter activity may reside in one or more of the proteins contaminating the brain ($Na^+ + K^+$)-ATPase preparation (Fig. 1). Thus, the brain may be useful for purifying the antiporter protein following its specific activity in proteoliposomes.

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