Biochimica et Biophysica Acta, 772 (1984) 127–134
Elsevier

BBA 72078

RECONSTITUTION OF THE SODIUM-CALCIUM EXCHANGER FROM CARDIAC SARCOLEMMAL VESICLES

SISTO LUCIANI *

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138 (U.S.A.)

(Received January 4th, 1984)

Key words: Na^+-Ca^{2+} exchange; Ca^{2+} transport; Protein-lipid interaction; Membrane reconstitution; (Dog cardiac sarcolemma)

The Na^+-Ca^{2+} exchanger was extracted from cardiac sarcolemmal vesicles and reconstituted into phospholipid vesicles by a cholate-dialysis method. Reconstitution was attempted with different phospholipids. Phosphatidylcholine alone was ineffective, whereas phosphatidylcholine and phosphatidylethanolamine (1:1, w/w) showed high activity, but a significant Ca^{2+} uptake in the absence of Na^+ gradient. Optimal reconstitution was obtained with a mixture of phosphatidylcholine and phosphatidylserine (9:1, mol/mol). The reconstituted proteoliposomes showed an ouabain-sensitive ($Na^+ + K^+$)-ATPase activity and a Na^+-Ca^{2+} exchange with a specific activity comparable to that of the original vesicles. The specificity toward Na^+ was also recovered. A partial purification of the exchanger was obtained by the method of transport-specificity fractionation (Goldin, S.M. and Rhoden, V. (1978) J. Biol. Chem. 253, 2575–2583). When proteoliposomes were reconstituted with sodium oxalate inside and incubated with calcium in the presence of an outwardly directed Na^+ gradient, the vesicles containing the Na^+-Ca^{2+} exchanger specifically accumulated calcium which precipitated inside as calcium oxalate. The resulting increase in density allowed separation of the proteoliposomes containing the Na^+-Ca^{2+} exchanger from the rest of the vesicles on a sucrose density gradient.

Introduction

Calcium has an important role in heart-muscle contraction. In fact it has been known for a century (Ringer, 1883) that heart contraction in vitro depends upon the presence of calcium in the external bathing medium [1].

Three transport systems have been found that allow the entrance and exit of calcium across the sarcolemma during the contration-relaxation cycle in cardiac muscle. These systems are the slow Ca^{2+} channel sensitive to verapamil, an ATP-dependent Ca^{2+} pump, and the Na^+-Ca^{2+} antiport catalyzing Ca^{2+} entrance and/or exit in exchange for Na^+ [2–4]. The third system is regulated by the sodium gradient across the sarcolemmal membrane. The Na^+-Ca^{2+} exchange might be used either to maintain a low concentration of free intracellular calcium or to bring Ca^{2+} into the cell during the systolic phase of contraction, or both. The exchange may function in either direction, depending upon the direction of the Na^+ and Ca^{2+} gradients.

The evidence for Na^+-Ca^{2+} exchange across the cardiac sarcolemma is mainly derived from experiments performed on isolated heart-muscle preparations. Recently, Reeves and Sutko [5] were able to demonstrate a Na^+-Ca^{2+} exchange activity

* Present address: Department of Pharmacology, University of Padova, Largo E. Meneghetti, 2 - 35100 Padova, Italy.

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

in resealed vesicles derived from cardiac sarcolemma. Following a partial purification of this system [6–8] from heart sarcolemma, a series of studies on its properties have been conducted [8–10].

The study of the kinetic of Na^+-Ca^{2+} exchange activity indicates that in sarcolemmal vesicles [8–10] more than two Na^+ ions are exchanged for each Ca^{2+}, thus implying that the exchange is electrogenic. An electrogenic Na^+-Ca^{2+} exchange has also been demonstrated in squid axon [11]. However, the evaluation of the stoichiometry of the Na^+-Ca^{2+} exchange is complicated by the presence of other mechanisms that regulate intracellular Ca^{2+} concentration. In order to characterize the kinetic properties and the physiological role of the Na^+-Ca^{2+} exchange, the reconstitution of the exchange activity into phospholipid vesicles was attempted. The reconstitution has also been used as a tool for the purification of the Na^+-Ca^{2+} exchanger.

The results indicate that Na^+-Ca^{2+} exchange can be extracted and reconstituted in artificial phospholipid vesicles. A partial purification was also achieved.

Experimental procedures

Chemicals. $^{45}CaCl_2$ was from New England Nuclear (Boston, MA), ouabain from Sigma (St. Louis, MO). Amiloride, originally from Merck Sharp & Dohme (NJ), was a gift from Lewis Cantley, Harvard University. Alamethicin was kindly provided by Dr. Joseph E. Grady (Upjohn Compnay, Kalamazoo, MI). Verapamil was kindly supplied by Knoll Pharmaceutical Company (NJ). Cholic acid was crystallized from 95% ethanol. Phosphatidylcholine was purified from fresh egg-yolk by chloroform + methanol extraction (2 : 1, v/v) followed by five successive acetone precipitations from diethyl ether (10 : 1, v/v). Purified lipid was stored as a suspension (100 mg/ml) in water containing 5 mM 2-mercaptoethanol at −70°C under nitrogen. Phosphatidylserine was obtained from PL-Biochemicals (Milwaukee, WI). All other reagents were of the highest analytical grade available.

Preparation of sarcolemmal vesicles. Dog heart sarcolemmal vesicles were prepared by a modification of the procedure described by Pitts [8]. Dog hearts were a gift of the Cardiovascular Research Department of Massachusetts General Hospital. Left ventricles (120–150 g) were thoroughly washed in ice-cold saline, freed of pericardium and endocardium, finely minced with scissors and homogenized in 400 ml of 0.6 M sucrose, 10 mM imidazole/HCl (pH 7) with a commercial Waring Blendor (5 × 10 s) followed by two strokes in a teflon-glass homogenizer. The homogenate was centrifuged at 12000 × *g* for 30 min. The supernatant was diluted with 600 ml of 160 mM NaCl/20 mM Mops-Tris (pH 7.4) and centrifuged at 96000 × *g* for 60 min. The sediment was resuspended in 0.25 M sucrose/0.3 M NaCl/50 mM sodium pyrophosphate/0.1 M Tris (pH 7.1) and laid over 0.6 M sucrose/0.3 M NaCl/50 mM sodium pyrophosphate/0.1 M Tris (pH 7.1) and centrifuged at 170000 × *g* for 90 min [6]. The white membrane banding at the interface was collected, diluted in 160 mM NaCl, 20 mM Mops-Tris (pH 7.4) and centrifuged at 170000 × *g* for 30 min. The pellet, resuspended in NaCl-Mops medium at a protein concentration of 3–4 mg/ml, was quickly frozen in liquid nitrogen and stored at −70°C for up to 3–4 weeks. The average yield was 4–5 mg of protein.

Reconstitution of the Na^+-Ca^{2+} transporter. Reconstitution was achieved by cholate extraction of the vesicles followed by dialysis as described by Goldin [12]. Sarcolemmal vesicles (1 mg protein) were added to a clarified dispersion (Vortex mixing under N_2) of phospholipids (20 mg/ml) in 0.5 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 20 mg/ml sodium cholate, 20 mM Tris-Mops buffer (pH 7.4). The mixture was incubated for 2 min at room temperature and for 25 min at 0°C. The rechilled mixture was spun-down at 100000 × *g* for 10 min, and the supernatant was dialyzed at 4°C in a hollow-fiber apparatus against 800 ml of 80 mM NaCl/40 mM sodium oxalate/20 mM Na-Hepes (pH 7.4)/1 mM dithiothreitol for 16 h. The vesicles formed were then dialyzed against 200 ml of 160 mM NaCl/0.8 mM sodium oxalate/20 mM Na-Hepes (pH 7.4)/1 mM dithiothreitol for 4 h to remove most of the external sodium oxalate.

Gradient centrifugation of the reconstituted proteoliposomes. Linear gradients were formed in 5-ml ultracentrifuge tubes from (a) equal volumes of a

low density solution of 325 mM glycerol/75 mM sucrose/20 mM Tris-Mops (pH 7.4) and a high density solution of 400 mM sucrose/20 mM Tris-Mops (pH 7.4) or (b) equal volumes of a low density solution of 150 mM choline chloride/10 mM Tris-Hepes and a high density solution of 300 mM sucrose/10 mM Tris-Hepes.

The gradients were centrifuged for 5 h at 49000 rpm at 3°C in an SW 50.1 Beckman rotor. 100–130-μl fractions were collected by piercing the bottom of the tubes.

The fractions collected were assayed for inorganic phosphate, ^{45}Ca, Na^+-Ca^{2+} exchange activity and polypeptide composition by SDS acrylamide gel electrophoresis.

$(Na^+ + K^+)$-ATPase. The activity of this enzyme was measured by a coupled spectrophotometric assay [13] using a Cary model 15 spectrophotometer with the following medium: 100 mM NaCl, 20 mM KCl, 1.0 mM ATP, 2.0 mM $MgCl_2$, 20 mM Tris-Mops (pH 7.4), 0.10 mM EDTA, 1.0 mM dithiothreitol, 1.5 mM phospho*enol*pyruvate, 0.25 mg/ml NADH, 20 μg/ml pyruvate kinase, 20 μg/ml lactate dehydrogenase, at 37°C. Total unmasked ATPase activity was measured in the presence of alamethicin [6], which produced a 3–4-fold stimulation of the basal activity. The ouabain-sensitive fraction of the total activity was defined as $(Na^+ + K^+)$-ATPase activity.

Measurement of Ca^{2+} uptake. (A) Sarcolemmal vesicles. 5 μl Na^+-loaded vesicles (10–15 μg protein) were diluted 1 : 100 either in NaCl, KCl or 160 mM choline chloride medium/20 mM Tris-Mops (pH 7.4)/40 μM $^{45}CaCl_2$ (100–200 cpm/pmol) in a total volume of 500 μl and incubated at 37°C. Samples of 50 μl were filtered through a Millipore filter (0.45 μm) washed three times with 3 ml of ice-cold 160 mM KCl/20 mM Tris-Mops (pH 7.4) 1 mM $LaCl_3$. The dried filters were counted by liquid scintillation spectroscopy (Aquasol). (B) Proteoliposomes. Reconstituted proteoliposomes (1–3 μg protein) were diluted in NaCl or KCl or 160 mM choline chloride medium/20 mM Tris-Mops (pH 7.4)/40 μM $^{45}CaCl_2$ (100–200 cpm/pmol) and incubated at 37°C. ^{45}Ca uptake was measured [12] by separation of the vesicles from the external solution on a Sephadex G-50 (coarse) column (0.7 × 20 cm, 0.6 ml/min). The column was equilibrated and eluted at 4°C with nonradioactive medium identical with that in which the samples were incubated. 16 fractions were collected at 30-s intervals, diluted in scintillation fluid (Aquasol) and counted. In some cases, ^{45}Ca uptake was measured by the method of Gasko et al. [14] as described by Levenson et al. [15].

Additional methods. Protein was estimated by the method of Lowry et al. [16] in the presence of 1% sodium dodecylsulphate. Inorganic phosphate was measured by using the ashing procedures of Ames [17]. Polyacrylamide SDS gel electrophoresis was performed according to the method of Laemmli [18].

Results

Sarcolemmal vesicles

A highly purified preparation of cardiac sarcolemma was obtained by modifications of recently published isolation procedures [6–8]. The preparation obtained had a $(Na^+ + K^+)$-ATPase specific activity of 89 μmol/mg protein/h, and a Na^+-Ca^{2+} exchange of 45 nmol of Ca^{2+} uptake per mg of protein at equilibrium.

The Na^+-Ca^{2+} exchange was sensitive to lanthanum (100 μM) and insensitive to verapamil

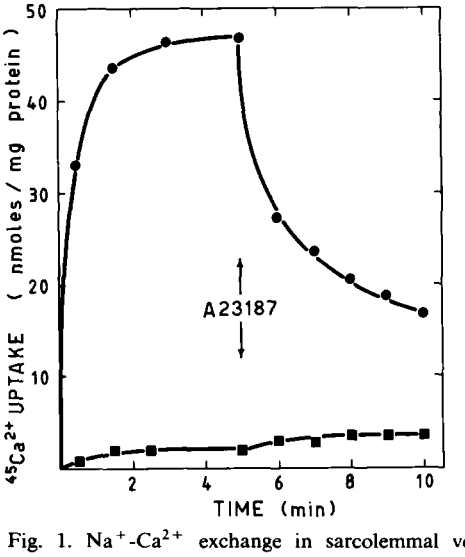

Fig. 1. Na^+-Ca^{2+} exchange in sarcolemmal vesicles. ^{45}Ca uptake was measured by diluting 1 : 100 sarcolemmal vesicles into a medium containing 40 μM $CaCl_2$ and either 160 mM KCl (●------●) or 160 mM NaCl (■------■) 20 mM Tris-Mops (pH 7.4). Temperature was 37°C. The concentration of calcium ionophore A23187 dissolved in dimethylsulfoxide was 1 μM.

in agreement with the results reported by others [5,9]. The rate of Ca^{2+} uptake is shown in Fig. 1. It can be seen that the uptake of calcium reaches equilibrium at 2.5 min and that the addition of calcium ionophore (A 23187) at the plateau induces the release of most of the calcium taken up. The uptake of calcium in the absence of a Na^+ gradient is less than 10% of that in the presence of a Na^+ gradient.

Amiloride, a known inhibitor of passive sodium transport [19], reduced the Na^+-Ca^{2+} exchange in sarcolemmal vesicles (Fig. 2). From the results reported in Fig. 2, it appears that the rate of Ca^{2+} uptake is 50% reduced by 200 μM amiloride.

The observed inhibition is in agreement with the results reported by Smith et al. [20] in murine erythroleukemia cells; the higher sensitivity of the latter preparation may be explained by the reported property of the cells of accumulating amiloride [20].

Reconstituted proteoliposomes

Reconstitution of Na^+-Ca^{2+} exchange from sarcolemmal vesicles in proteoliposomes was achieved by cholate extraction at high ionic strength followed by dialysis in a hollow-fiber apparatus [12]. This method was found advantageous with respect to the dilution procedure [21], because the latter increased the nonspecific Na^+-Ca^{2+} exchange, namely the uptake of calcium in the absence of Na^+ gradient. A large excess of added phospholipids (protein/phospholipid ratio 1 : 20) was required for optimal reconstitution, using the dialysis method. A similar protein to phospholipid dilution was also shown to be a requirement in the reconstitution of Ca^{2+}-ATPase [21].

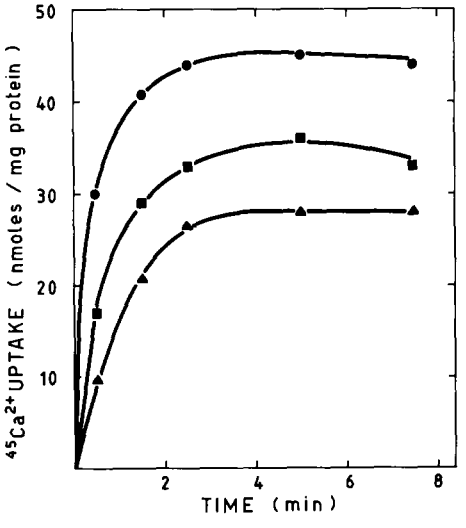

Fig. 2. Effect of amiloride on Na^+-Ca^{2+} exchange. Experimental conditions as in Fig. 1. Sarcolemmal vesicles were preincubated in the presence of amiloride for 20 min at 37°C before the addition to the $CaCl_2$/KCl medium. Control, (●———●); 190 μM amiloride, (■———■); 360 μM amiloride, (▲———▲).

It has been shown in several reconstitution experiments (see e.g. Ref. 26) that the presence of negatively charged phospholipids may be required for restoration of membrane-linked enzymatic activities.

It has been reported recently [25] that the presence of cholesterol and negatively charged lipids greatly affects the reconstitution of the acetylcholine receptor. The effect of defined phospholipids was therefore studied in the reconstitution of Na^+-Ca^{2+} exchange.

As shown in Table I, in the presence of phosphatidylcholine as the only phospholipid in the reconstitution medium, the exchange activity recovered was very low, whereas with a mixture of phosphatidylcholine and phosphatidylserine (9 : 1,

TABLE I

EFFECT OF PHOSPHOLIPID COMPOSITION ON RECONSTITUTION OF Na^+-Ca^{2+} EXCHANGE

Reconstitution was performed as described in experimental procedures with 26 μmol PC, or 24 μmol PC + 3 μmol PS, or 13 μmol PC + 13 μmol PE or 26 μmol acetone-washed asolectin. Ca^{2+} uptake was measured as described in the experimental procedure after 5 min of incubation at 37°C. The incubation medium was 160 mM choline chloride/20 mM Tris-Mops (pH 7.4) (+ Na^+ gradient) or 160 mM NaCl/20 mM Tris-Mops (pH 7.4) (− Na^+ gradient).

Phospholipids	Na^+-Ca^{2+} exchange (nmol Ca uptake/mg protein)		
	+ Na^+ gradient	− Na^+ gradient	Ratio
PC (3)	3 (2.0– 3.8)	1.0	3
PC + PS (5)	14(11.2–16.1)	1.5(1.1–1.9)	9.3
PC + PE (2)	15	9.0	1.5
Asolectin (3)	20(18.8–22.2)	4.0(3.1–4.8)	5

mol/mol) or with asolectin the exchange activity reconstituted was high. The mixture of phosphatidylcholine with phosphatidylethanolamine elicited a significant exchange activity, but also a very high Ca^{2+} uptake in the absence of Na^+ gradient.

A typical reconstitution experiment is reported in Table II. It can be seen that both Na^+-Ca^{2+} exchange and ouabain-sensitive ($Na^+ + K^+$)-ATPase have been reconstituted. The enzymatic activity ($Na^+ + K^+$)-ATPase (plasma membrane marker) is stimulated by alamethicin. This indicates that the enzymatic activity has been reconstituted in sealed vesicles. The fraction of Ca^{2+} uptake which is dependent on a Na^+ gradient is somewhat lower than in sarcolemmal vesicles.

The specificity of the exchange toward Na^+ was tested by dialyzing the membrane/phospholipid/cholate mixture against a medium containing K^+ or equimolar Na^+ and K^+ instead of Na^+. As shown in Table III, the uptake of Ca^{2+} in reconstituted proteoliposomes with K^+ inside was 20–25% compared to that in proteoliposomes containing Na^+, and only partially sensitive to the K^+ gradient. When proteoliposomes were reconstituted with both Na^+ and K^+ inside and the Na^+-Ca^+ exchange activity was tested either in the presence of a Na^+ gradient (K^+ outside) or in the presence of a K^+ gradient (Na^+ outside), they showed a remarkable specificity toward Na^+, with an uptake of Ca^{2+} in a K^+ gradient of 5% of that in a Na^+ gradient. No significant Na^+-dependent Ca^{2+} uptake could be detected when reconstitution was performed as described above but in the absence of membrane proteins.

TABLE III

Na^+-Ca^{2+} EXCHANGE IN RECONSTITUTED PROTEOLIPOSOME IN THE PRESENCE OF Na^+ OR K^+ GRADIENTS

These values are corrected for the uptake in liposomal vesicles prepared in the absence of protein. Proteoliposomes were reconstituted as described in the experimental procedures by using PC + PS (9:1, mol/mol).

Ion inside the proteoliposomes	Ion in the medium	Ca^{2+} uptake (nmol/mg protein)	
		2 min	5 min
Na^+	Na^+	1.1	1.5
Na^+	K^+	10.6	13.8
K^+	K^+	0.8	1.2
K^+	Na^+	2.5	4.3
$Na^+ + K^+$	Na^+	0.2	0.3
$Na^+ + K^+$	K^+	4.2	6.7

Purification of the Na^+-Ca^{2+} exchanger

In order to achieve a purification of the reconstituted Na^+-Ca^{2+} exchanger, a method was devised to separate the proteoliposomes containing the exchanger from the rest of the preparation. This method was a modification of the transport specificity fractionation procedure introduced by Goldin [22,23].

The method makes use of the property of proteoliposomes reconstituted in the presence of potassium oxalate to take up Ca^{2+} in exchange for Na^+. In this preparation with a high phospholipid to protein ratio (20:1), it is likely that the cholate solubilizable components of the membrane are randomly distributed. In these systems each vesicle is able to incorporate only one or at most a few

TABLE II

Na^+-Ca^{2+} EXCHANGE AND ($Na^+ + K^+$)-ATPase OF SARCOLEMMAL VESICLES AND RECONSTITUTED PROTEOLIPOSOMES

Alamethicin was 1 mg/mg protein, ouabain 1 mM. Other conditions were as described in Experimental procedures.

	Na^+-Ca^{2+} exchange (nmol Ca^{2+} uptake/mg protein)		($Na^+ + K^+$)-ATPase (μmol/mg protein)		
	+ Na^+ gradient	− Na^+ gradient	none	alamethicin	alamethicin + ouabain
Sarcolemmal vesicles	27.2	1.4	30.6	98.2	16.0
Proteoliposomes	20	4	6	50	5

membrane proteins. Therefore, only a fraction of the reconstituted proteoliposomes will contain the Na^+-Ca^{2+} exchanger.

The proteoliposomes containing the Na^+-Ca^{2+} exchagner will take up calcium which precipitates as calcium oxalate, increasing the density of the proteoliposomes and leading to their more rapid movement in a sucrose gradient as compared to proteoliposomes without the transporter. Therefore, if the Ca^{2+} content (^{45}Ca) and the phospholipid phosphorus are measured in the fractions recovered after a sucrose gradient, the ^{45}Ca profile should show a shift with respect to the main phospholipid peak. As shown in Fig. 3, a small shift of the calcium peak with regard to the phospholipid peak has been obtained, indicating that the calcium loaded vesicles migrate in a denser region of the gradient.

In order to test the activity of the separated fraction, the proteoliposomes were partially loaded with calcium by a short term (3 min) incubation in the presence of unlabeled Ca^{2+}, before being subjected to the sucrose gradient. The procedure was sufficient to obtain the separation and allowed at the same time the determination of the exchange

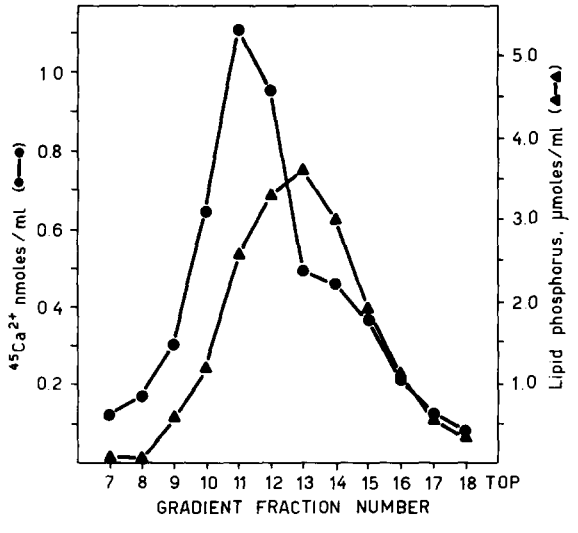

Fig. 3. Distribution of ^{45}Ca within vesicles separated on a sucrose gradient (gradient (a) of experimental procedures). Reconstituted proteoliposomes were incubated for 7 min in a medium comprising 160 mM KCl/40 µM $CaCl_2$/20 mM Tris-Mops. External $^{45}CaCl_2$ was removed by filtering the vesicles through Chelex before layering on top of the gradient.

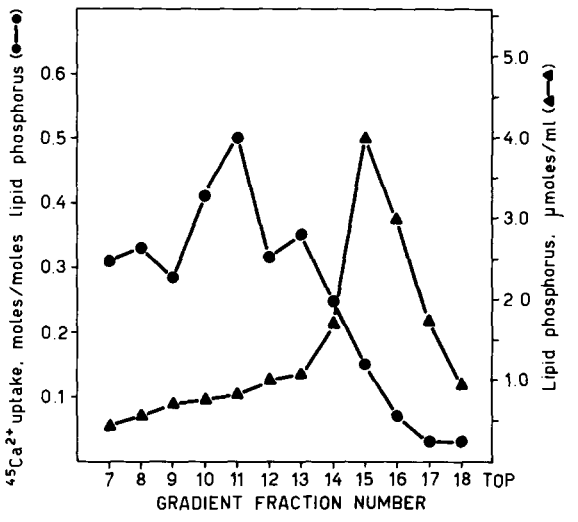

Fig. 4. Na^+-Ca^{2+} exchange distribution on a sucrose gradient (gradient (b) of experimental procedures). Before being placed on the gradient, the vesicles were incubated for a short time (3 min) with unlabeled calcium in the presence or in the absence of Na^+ gradient. Na^+-Ca^{2+} exchange activity was measured on the fractions after the sucrose gradient. Na^+-Ca^{2+} exchange was measured as described in the experimental procedures. The values of Na^+-Ca^{2+} exchange represent the difference between the uptake of $^{45}Ca^{2+}$ in the presence of Na^+ gradient and that in the absence of Na^+ gradient.

activity. As shown in Fig. 4, the main peak of the Na^+ gradient-dependent exchange activity appeared to be shifted from the bulk of the reconstituted proteoliposomes to a position coincident with that of the fraction showing the highest ^{45}Ca content (see Fig. 3).

Most (80%) of the added protein remained associated with the main proteoliposome peak.

Fig. 4 also shows that the Na^+-Ca^{2+} exchange activity of the peak fraction after the gradient was 500 nmol of $^{45}Ca^{2+}$/µmol lipid phosphorus. This figure can be compared with the activity of 1 nmol Ca^{2+}/µmol lipid phosphorus observed in the reconstituted proteoliposomes before the sucrose gradient. Although the extreme dilution of protein prevented the evaluation of the specific activity of the fractions showing $^{45}Ca^{2+}$ uptake, the observation reported above clearly indicates a partial purification of the Na^+-Ca^{2+} exchanger.

Taken together, these results indicate that the functionality of the Na^+-Ca^{2+} transporter allows the movement of a small fraction of the recon-

stituted proteoliposomes to a position of higher density in the sucrose gradient. Thus leading to a partial purification of the exchanger.

The fractions enriched in Na^+-Ca^{2+} exchange activity have been analyzed by SDS gel electrophoresis in order to identify the component(s) of the exchanger. However, the partial purification achieved does not permit a clear identification of the specific component(s) of the Na^+-Ca^{2+} exchanger, due to incomplete resolution of the peak containing the Na^+-Ca^{2+} exchange activity.

Discussion

The results reported in this paper indicate that the Na^+-Ca^{2+} exchanger has been extracted from cardiac sarcolemma and reconstituted into phospholipid vesicles, where it retains the characteristic Na^+ specificity which it has in the original membrane.

The specific activity of the reconstituted proteoliposomes is comparable to the activity of the native membrane, indicating that the carrier has not been denaturated during the solubilization. However, since 50–60% of the original membrane protein is extracted by cholate, it is also evident that only a fraction of the total Na^+-dependent Ca^{2+} uptake is recovered. A partial incorporation of the exchanger into phospholipid vesicles may explain this observation.

The main objective of this work was to achieve a partial purification of the Na^+-Ca^{2+} exchanger by extraction of the membrane protein and its reconstitution into phospholipid vesicles.

Unlike the situation with the membrane ATPases which have enzymatic activity which can be measured independently from transport, in the case of an exchange protein (antiport) the estimation of ion exchange is the only 'proof' of reconstitution. Therefore, it was essential for the reconstitution of Na^+-Ca^{2+} exchange activity to incorporate the transporter into tight phospholipid vesicles in a way which allowed measurement of the transport function.

The results of this paper indicate that the lipid composition of the reconstitution mixture is important to achieve proper reconstitution. In fact, no reconstitution was obtained in the presence of PC alone, whereas the use of the mixture of PC and PE (1 : 1) resulted in proteoliposomes with an exchange activity poorly sensitive to the presence of a Na^+ gradient. The best reconstitution was obtained with a mixture of PC and PS. Even in the absence of a more complete investigation on the specific lipid requirement for the reconstitution of the Na^+-Ca^{2+} exchanger, the observations reported above deserve some comment.

The importance of the negatively charged phospholipids for the reconstitution of membrane-bound enzymes has been demonstrated in several cases [26].

It is possible that the presence of negatively charged phospholipids in the bilayer induces an expanded bilayer more suitable to accommodate protein conformational changes and therefore an active Na^+-Ca^{2+} exchange. This hypothesis is supported by a recent paper [31] in which it is shown that phospholipase C enhances Na^+-Ca^{2+} activity of sarcolemmal cardiac membranes.

The effect of phospholipase C is associated with an enrichment of negatively charged phospholipids. However, an effect of diacylglycerol generated from phospholipid hydrolysis cannot be excluded under these conditions.

Observations indicating preferential requirement for negatively charged phospholipids have already been reported in the case of other integral proteins, like the mitochondrial ATPase complex [27] and the adenine nucleotide carrier [28].

When the PC/PE mixture is used, it appears that a leaky proteoliposome is formed which is able to take up calcium even in the absence of a sodium gradient. This might be due to the tendency of PE containing structures to reduce the membrane bilayer integrity [32].

The reconstitution of the Na^+-Ca^{2+} exchanger in the presence of sodium oxalate with a large excess of phospholipid was useful in the partial purification of the reconstituted proteoliposomes by the transport-specificity fractionation procedure. The increase of the relative density of the proteoliposomes containing the exchanger induces a shift of the proteoliposomes with a high Na^+-Ca^{2+} exchange activity toward the lower portion of the gradient.

A partial purification of the Na^+-Ca^{2+} exchange activity from bovine heart has been recently reported by Miyamoto and Racker [24].

Although the results reported by these investigators generally agree with those reported in this paper, no direct comparison can be made, because the starting material of Miyamoto and Racker was a crude preparation of plasma membrane (spec. act. 6 nmol/mg per min) whereas a purified preparation (spec. act. 35 nmol/mg per min) has been used in this work.

While this manuscript was in preparation, two reports appeared showing the reconstitution of Na^+-Ca^{2+} exchange from rat brain [29] and chick heart [30].

Acknowledgments

I thank Guido Guidotti for his kind hospitality and support during the course of this work, Lewis Cantley, Gilbert Chin, Michael Forgac (Harvard), Alessandro Bruni and Franca Carpenedo (Padova) for helpful discussions and Annamaria Malosti, Roberta Sato and Roberto Varotto for editing. This work was done during the tenure of NATO-Senior and Fulbright fellowships and supported by NIH Grant HL 08893 and NSF Grant PCM-04364 (to Guido Guidotti) and CNR Italia-USA Grant CT. 81.00252.04 (to S.L.).

References

1 Ringer, S. (1883) J. Physiol. (Lond.) 3, 29–42
2 Reuter, H. and Seitz, N. (1968) J. Physiol. (Lond.) 195, 451–470
3 Langer, G.A. (1968) Physiol. Rev. 48, 708–757
4 Sulake, P.V. and St. Louis, P.J. (1980) Prog. Biophys. Mol. Biol. 35, 135–195
5 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 590–594
6 Jones, L.R., Maddock, S.W. and Besh, H.R. (1980) J. Biol. Chem. 255, 9971–9980
7 Bartshat, D.K. and Lindenmayer, G.E. (1980) J. Biol. Chem. 255, 9626–9634
8 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235
9 Caroni, P., Reinlib, L. and Carafoli, E. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6354–6358
10 Philipson, K.D. and Nishimoto, A.Y. (1980) J. Biol. Chem. 255, 6880–6882
11 Blaustein, M.P. (1977) Biophys. J. 20, 79–111
12 Goldin, S.M. (1977) J. Biol. Chem. 252, 5630–5642
13 Forgac, M.D. (1979) J. Biol. Chem. 255, 1547–1553
14 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Soulinna, E.M. and Racker, E. (1976) Anal. Biochem. 72, 57–65
15 Levenson, R., Housman, D. and Cantley, L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5948–5952
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Ames, B.N. (1966) Methods Enzymol. 8, 115–117
18 Laemmli, U.K. (1970) Nature 227, 680–685
19 Bentley, P.S. (1968) J. Physiol. (Lond.) 195, 317–330
20 Smith, R.L., Macara, I.G., Levenson, R., Housman, D. and Cantley, L. (1982) J. Biol. Chem. 257, 773–780
21 Knowles, A.F., and Racker, E. (1975) J. Biol. Chem. 250, 3538–3544
22 Papazian, D., Rahamimoff, H. and Goldin, S.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3708–3712
23 Goldin, S.M. and Rhoden, V. (1978) J. Biol. Chem. 253, 2575–2583
24 Miyamoto, H. and Racker, E. (1980) J. Biol. Chem. 255, 2656–2658
25 Ochoa, E.L.M., Dalziel, A.W. and McNamee, M.G. (1983) Biochim. Biophys. Acta 727, 151–162
26 Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209–237
27 Bruni, A., Pitotti, A., Palatini, P., Dabbeni-Sala, F. and Bigon, E. (1979) Biochim. Biophys. Acta 545, 404–414
28 Krämer, R. and Klingenberg, M. (1980) FEBS Lett. 56, 257–260
29 Schellenberg, G.D. and Swanson, P.D. (1982) Biochim. Biophys. Acta 690, 133–144
30 Wakabayashi, S. and Goshima, K. (1983) Biochim. Biophys. Acta 693, 125–133
31 Philipson, K.D., Frank, J.S. and Nishimoto, A.Y. (1983) J. Biol. Chem. 258, 5905–5910
32 Cullis, P.R., De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420