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PARTIAL PURIFICATION OF Na^+-Ca^{2+} ANTIPORTER FROM PLASMA MEMBRANE OF CHICK HEART

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To study Na^+-Ca^{2+} exchange, proteins of membrane vesicles from chick hearts were solubilized with cholate in the presence of phospholipids and the cholate extract was treated with pronase. These purified proteoliposomes, reconstituted by subsequent dilution and centrifugation to eliminate the cholate, catalyzed Ca^{2+} uptake depending on the intraliposomal Na^+ (Na_i^+) concentration. The maximal amount of Ca^{2+} accumulating in the liposomes was 140 nmol/mg protein and the initial rates of Na_i^+-dependent Ca^{2+} uptake were routinely 20 to 40 nmol/mg per 3 s at 25°C, but only 2 to 4 nmol/mg per 3 s for the crude proteoliposomes from the cholate extract not treated with pronase. Thus the pronase treatment resulted in 10-fold purification. Na_i^+-dependent Ca^{2+} uptake by purified proteoliposomes was 30- to 50-fold higher than that by the initial membrane vesicles. The fundamental properties of Na_i^+-dependent Ca^{2+} uptake in purified proteoliposomes such as K_m for Ca^{2+}, the sensitivity for Na^+ and pH dependency, were nearly equal to those in membrane vesicles and crude proteoliposomes. Thus, pronase treatment was very useful for obtaining reconstituted liposomes containing highly enriched Na^+-Ca^{2+} antiporters which were functionally intact.

Introduction

In many tissues, the intracellular concentration of free Ca^{2+} is maintained at a low value ($\leqq 10^{-7}$ M) by extrusion of Ca^{2+} from the cytoplasm, while the extracellular concentration of Ca^{2+} is high ($\geqq 10^{-3}$ M). Two systems in the plasma membrane extrude Ca^{2+}, the ATP-driven Ca^{2+} pump system [1–5] and the Na^+-Ca^{2+} exchange system [6,8]. A concentration gradient of Ca^{2+} generated by Na^+-Ca^{2+} exchange is supported by energy from both a gradient of Na^+ and membrane potential.

Recent extensive studies on cardiac muscle have concentrated on simple experimental systems, such as cultured cells [9–12] and sarcolemma vesicles [13–22], and several reports have appeared on the properties of Na^+-Ca^{2+} exchnage. It was found to exist specifically in the sarcolemma membrane [13–15]. It is electrogenic [11,13,15,17,18] and mediates the exchange of three or more Na^+ for one Ca^{2+} [10,12,14]. Ca^{2+} can also be exchanged for Ca^{2+} through the Na^+-Ca^{2+} exchange system [19,20], which is inhibited by low pH [10,22] and various di- or trivalent cations, such as Co^{2+}, Sr^{2+} and La^{3+} [9,10,15]. These findings are important for understanding the regulation of Ca^{2+} in vivo, but the molecular entity of Na^+-Ca^{2+} exchange remains to be studied. Miyamoto and Racker [23] recently reported the reconstitution of Na^+-Ca^{2+} antiporter solubilized with cholic acid from bovine heart membrane. They also reported

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

that the activity of Na^+-Ca^{2+} exchange was enhanced to severalfold that of the native membrane by incorporating the membrane proteins into artificial liposomes.

Based on the reports of many studies, there seems to be a carrier in the plasma membrane which mediates Na^+-Ca^{2+} exchange. Thus, this Na^+-Ca^{2+} antiporter should be purified to clarify the physiological role and molecular mechanism of Na^+-Ca^{2+} exchange. The purpose of our study was to purify the Na^+-Ca^{2+} antiporter and we found that the specific activity of Na^+-Ca^{2+} exchange markedly increased if the membrane proteins solubilized with cholic acid were treated with pronase, though we could not achieve a complete purification. We also examined whether the fundamental properties of Na^+-Ca^{2+} exchange are maintained during the purification procedure including pronase treament.

Methods

Materials. Cholic acid (from Wako Chem. Co.) was dissolved in ethanol, and then recrystallized by adding distilled water. Soybean phospholipids (L-α-lecithin, type II-S) were obtained from Sigma Chem. Co. and sonicated for 20 min with a bath-type sonicator (Kubota, 200 M) in a buffer containing 20 mM 4-morpholinepropanesulfonic acid (Mops)/Tris (pH 7.4). Ouabain and valinomycin were purchased from Sigma Chem. Co., pronase from Kaken Chem. Co., α-chymotrypsin and trypsin from P-L Chem. Co., and $^{45}CaCl_2$ and $^{22}NaCl$ from New England Nuclear.

Preparation of membrane vesicles. Membrane vesicles were prepared from 1-day-old chick heart by a method similar to that described previously [11]. Hearts were removed from the chicks and homogenized in a solution of 0.6 M sucrose, 30 mM imidazole/ HCl (pH 7.0) for 20 s in an Ultra Turrax Tissuemizer (IKA Werk). The homogenate was centrifuged at $12000 \times g$ for 30 min and the supernatant was diluted with one volume of a solution containing 140 mM KCl, 20 mM Mops/Tris and 1 mM EGTA (pH 7.4), and then centrifuged at $70000 \times g$ for 30 min. The pellet (membrane vesicles) was used for the subsequent purification procedure. If the pellet was used for measurement of Ca^{2+} uptake, it was suspended in a solution containing 140 mM NaCl or KCl, 20 mM Mops/Tris (pH 7.4). After centrifugation at $70000 \times g$ for 30 min, the pellet was resuspended in the same solution as that before centrifugation. The resulting membrane vesicles were preloaded with 140 mM NaCl or KCl by incubation for 50 min or more at 37°C. Membrane vesicles preloaded with various concentrations of 0 to 140 mM NaCl (isosmotically substituted for 0 to 140 mM KCl) were prepared in a similar way [11].

Solubilization, reconstitution and partial purification of Na^+-Ca^{2+} antiporter. Solubilization and reconstitution of Na^+-Ca^{2+} antiporter were performed by a method similar to that described previously [23]. The membranes (2 mg/ml protein) prepared as described above, were solubilized with 2% cholic acid (adjusted to pH 8.0 with NaOH) in the presence of 2.5% soybean phospholipid, 0.5 M NaCl and 20 mM Mops/Tris (pH 7.4) at 0–4°C. After centrifugation at $160000 \times g$ for 60 min, the supernatant was treated with 1 mg/ml pronase for 10 min at 37°C and then cooled in an ice bath to stop the treatment. Next, the cholate extract treated with pronase was diluted with five volumes of a solution containing 140 mM NaCl, 20 mM Mops/Tris (pH 7.4) and centrifuged at $160000 \times g$ for 30 min. Proteoliposomes were preloaded with Na^+ or K^+ by solubilizing the pellet containing reconstituted proteoliposomes with a solution containing 1% cholic acid (neutralized with NaOH or KOH), 140 mM NaCl or KCl and 20 mM Mops/Tris (pH 7.4) and then diluting this with five to seven volumes of a solution containing 140 mM NaCl or KCl, 20 mM Mops/Tris (pH 7.4). After centrifugation at $160000 \times g$ for 30 min, the pellet (purified proteoliposomes) was resuspended in the same diluting solution as before centrifugation. Crude proteoliposomes preloaded with Na^+ or K^+ were obtained in a similar way from the cholate extract not treated with pronase. Proteoliposomes preloaded with various concentrations of NaCl (0 to 140 mM in place of KCl) were also prepared in a similar way. All procedures except pronase treatment were performed at 0–4°C.

Measurement of Na^+-Ca^{2+} exchange. Na^+-Ca^{2+} exchange in membrane vesicles or proteoliposomes was measured at 25°C essentially as described previously with minor modifications [11]. In the present experiment, we measured Ca^{2+}

uptake using a rapid mixing apparatus. The uptake reaction was initiated by injecting 20 μl of sample suspended in a solution containing 0 to 140 mM NaCl (usually 0 and 140 mM) and 20 mM Mops/Tris (pH 7.4) from a micropipette associated with a solenoid-driven syringe into 500 μl of the uptake solution on the side of a plastic tube at 25°C and a timer was simultaneously started. Purified and crude proteoliposomes contained 0.3–0.5 and 1.0–1.5 mg/ml protein, respectively. Membrane vesicles contained 4–6 mg/ml protein. The uptake solution contained 140 mM KCl, 1 μCi/ml $^{45}CaCl_2$, 10 to 500 μM $^{40}CaCl_2$ (usually 50 μM) and 20 mM Mops/Tris (pH 7.4). The reaction mixture in the tube was vigorously mixed with a Vortex Mixer during the uptake periods. At a preset time, the reaction was stopped by injecting 100 μl of 80 mM $LaCl_3$ into the tube in response to a signal from the timer. Then 5 ml of the washing solution containing 100 mM $CoCl_2$ and 1 mM $LaCl_3$ was added to the reaction mixture and the total solution was applied to a Millipore filter (pore size: 0.45 μm) under suction at 25°C. This filter was washed twice with 5 ml of the washing solution and dried. The ^{45}Ca remaining on the filter was measured with a liquid scintillation spectrometer (Aloka). We routinely measured Ca^{2+} uptake by KCl-loaded vesicles or KCl-loaded proteoliposomes as control experiments. In the present experiment, we used $LaCl_3$ and $CoCl_2$ to both stop the reaction and wash the filter in order to: (1) remove surface-bound Ca^{2+}, (2) inhibit Ca^{2+}-leak from vesicles or proteoliposomes, and (3) inhibit non-specific binding of ^{45}Ca of the filter. When ^{45}Ca uptake by proteoliposomes was measured for various protein contents, the intraliposomal ^{45}Ca-radioactivity was linear to the protein content applied at least in the range of less than 30 μg/20 μl ($A_{400} = 60$). Therefore, all experiments of ^{45}Ca uptake were performed using proteoliposomes adjusted to the suitable protein concentration and suitable turbidity in the range of such linearity.

The activities of Ca^{2+} uptake by membrane vesicles and crude and purified proteoliposomes all were stable for at least 5 h at room temperature during the experiments.

Polyacrylamide gel electrophoresis. Gel electrophoresis of the proteins in the proteoliposomes was performed as described previously [24]. Membrane proteins were solubilized at room temperature in a dissociation medium containing 62.5 mM Tris/HCl, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol and 0.001% Bromphenol blue (pH 6.8), and then boiled for 1.5 min. Electrophoresis was performed on slabs 0.7 mm thick. Gels usually contained 10% acrylamide. After electrophoresis, protein profiles were monitored by the silver-staining method as described previously [25]. The molecular weights of protein standards used (from Pharmacia Fine Chem. Co.) were phosphorylase *b* (94000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactoalbumin (14400).

Other measurements. $(Na^+ + K^+)$-ATPase assay was performed by spectrophotometric measurement coupled with pyruvate kinase as described previously [26]. Azide-sensitive ATPase was measured as the fraction of ATPase activity inhibited by 10 mM sodium azide in the course of $(Na^+ + K^+)$-ATPase assay. ATP-dependent Ca^{2+} uptake (in the presence of 5 mM oxalate) was measured by a method similar to that described previously [27]. Total sugar content was measured by phenol-sulfonic acid reaction as described previously [28]. Protein was measured by the method of Lowry et al. [29].

Results

Ca^{2+} transport in proteoliposomes containing highly enriched Na^+-Ca^{2+} antiporter

Na^+- or K^+-preloaded proteoliposomes from cholate extract treated with pronase (purified proteoliposomes) were diluted with an Na^+-free solution containing ^{45}Ca. Thus, Ca^{2+} uptake was measured in the presence or absence of Na^+ gradient from the inside to the outside of liposomes (Fig. 1). Ca^{2+} uptake was rapid in the presence of Na^+ gradient, but only slight in its absence, showing that an Na^+ gradient was required for Ca^{2+} uptake. To determine only Na_i^+-dependent Ca^{2+} uptake, all data in the text were corrected for the Ca^{2+} uptake by K^+-preloaded proteoliposomes which must occur through systems other than Na^+-Ca^{2+} exchange, such as passive diffusion and the remaining of bound Ca^{2+}. Our apparatus (see

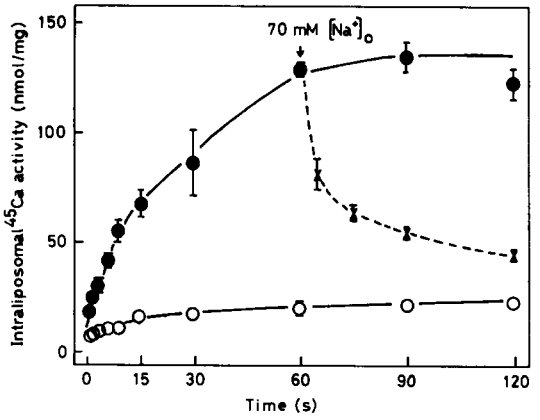

Fig. 1. Time-course of Ca^{2+} uptake by proteoliposomes from cholate extract treated with pronase. At time 0, 20 μl of proteoliposomes was rapidly added to 500 μl of a solution containing 140 mM KCl, 20 mM Mops/Tris (pH 7.4) and 50 μM labeled Ca^{2+} at 25°C. The reaction was stopped at the appropriate time by addition of 100 μl of 80 mM $LaCl_3$ (see Methods). (●) and (○) show the time-course of Ca^{2+} uptake by Na^+- and K^+-loaded proteoliposomes, respectively. At the time shown by the arrow, 500 μl of a medium containing 140 mM NaCl, 1 mM EGTA, 20 mM Mops/Tris (pH 7.4) was added to the reaction mixture and then the time course of decrease of intraliposomal ^{45}Ca activity was measured. Points and bars represent means ± S.D. for three to five determinations.

Methods) allowed rapid measurement of Ca^{2+} uptake (minimum period = 1 s). As shown in Fig. 1, the linearity of Na_i^+-dependent Ca^{2+} uptake was maintained for at least 3 s. It was also maintained for at least 3 s in both membrane vesicles and proteoliposomes from cholate extract not treated with pronase (data not shown). Thus, in the present study, the initial rate was measured as the Ca^{2+} uptake after 3 s of reaction.

In addition to the presence of Na_i^+-dependent Ca^{2+} uptake, the following results showed that the purified proteoliposomes contained Na^+-Ca^{2+} antiporters: (1) The maximal amount of Ca^{2+} uptake accumulated by Na^+-preloaded proteoliposomes reached 140 nmol/mg (Fig. 1). Proteoliposomes loaded with 50 μM Ca^{2+} and ^{45}Ca by a method similar to that of Na^+-preloading (see Methods) had a Ca content of about 24 nmol/mg, showing that under the conditions of Fig. 1, the Na^+-Ca^{2+} exchange system can concentrate Ca^{2+} to more than 5-fold the level of the external medium with the support of an Na^+-gradient. (2) Ca^{2+} accumulated inside the proteoliposomes was rapidly discharged when 70 mM Na^+ was added to the incubation solution, but slowly when a solution containing no Na^+ was added. (3) ^{22}Na was rapidly released from the purified proteoliposomes preloaded with 1 mM labeled Na in the solution containing 10 mM Ca^{2+}, but little was released in the Ca^{2+}-free solution (data not shown), suggesting that Na^+ efflux is coupled to Ca^{2+} uptake. (4) Monovalent cations such as K^+, Li^+, choline$^+$ (except Na^+), which were loaded in the purified proteoliposomes, did not stimulate Ca^{2+} uptake (data not shown).

Partial purification of Na^+-Ca^{2+} antiporter

We first tested the thermal stability of the cholate extract. Incubating it at a high temperature (≧ 37°C), markedly reduced the resulting Ca^{2+} uptake by reconstituted proteoliposomes. Proteoliposomes prepared from the cholate extract incubated for 10 min at 60°C completely lost Na_i^+-dependent Ca^{2+} uptake activity. After incubation of the extract at 37°C for 30 and 90 min, only 60% and 30% activity remained, respectively. However, after incubation for 10 min at 37°C, 90% or more activity remained. Therefore, the cholate extract was subjected to pronase treatment for 10 min at 37°C.

The initial rates of Na_i^+-dependent Ca^{2+} uptake for membrane vesicles and crude and purified proteoliposomes were compared (Table I). Equal volumes of vesicles suspensions or cholate extract were used for Na^+- or K^+-preloading (for control) in each preparation, and the resulting protein contents were equivalent for both preparations. In crude proteoliposomes from cholate extract not treated with pronase, the specific activity of Na_i^+-dependent Ca^{2+} uptake was about 5-fold higher than that of the native membrane vesicles, as reported earlier by Miyamoto and Racker [23]. On the other hand, proteoliposomes from cholate extract treated with pronase exhibited a high specific activity of Na_i^+-dependent Ca^{2+} uptake which was about 10-fold that in crude proteoliposomes and 50-fold that in membrane vesicles.

Proteoliposomes from cholate extract treated with pronase and subjected to dilution and centrifugation only once (without the preloading procedure, see Methods) had a Ca^{2+} uptake activity

TABLE I

PARTIAL PURIFICATION OF Na^+-Ca^{2+} ANTIPORTER

Preparation was started from 100 chick hearts (30 g tissue). The protein content in each fraction was adjusted to the value originating from 100 hearts. Enzyme treatment was performed for 10 min at 37°C at a concentration of 1 mg/ml.

Preparation	Enzyme treatment of cholate extract	Protein		Na_i^+-dependent Ca^{2+} uptake			
		mg	%	Specific activity		Total activity	
				nmol/mg/3 s	-fold	nmol/3 s	%
Membrane vesicles		41.1	100	0.63 ± 0.06 [a]	1.0	25.9	100
Proteo-liposomes	None	3.97	10	3.05 ± 0.33	4.8	12.1	47
	Pronase	0.43	1	29.7 ± 2.7	47.1	12.8	49
	α-Chymotrypsin	0.88	2	14.3 ± 1.2	22.7	12.6	49
	Trypsin	0.72	2	11.7 ± 0.7	18.6	8.4	32

[a] The uptake solution contained 50 μM $^{45}CaCl_2$ at 25°C. Values are means ± S.D. for four determinations.

which was not very high (about 10 nmol/mg per 3 s). The value (25–45 nmol/mg per 3 s) was higher for purified proteoliposomes after the Na^+-preloading procedure including solubilization, dilution and centrifugation. Thus, the preloading procedure was also indispensable to better purification of the Na^+-Ca^{2+} antiporter. The specific activity of Ca^{2+} uptake is probably enhanced by eliminating amino acids and peptide fragments which appeared with the pronase treatment. Subsequent repetitions of the preloading procedure did not cause further enhancement of Ca^{2+} uptake.

When the membrane vesicles were directly treated with pronase for 10 to 30 min at 37°C, the specific activity of Na_i^+-dependent Ca^{2+} uptake by the vesicles did not increase (data not shown). Moreover, proteoliposomes reconstituted from the vesicles treated with pronase did not exhibit as high an activity (only 4 to 5 nmol/mg per 3 s). This shows that pronase treatment of the cholate extract but not the vesicles suspensions is essential for obtaining good purification.

Recovery of the total activity was also high in the purified proteoliposomes. About 50% of the total activity in the membrane vesicles was recovered even when the cholate extract was treated with pronase (Table I), suggesting that Na^+-Ca^{2+} antiporter is not decomposed to an inactive form by pronase treatment.

High specific activity was also observed in proteoliposomes from the cholate extract treated with other proteases such as α-chymotrypsin and trypsin (Table I). However, α-chymotrypsin and trypsin was less favorable than pronase.

Table II shows the sugar contents and several enzymatic activities of membrane vesicles and crude and purified proteoliposomes. Both sugar content per mg protein and the specific activity of $(Na^+ + K^+)$-ATPase were higher in purified proteoliposomes than initial membrane vesicles, although the recoveries of these activities were only a few percent. No activities of azide-sensitive ATPase and ATP-dependent Ca^{2+} uptake could be detected in the purified proteoliposomes.

Fig. 2. shows polyacrylamide gel electrophoresis of crude and purified proteoliposomes. The silver-staining method employed is about 100-times more sensitive than the Coomassie blue-staining method [30]. More than 50 bands could be detected in crude proteoliposomes, while the purified proteoliposomes containing highly enriched Na^+-Ca^{2+} antiporter showed only five major bands corresponding to relatively small apparent molecular weights of 20 000, 21 000, 30 000, 32 000 and 36 000. Some minor additional bands were also seen in several regions. Polyacrylamide gel electrophoresis also showed that pronase did not contaminate the purified proteoliposomes (data not shown). Unfor-

TABLE II

SUGAR CONTENT AND SEVERAL ENZYMATIC ACTIVITIES

Preparation was started from 100 chick hearts (30 g tissue). Crude and purified proteoliposomes mean the preparations reconstituted from cholate extract not treated and treated with pronase (1 mg/ml), respectively. Values presented are the relative sugar content or specific enzyme activities. In parentheses percentage of total sugar content and total enzymatic activities in the membrane vesicles is shown. n.d., not detectable.

	Membrane vesicles	Crude proteoliposomes	Purified proteoliposomes
Sugar (mg/mg protein)	0.25 (100)	0.38 (19)	0.78(4)
$(Na^+ + K^+)$-ATPase (μmol/mg/h)	6.8 (100)	8.0 (15)	10.8 (2)
Azide-sensitive ATPase (μmol/mg/h)	3.2 (100)	n.d. (–)	n.d. (–)
ATP-dependent Ca^{2+} uptake (nmol/mg/min)	42.7 (100)	< 4.0 (< 1)	n.d. (–)

tunately, we could not determine which band was the Na^+-Ca^{2+} antiporter.

Comparison of funamental properties of Na^+-Ca^{2+} exchange between native membrane vecicles and crude and purified proteoliposomes

Membrane vesicles and crude and purified proteoliposomes all exhibited the following similar properties of Na_i^+-dependent Ca^{2+} uptake rates: (1) The initial rate of Ca^{2+} uptake was saturable with respect to external Ca^{2+} concentration ($[Ca^{2+}]_o$) (data not shown). (2) Ca^{2+} uptake markedly increased with an increase of the internal Na^+ concentration ($[Na^+]_i$) in the range of 0 to 20 mM, reaching saturation at 20 to 140 mM (Fig. 3). (3) The external Na^+ concentration ($[Na^+]_o$) greatly inhibited Ca^{2+} uptake (data not shown). The degrees of inhibition by 140 mM $[Na^+]_o$ were 80, 83 and 92%, respectively, for membrane vesicles and crude and purified proteoliposomes. (4) Ca^{2+} uptake was inhibited at external low pH but stimulated at high pH (Fig. 4). The curves seemed to be sigmoidal. (5) Ca^{2+} uptake rate was slow at low temperature but rapid at high temperature. Arrhenius plots displayed two components and one distinct inflection point at 21–24°C (data not shown). (6) The K^+-specific ionophore, valinomycin, markedly stimulated Ca^{2+} uptake (150 to 220% of control), suggesting that Na^+-Ca^{2+} exchange is electrogenic.

Table III gives several kinetic parameters. $K_{1/2}$ for Na_i^+ means $[Na^+]_i$ for half maximal velocity of Na_i^+-dependent Ca^{2+} uptake. $K_{1/2}$ for Na_o^+ and $pK_{1/2}$ for pH mean $[Na^+]_o$ and external pH for half inhibitions of maximal velocities of Na_i^+-dependent Ca^{2+} uptake, respectively. The kinetic

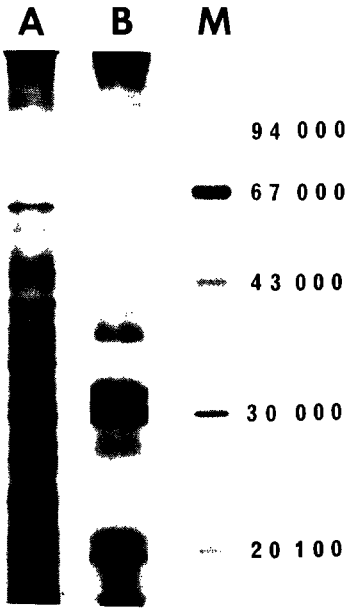

Fig. 2. SDS-gel electrophoresis. Electrophoresis was performed as shown in Methods. A and B correspond to protein profiles of proteoliposomes from cholate extract not treated and treated with pronase, respectively. M shows marker proteins described in Methods. The gel contained 10% acrylamide. The same protein content (15 μg) was applied for both A and B.

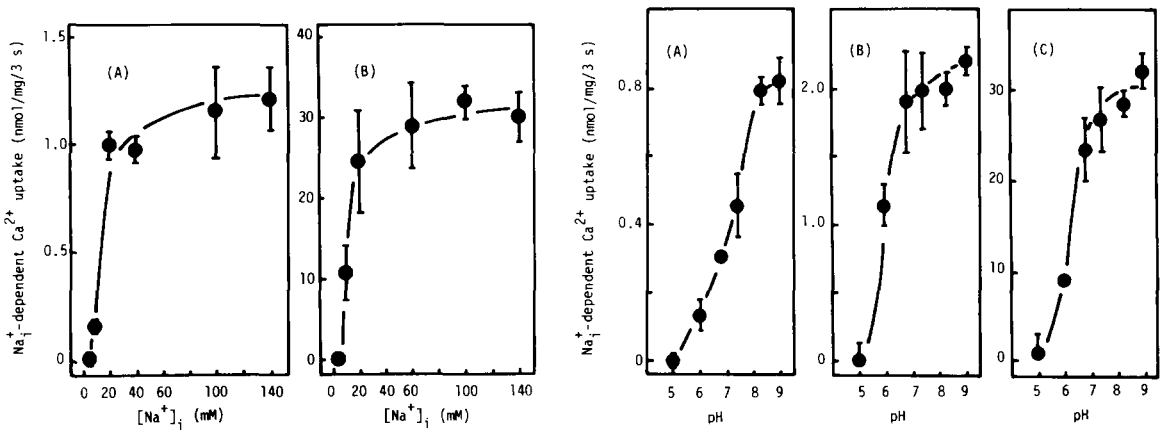

Fig. 3. Internal Na^+-dependency of the initial rate of Na_i^+-dependent Ca^{2+} uptake by membrane vesicles (A) or proteoliposomes (B) from cholate extract treated with pronase. Membrane vesicles and proteoliposomes loaded with various concentrations of Na^+ were prepared as given in Methods. The uptake solution contained 50 μM labeled Ca^{2+}. The exchange reaction in proteoliposomes was stopped after 1.5 s of reaction and the data was recalculated as nmol/mg per 3 s. Points and bars represent means $\pm$ S.D. for five determinations.

Fig. 4. PH-Dependency of Na_i^+-dependent Ca^{2+} uptake in membrane vesicles (A), proteoliposomes from cholate extract not treated (B) and treated (C) with pronase. The uptake solution was buffered with 10 mM Tris/maleate instead of 20 mM Mops/Tris at each pH and contained 50 μM labeled Ca^{2+}. Points and bars represent means $\pm$ S.D. for four determinations.

parameters, given in Table III, were almost equal for crude and purified proteoliposomes, suggesting that these kinetic properties did not change at least by pronase treatment. However, slight differences in K_m, $pK_{1/2}$ for pH and activation energy were detected between the membrane vesicles and the two proteoliposomal preparations.

Discussion

Cholate-extracted membrane proteins treated with pronase were incorporated into artificial liposomes in this study and the resulting purified proteoliposomes contained functional Na^+-Ca^{2+} antiporters based on the following observations:

TABLE III

VARIOUS KINETIC PARAMETERS

Crude and purified proteoliposomes mean preparations reconstituted from cholate extract not treated and treated with pronase (1 mg/ml), respectively. $K_{1/2}$ for Na_i^+, $K_{1/2}$ for Na_o^+ and $pK_{1/2}$ for pH mean the internal and external concentrations of Na^+ and the external pH for the half maximal velocities of Na_i^+-dependent Ca^{2+} uptake, respectively.

Preparation	V_{max} (nmol/mg/3 s)	K_m for Ca_o^{2+} (μM)	$K_{1/2}$ for Na_i^+ (mM)	$K_{1/2}$ for Na_o^+ (mM)	$pK_{1/2}$ for pH (pH)	Activation energy (kcal/mol)
Membrane vesicles	1.96	52	13	15	7.2	40.0 (12–24°C) 12.3 (24–38°C)
Crude proteoliposomes	3.53	21	-	25	6.1	26.0 (12–24°C) 5.6 (24–38°C)
Purified proteoliposomes	36.9	20	11	8	6.3	25.2 (5–21°C) 6.7 (21–38°C)

(1) Ca^{2+} uptake was selectively sensitive to Na^+, i.e., internal Na^+ markedly stimulated Ca^{2+} uptake (see Fig. 1 and Fig. 2), while external Na^+ strongly inhibited it (data not shown). Also, Ca^{2+} accumulated was rapidly released when Na^+ was added to the external medium (see Fig. 1). (2) Ca^{2+} uptake was saturable with respect to the concentrations of both internal Na^+ and external Ca^{2+}, suggesting that there is a carrier possessing high affinity for both Na^+ and Ca^{2+} in artificial liposomes. (3) Na^+(^{22}Na) efflux was stimulated by Ca^{2+} added to the external solution (data not shown), showing that Na^+ is exchanged for Ca^{2+}. This Na^+-Ca^{2+} exchange was electrogenic. These fundamental properties of Ca^{2+} uptake did not change during purification including pronase treatment.

Let us next consider the quantitative properties of Na_i^+-dependent Ca^{2+} uptake in membrane vesicles and crude and purified proteoliposomes. Various kinetic parameters (K_m for Ca_o^{2+}, $K_{1/2}$ for Na_i^+ and Na_o^+, $pK_{1/2}$ for pH and activation energy) in purified proteoliposomes were close to those in membrane vesicles and crude proteoliposomes, though slight differences in K_m, $pK_{1/2}$ for pH and activation energy were detected between the membrane vesicles and the two proteoliposomal preparations (see Table III). However, the small difference of K_m is probably insignificant if we take into account the complexity of the membrane vesicles. In fact, the K_m value (20 μM) we found using proteoliposomes was similar to those reported for sarcolemma vesicles isolated from native membranes: 31 μM by our laboratory [11], 18 μM by Reeves and Sutko [13], 30 μM by Bers et al. [15], 28 μM by Philipson et al. [22]. Philipson et al. [22] suggested that the K_m for Ca^{2+} depends on the range of Ca^{2+} concentrations used in the uptake solution: it is low at less than 40 μM $[Ca^{2+}]_o$, but high above 40 μM. This may explain the different values between the membrane vesicles and proteoliposomes. The $pK_{1/2}$ for pH was also slightly higher in the membrane vesicles than in the two proteoliposomes preparations. Reports from our laboratory [10] and Philipson et al. [22] stated that H^+ appears to competitively interact with Ca^{2+} at least at higher Ca^{2+} concentrations ($[Ca^{2+}]_o > 25$ μM). This shows that the position of the sigmoidal curve for pH (see Fig. 4) may shift to either the acidic or basic side in a K_m (for Ca^{2+})-dependent manner when Ca^{2+} uptake is measured with constant $[Ca^{2+}]_o$. Therefore, the slightly high K_m value in the membrane vesicles (see Table III) probably causes the shift of the sigmoidal curve to the basic side.

We achieved 30- to 50-fold purification of the activity of Na_i^+-dependent Ca^{2+} uptake. The activity was 10-fold higher in purified than in crude proteoliposomes (see Table I). Several possible reasons can be considered for this enhancement: (1) Na^+-Ca^{2+} antiporter is activated by cleavage of its primary structure with pronase. (2) Na^+-Ca^{2+} antiporter is activated by elimination of endogeneous inhibitors with pronase. (3) Na^+-Ca^{2+} antiporter in the cholate extract is more resistant to pronase than other proteins. Consequently, the specific activity is enhanced as pronase eliminates proteins other than Na^+-Ca^{2+} antiporter. The functional intactness of Na^+-Ca^{2+} antiporter discussed above supports the third possibility, as does the high recovery (see Table I).

We also obtained the information about the size of a detergent-micelle from the preliminary data from gel filtration. When the cholate extract was applied to a column of Sephadex G-200 (from Pharmacia Fine Chem. Co.), more than 80% of the total protein was recovered in the void volume (data not shown). The total activity of Na_i^+-dependent Ca^{2+} uptake was recovered from this fraction, though the activity was reduced considerably during chromatography. This shows that the size of a detergent-micelle containing Na^+-Ca^{2+} antiporters is more than 800 kDa, according to the Sephadex G-200 exclusion limit. This result suggests that the detergent-micelle containing Na^+-Ca^{2+} antiporters also contains a number of lipids and/or other proteins. Thus, the lipids may protect Na^+-Ca^{2+} antiporters against pronase. In addition, these results suggest that Na^+-Ca^{2+} antiporter is more hydrophobic than other proteins. Note also that pronase treatment of the cholate extract but not of the vesicle suspension is important to achieve high purification and recovery. Thus, exogenous lipids added may play an important role in the protection against pronase.

The maximal amount of Ca^{2+} accumulated by the purified proteoliposomes in the present work was 140 nmol/mg protein (120 nmol/mg for Na_i^+

-dependent Ca^{2+} uptake) at 25°C and initial rates of 25–45 nmol/mg per 3 s (20–40 nmol/mg per 3 s for Na_i^+-dependent Ca^{2+} uptake) were routinely obtained. These values are much higher than those reported previously by several laboratories [11,13–15,17,19–21] using isolated sarcolemma vesicles. But the maximal amounts of Ca^{2+} accumulated, which were reported more recently as 80 nmol/mg by Caroni et al. [16], 60–100 nmol/mg by Philipson et al. [22], 90 nmol/mg by Reeves and Sutko [18], are comparable to our value, although direct comparison may be difficult because of differences in experimental conditions such as temperature, Ca^{2+} concentration and animal species employed.

In conclusion, we found that pronase treatment is very useful for obtaining a preparation containing highly enriched Na^+-Ca^{2+} antiporters which are functionally intact. Our final preparation did not exhibit ATP-dependent Ca^{2+} uptake activity (see Table II), another Ca^{2+} transport pathway of the sarcolemma membrane. Thus, our preparation can serve as a simple experimental system for studies of Na^+-Ca^{2+} exchange as well as further purification.

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