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Phospholipid composition modulates the Na^+–Ca^{2+} exchange activity of cardiac sarcolemma in reconstituted vesicles

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Na^+–Ca^{2+} exchange activity in cardiac sarcolemmal vesicles is known to to be sensitive to charged, membrane lipid components. To examine the interactions between membrane components and the exchanger in more detail, we have solubilized and reconstituted the Na^+–Ca^{2+} exchanger into membranes of defined lipid composition. Our results indicate that optimal Na^+–Ca^{2+} exchange activity requires the presence of certain anionic phospholipids. In particular, phosphatidylserine (PS), cardiolipin, or phosphatidic acid at 50% by weight results in high Na^+–Ca^{2+} exchange activity, whereas phosphatidylinositol and phosphatidylglycerol provide a poor environment for exchange. In addition, incorporation of cholesterol at 20% by weight greatly facilitates Na^+–Ca^{2+} exchange activity. Thus, for example, an optimal lipid environment for Na^+–Ca^{2+} exchange is phosphatidylcholine (PC, 30%)/PS (50%)/cholesterol (20%). Na^+–Ca^{2+} exchange activity is also high when cardiac sarcolemma is solubilized and then reconstituted into asolectin liposomes. We fractionated the lipids of asolectin into subclasses for further reconstitution studies. When sarcolemma is reconstituted into vesicles formed from the phospholipid component of asolectin, Na^+–Ca^{2+} exchange activity is low. When the neutral lipid fraction of asolectin (including sterols) is also included in the reconstitution medium, Na^+–Ca^{2+} exchange activity is greatly stimulated. This result is consistent with the requirement for cholesterol described above. Proteinase treatment, high pH, intravesicular Ca^{2+} and dodecyl sulfate all stimulate Na^+–Ca^{2+} exchange in native sarcolemmal vesicles. We examined the effects of these interventions on exchange activity in reconstituted vesicles of varying lipid composition. In general, Na^+–Ca^{2+} exchange could be stimulated only when reconstituted into vesicles of a suboptimal lipid composition. That is, when reconstituted into asolectin or PC/PS/cholesterol (30:50:20), the exchanger is already in an activated state and can no longer be stimulated. The one exception was that the Na^+–Ca^{2+} exchanger responded to altered pH in an identical manner, independent of vesicle lipid composition. The mechanism of action of altered pH on the exchanger thus appears to be different from other interventions.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycolbis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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Introduction

Regulation of intracellular Ca^{2+} is of utmost importance during the cardiac contraction-relaxation cycle. A highly active Na^+–Ca^{2+} exchange system has been well characterized in cardiac sarcolemmal vesicles [1,2] and is probably important in cellular Ca^{2+} homeostasis. Na^+–Ca^{2+} exchange can mediate either cellular Ca^{2+} influx or efflux, depending on membrane potential and ion gradients, but an exact role in excitation-contraction coupling remains controversial [1,3,4]. In vitro, several interventions modulate Na^+–Ca^{2+} exchange activity. Examples include altered pH [5], proteinase treatment [6] and intravesicular Ca^{2+} [7]. Also, exchange activity is sensitive to membrane lipid composition. In general, anionic amphiphiles stimulate exchange activity. The stimulation can be induced by incorporation of amphiphiles into native sarcolemmal vesicles [8–10] or through the actions of phospholipases [11,12].

Several groups have successfully solubilized and reconstituted the Na^+–Ca^{2+} exchange protein(s) [13–19]. Properties of exchange in reconstituted vesicles, are generally similar to those in the native sarcolemmal vesicles, although the reconstitution process itself may stimulate exchange activity [17,18]. In most cases, the Na^+–Ca^{2+} exchanger was reconstituted into vesicles composed of asolectin, a crude soybean lipid extract.

In the present study, we employ reconstitution techniques to investigate further the interactions of lipid components with the Na^+–Ca^{2+} exchange process. We have reconstituted the exchanger into vesicles of defined lipid composition and find that inclusion of both specific anionic phospholipids and cholesterol are required for optimal activity. In addition, we reinvestigate the effects of interventions, such as proteinase treatment and altered pH, and report on their dependence on lipid composition.

Materials and Methods

Phospholipids (phosphatidylcholine (PC) (egg), phosphatidylethanolamine (PE) (egg), phosphatidylserine (PS) (bovine brain), phosphatidylinositol (PI) (bovine liver), phosphatidic acid (egg), phosphatidylglycerol (PG) (egg), cardiolipin (bovine liver), sphingomyelin) were purchased from Avanti Polar Lipids. Each lipid was identified as a single spot by thin-layer chromatography (TLC). Asolectin was obtained from Associated Concentrates, cholesterol from Sigma, Bio-Beads SM-2 from Bio-Rad. All organic solvents were analytical grade.

Highly enriched sarcolemmal vesicles were prepared from dog ventricles as described elsewhere [10,20].

Cardiac sarcolemmal vesicles were solubilized using Triton X-100 or cholate in the presence of asolectin (soybean phospholipids) or various combinations of commercial phospholipids. To first prepare the detergent/lipid suspension, mixtures of lipids (10 mg) in chloroform were dried under a stream of nitrogen. If the detergent to be used was Triton X-100, we then added 188 μl of 10% Triton X-100 and 672 μl of water to the dried lipid. After brief sonication, 40 μl of 500 mM Mops-Tris (pH 7.4) and 100 μl of 5 M NaCl were added and the mixture was sonicated once again until clarity. About 30 μl of native sarcolemmal vesicles (3 mg protein/ml) were then solubilized with 4 vol. of the Triton X-100/phospholipid mixture. Final concentrations were 1.5% Triton X-100, 8 mg/ml phospholipid, 18 mM Mops-Tris, 428 mM NaCl. After a 5 min incubation at 4° C, the mixture was spun in a Beckman airfuge for 15 min. To reconstitute the solubilized proteins into vesicles, 1 vol. of 0.5 M NaCl was added to the supernatant and the mixture was agitated in the presence of Bio-Beads SM-2 for 20 min at room temperature to remove the Triton X-100 [21]. In order to ensure complete removal of the detergent, this step was repeated with fresh Bio-Beads. The supernatant was then diluted 5-fold with 140 mM NaCl/10 mM Mops-Tris (pH 7.4) and spun at $140000 \times g$ for 90 min in Beckman L5-50 ultracentrifuge. The resulting pellet was suspended in about 40 μl of 140 mM NaCl/10 mM Mops-Tris (pH 7.4). Soldati et al. [18] have also employed Triton X-100 for solubilization and reconstitution of sarcolemmal Na^+–Ca^{2+} exchange activity.

Solubilization of vesicles using cholate followed a similar procedure. Final concentrations after mixing 1 vol. sarcolemma (3 mg/ml) with 4 vol. cholate/phospholipid mixture were 25 mg/ml

phospholipid, 2% Tris cholate, 482 mM NaCl, 18 mM Mops-Tris (pH 7.4). In this case, solubilized proteins were reconstituted into vesicles by the cholate-dilution method [13]. After removing non-solubilized material by centrifugation, the clear supernatant was diluted 6-fold with cold 124 mM sodium phosphate (pH 7.4). Reconstituted vesicles were spun as above and resuspended in the sodium phosphate solution. The reconstituted vesicles were freshly prepared for each experiment and were stable at room temperature for at least a few hours.

For intravesicular volume measurements, the native sarcolemmal vesicles were solubilized and then reconstituted in the presence of [^{14}C]sucrose. The [^{14}C]sucrose trapped within the reconstituted vesicles was determined by filtration. Blanks for which the [^{14}C]sucrose was added only after reconstitution were subtracted.

Total lipids were extracted from native sarcolemmal vesicles or from asolectin by the method of Bligh and Dyer [22]. The chloroform phase was evaporated to dryness under a slow stream of nitrogen and the lipids were either used directly in reconstitution experiments or separated into subclasses. In the latter case, the dried lipids were dissolved in chloroform/methanol (2 : 1, v/v), applied to an activated silicic acid column and eluted successively with chloroform, acetone and methanol [23]. The eluted fractions, containing neutral lipids, glycolipids and phospholipids, respectively, were dried and used in reconstitution experiments as described in Results. Samples of the different lipid classes were checked for possible cross-contamination by TLC on silica-coated plates using chloroform/methanol/water (65 : 25 : 4, v/v) for phospholipids and hexane/ethyl ether/acetic acid (80 : 20 : 1, v/v) for neutral lipids. Lipid-bound phosphate was determined as described in Ref. 11.

Na^+–Ca^{2+} exchange was measured by adding 5 μl of Na^+-loaded vesicles to 245 μl of uptake medium at 37°C. This medium contained 140 mM KCl, 10 mM Mops-Tris (pH 7.4), 0.3 μCi $^{45}CaCl_2$, $CaCl_2$ (10 μM, unless stated otherwise) and 0.4 μM valinomycin. Blanks were obtained by diluting vesicles into uptake solution containing 140 mM NaCl instead of KCl. Blanks represent non-specific Ca^{2+} uptake that does not require a Na^+ gradient. All data were corrected for this nonspecific Ca^{2+} uptake which was never more than 10% of the total counts. The uptake reaction was stopped at 1.5 s, unless stated otherwise, by the automatic addition of 30 μl of 140 mM KCl/10 mM EGTA. Immediately thereafter, 1 ml of cold 140 mM KCl/1 mM EGTA was added. 1 ml of this solution was applied to a 0.22-μm Sartorius nitrocellulose filter under suction. The filter was washed with two 3-ml aliquots of ice-cold 140 mM KCl/1 mM EGTA. Radioactive Ca^{2+} on the filters was detected by liquid scintillation techniques. These procedures have been described previously in detail [10,19].

Passive Ca^{2+} efflux measurements were performed as described elsewhere [10].

All experiments were performed in duplicate, with an n of at least 3. Data are expressed as means ± S.E.

Results

We studied the interaction of the Na^+–Ca^{2+} exchanger with the membrane lipid environment by solubilizing native sarcolemmal vesicles and reconstituting exchange activity into vesicles of defined lipid composition. We began with vesicles composed of mixtures of PC and PS (Fig. 1, lower curve). When the exchanger was reconstituted in 100% PC, exchange activity was barely detectable. Activity increased as PS content increased and reached a maximum at 50% PS. Activity was still low (approx. 1 nmol/mg per s) compared to that of native vesicles (approx. 4 nmol/mg per s). Na^+–Ca^{2+} exchange activity was greatly stimulated by including cholesterol in the lipid mixture (Fig. 1, upper curve, and Fig. 2). The optimal cholesterol content was 20% by weight (Fig. 2). As in the absence of cholesterol, highest exchange activity occurred when PS was 50% (Fig. 1, upper curve).

To determine whether high Na^+–Ca^{2+} exchange activity specifically required PS, we measured exchange activity in vesicles reconstituted from several phospholipid mixtures (Table I). We investigated whether other anionic phospholipids could substitute for PS. Both phosphatidic acid and cardiolipin could take the place of PS in maintaining high exchange activity. In contrast,

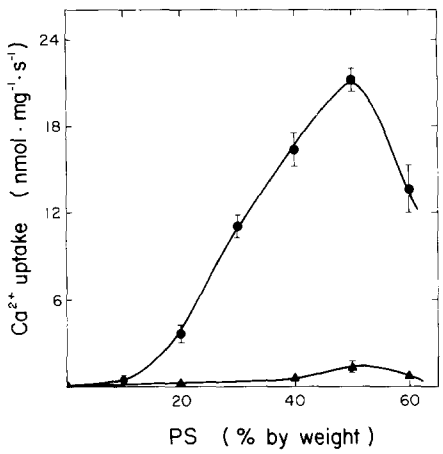

Fig. 1. Initial rate of Na^+–Ca^{2+} exchange as a function of PS content. Amount of PS included is expressed as % by weight of the lipid in the reconstitution medium. Ca^{2+} uptake in vesicles as reconstituted as follows: (▲, PS (as indicated)/PC (remaining %); ●, cholesterol (20%)/PS (as indicated)/PC (remaining %). [Ca^{2+}] in uptake media was 10 μM. The uptake reaction proceeded for 1.5 s. All data are corrected for Na^+-independent Ca^{2+} uptake.

vesicles reconstituted with two other anionic phospholipids, PI and PG, had low exchange activities. In each case, optimal activity required 20% cholesterol and 50% anionic phospholipid (not shown). Soldati et al. [18] have also reported higher exchange activity after reconstituting sarcolemma with PC : PS than with PC : PI. When PE was used in place of PC, the reconstituted vesicles had lower Na^+–Ca^{2+} exchange activities (Table I). In this case, however, unilamellar vesicles may not have formed. This was suggested by the appearance of a white turbid pellet after centrifugation, instead of the usual translucent pellet. Replacing PC with sphingomyelin did not influence exchange rates significantly.

We also reconstituted sarcolemma into vesicles using asolectin. As previously observed [13,17], the Na^+–Ca^{2+} exchange activity of vesicles reconstituted with asolectin was 4–5-fold higher than that of native sarcolemmal vesicles and was also higher than that of any reconstituted vesicles formed from defined lipid mixtures (Table I). We also reconstituted sarcolemma into vesicles with a phospholipid composition similar to that in asolectin as reported in the literature. These vesicles had low Na^+–Ca^{2+} exchange activity (Table I), perhaps due to the absence of sterols and

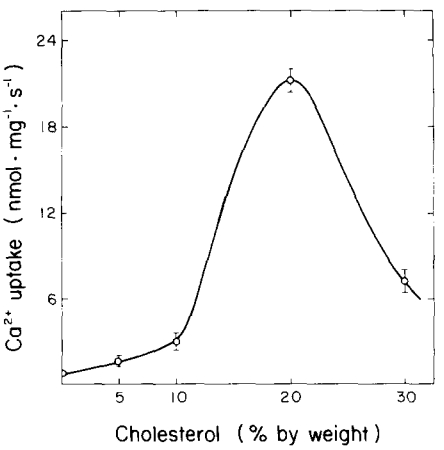

Fig. 2. Initial rate of Na^+–Ca^{2+} exchange as a function of cholesterol content. Ca^{2+} uptake in reconstituted vesicles was PS (50%)/cholesterol (as indicated)/PC (remaining %). The composition is given as % by weight.

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Na^+–Ca^{2+} EXCHANGE AS A FUNCTION OF PHOSPHOLIPID COMPOSITION

Reconstitution lipid	Na_i^+-dependent Ca^{2+} uptake (nmol/mg per s)
Asolectin	19.1 ± 1.4
PC/PS/cholesterol (5 : 3 : 2)	4.3 ± 0.2
PC/PS/cholesterol (3 : 5 : 2)	15.8 ± 1.6
PC/phosphatidic acid/cholesterol (5 : 3 : 2)	6.3 ± 1.2
PC/phosphatidic acid/cholesterol (3 : 5 : 2)	11.9 ± 1.4
PC/PG/cholesterol (5 : 3 : 2)	2.9 ± 0.7
PC/PG/cholesterol (3 : 5 : 2)	2.9 ± 0.5
PC/PI/cholesterol (5 : 3 : 2)	1.3 ± 0.3
PC/PI/cholesterol (3 : 5 : 2)	1.4 ± 0.3
PC/cardiolipin/cholesterol (5 : 3 : 2)	6.7 ± 0.2
PC/cardiolipin/cholesterol (3 : 5 : 2)	16.9 ± 0.6
PE/PS/cholesterol (3 : 5 : 2)	9.4 ± 2.1
PE/phosphatidic acid/cholesterol (3 : 5 : 2)	8.6 ± 0.3
Sphingomyelin/PS/cholesterol (3 : 5 : 2)	12.7 ± 1.1
Phospholipid similar to asolectin [a]	2.7 ± 0.5

[a] PC, 37%; PE, 34%; PI, 14%; PS, 3%; lyso-PC, 3%; cardiolipin, 6%. This composition is an approximate average of determinations found in the literature [24,25].

other factors present in asolectin. We have not quantitated the amounts of different phospholipids in the lot of asolectin used in our laboratory.

The role of sterols in increasing Na^+–Ca^{2+} exchange activity was further examined by reconstituting native sarcolemma into lipids extracted from either native sarcolemma or asolectin (Table II). Lipid extracts were separated into subclasses as described in Materials and Methods. Vesicles reconstituted with the phospholipid components alone had relatively low exchange rates. Including the neutral lipid fraction greatly increased exchange activity. When all lipid constituents (phospholipids + neutral lipids + glycolipids) were included in the reconstitution medium a further small increase in activity occurred. When Na^+–Ca^{2+} exchange was reconstituted in the separated and subsequently recombined components of sarcolemmal lipids, the activity was substantially higher than when the initial total lipid extract was used for the reconstitution (compare lines 1 and 4 of Table II). We have no explanation for this striking phenomenon, though perhaps the silicic acid column removes an inhibitory factor present in the crude lipid extract.

Fig. 3 shows the time course of Na^+–Ca^{2+} exchange reconstituted into four different lipid mixtures. The Na_i^+-dependent Ca^{2+} uptake is rapid and a plateau of uptake occurred after approx. 30 s. Asolectin reconstituted vesicles exhibited the highest activity, followed by vesicles with 50, 30 and 10% PS. Fig. 4 shows, in more detail, the Na^+–Ca^{2+} exchange of these same vesicles at short times. Na_i^+-dependent Ca^{2+} uptake is approximately linear for at least 1.5 s. Differences in the Ca^{2+} uptakes of the different vesicles are more pronounced at initial times than at longer time periods. For example, the Ca^{2+}

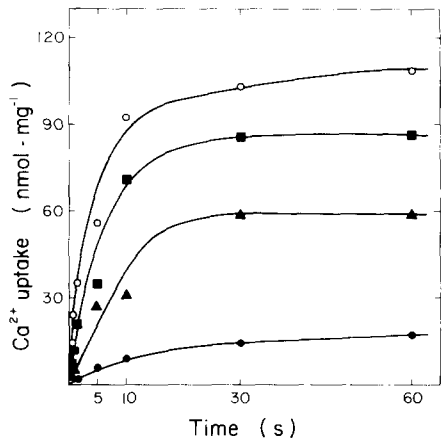

Fig. 3. Time course for Na_i^+-dependent Ca^{2+} uptake. Vesicles reconstituted with the following lipid mixtures: ○, asolectin; ■, PC/PS/cholesterol (3 : 5 : 2); ▲, PC/PS/cholesterol (5 : 3 : 2); ●, PC/PS/cholesterol (7 : 1 : 2).

TABLE II

Na^+–Ca^{2+} EXCHANGE IN VESICLES RECONSTITUTED WITH LIPIDS EXTRACTED FROM NATIVE SARCOLEMMAL VESICLES OR ASOLECTIN

The sarcolemmal Na^+–Ca^{2+} exchanger was reconstituted using the total Bligh-Dyer lipid extract from either native sarcolemma or asolectin (line 1), or lipid components fractionated from the Bligh-Dyer extract on a silicic acid column. See Materials and Methods for details. Values represent Na_i^+-dependent Ca^{2+} uptake (nmol/mg per s).

Reconstitution lipid	Lipid source	
	native sarcolemma	asolectin
Total lipids	5.5 ± 0.4	22.2 ± 1.4
Phospholipids	1.4 ± 0.1	7.4 ± 1.4
Phospholipids + neutral lipids	11.9 ± 1.2	17.4 ± 0.9
Phospholipids + neutral lipids + glycolipids	13.3 ± 1.0	22.8 ± 1.3

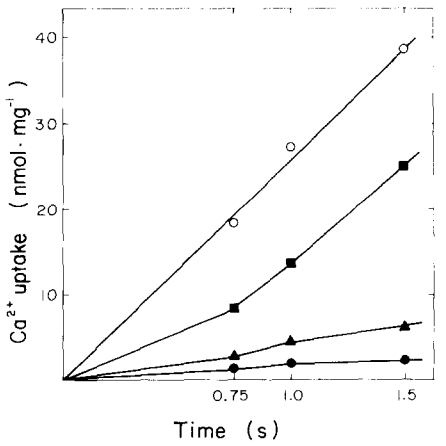

Fig. 4. Initial rates of Ca^{2+} uptake. Data are from Fig. 3 on an expanded scale. See legend to Fig. 3 for details.

uptake of asolectin vesicles is approx. 6-fold higher than that of PC/PS/cholesterol (5 : 3 : 2) vesicles after 1.5 s, but less than twice as high after 30 s. The result indicates that the PC/PS/cholesterol (5 : 3 : 2) vesicles have the capacity to accumulate Ca^{2+} and the low initial rate of the Na^+–Ca^{2+} exchange is due to low exchange activity and not to vesicle leakiness (see also Fig. 5). Qualitatively similar behavior is seen in vesicles reconstituted without cholesterol. For example, PC/PS (5 : 5) vesicles, which demonstrate very low initial rates of exchange (Fig. 1), have substantial Na_i^+-dependent Ca^{2+} uptake after 60 s (approx. 30 nmol/mg). The plateau levels of Ca^{2+} uptake are a complex function of several factors (e.g., initial Ca^{2+} uptake rate, maintenance of the potassium diffusion potential, dissipation of the Na^+ gradient, passive Ca^{2+} permeability) and are difficult to interpret. Thus, we are usually concerned only with initial rates of Na^+–Ca^{2+} exchange activity.

One possible explanation for different activities of the Na^+–Ca^{2+} exchange after reconstitution of sarcolemma into different lipid environments is that selective solubilization and reconstitution of the Na^+–Ca^{2+} exchange protein occurs. We addressed this possibility in two ways. First, we did SDS-polyacrylamide gel electrophoresis on different reconstituted vesicles. The silver-stained gel patterns were identical, and independent of the lipids used for reconstitution (not shown). Second, we reconstituted sarcolemma using lipids giving suboptimal Na^+–Ca^{2+} exchange activity (PC/PS/cholesterol (7 : 1 : 2)). These reconstituted vesicles were again solubilized and reconstituted with asolectin. The twice-reconstituted vesicles demonstrated a high rate of Na^+–Ca^{2+} exchange, indicating that the exchange protein was present in the initial, reconstituted vesicles, which had low activity. In addition, the total protein recovered in vesicles reconstituted with different phospholipids did not vary significantly (less than 10% variation).

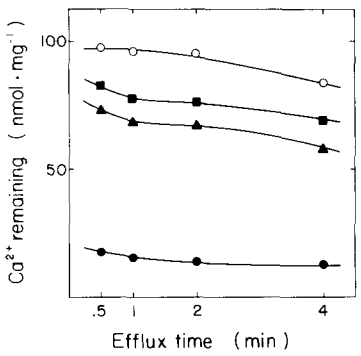

Fig. 5. Passive Ca^{2+} efflux from reconstituted vesicles. Vesicles were Ca^{2+}-loaded by Na^+–Ca^{2+} exchange for 1 min. Ca^{2+}-loaded vesicles were diluted into an equal volume (250 μl) of 140 mM KCl/10 mM Mops/1 mM EGTA (pH 7.4) and incubated at 37 °C. Aliquots were filtered at given times. The results are expressed as the amount of Ca^{2+} remaining in the vesicles following different efflux periods. Vesicles reconstituted with the following lipid mixtures: ○, asolectin; ■, PC/PS/cholesterol (3 : 5 : 2); ▲, PC/PS/cholesterol (5 : 3 : 2); ●, PC/PS/cholesterol (7 : 1 : 2).

All of the above results were obtained using Triton X-100 as the detergent to solubilize the sarcolemma. However, these results were not dependent upon the use of this detergent. We observed qualitatively similar behavior when the cholate-dilution method [13] was used for solubilization and reconstitution. For example, time courses of Na^+–Ca^{2+} exchange, very similar to those in Figs. 3 and 4, were obtained when vesicles of the same lipid compositions were formed by the cholate-dilution technique (not shown). In addition, we also studied the relationship between the size of the reconstituted vesicles and exchange activity. Intravesicular volume was measured by solubilizing sarcolemma and reconstituting vesicles with various lipid combinations in the presence of [^{14}C]sucrose. As shown in Table III, the intravesicular volumes did not vary significantly as a function of lipid composition. Vesicles reconstituted with PC/PI/cholesterol had a somewhat smaller intravesicular volume, and this may be a factor in the lower exchange activity of these vesicles. Also shown in Table III is evidence that the recovery of protein did not vary with lipid composition.

We measured Ca^{2+} efflux from the reconstituted vesicles to determine their passive Ca^{2+} permeability (Fig. 5). Vesicles were loaded with Ca^{2+} by Na^+–Ca^{2+} exchange, EGTA was added to inhibit further Ca^{2+} uptake, and passive Ca^{2+} loss was monitored. Similar low rates of passive Ca^{2+} efflux were observed for the different reconstituted vesicles. The results indicate that differences in initial rates of Na^+–Ca^{2+} exchange are not due to differences in vesicle leakiness. In

TABLE III

INTRAVESICULAR VOLUME AND PHOSPHOLIPID CONTENT OF RECONSTITUTED VESICLES

Solubilized sarcolemmal vesicles were reconstituted with various phospholipids in the presence of [^{14}C]sucrose. The trapped sucrose was measured as described in Materials and Methods.

Lipid mixture	Intravesicular volume (nl/μmol phospholipid)	Phospholipid/protein (μmol/mg)
Asolectin	568 ± 53	21.2 ± 1.8
PC/PS/cholesterol (3 : 5 : 2)	546 ± 52	23.7 ± 2.3
PC/PS/cholesterol (5 : 3 : 2)	571 ± 109	22.8 ± 4.3
PC/PS/cholesterol (7 : 1 : 2)	401 ± 89	20.5 ± 3.7
PC/PG/cholesterol (3 : 5 : 2)	379 ± 174	21.8 ± 1.4
PC/PI/cholesterol (3 : 5 : 2)	176 ± 30	19.1 ± 1.7
PC/cardiolipin/cholesterol (3 : 5 : 2)	494 ± 102	24.1 ± 4.1

separate experiments, Ca^{2+}-loaded vesicles were exposed to the Ca^{2+} ionophore A23187 during the efflux period. A 3-min exposure to A23187 (0.5 μM) resulted in near total loss (over 96%) of Ca^{2+}. This demonstrates that the Ca^{2+} was intravesicular and not associated with the membrane surface.

The dependence of the initial rate of Na^+–Ca^{2+} exchange on Ca^{2+} concentration is shown in Fig. 6 for reconstituted vesicles with different lipid compositions. As seen above, activities are highest for asolectin vesicles and for PC/PS/cholesterol vesicles with 50% PS. For these two cases, activity is half maximal at a $[Ca^{2+}] < 10$ μM. For the other two cases (PC/PS/cholesterol with PS equal to 10 or 30%), the apparent K_m (Ca^{2+}) is substantially higher, but cannot be estimated accurately from our limited data.

Reeves and Poronnik [7] have reported that intravesicular Ca^{2+} stimulates the Na^+–Ca^{2+} exchange of native sarcolemmal vesicles. We investigated whether this phenomenon also occurs after solubilization and reconstitution. Sarcolemmal proteins were reconstituted in asolectin or PC/PS/cholesterol (3 : 5 : 2) vesicles. Preincubation of these vesicles with 100 μM EGTA or 100 μM Ca^{2+} for 3 h at 4°C resulted in modest inhibitions (30%) or stimulations (40%), respectively, of Na^+–Ca^{2+} exchange. In contrast, vesicles with initially very low activity (PC/PS/cholesterol (7 : 1 : 2)) could be stimulated to a much greater extent. In this case, preincubation with EGTA inhibited activity by 48 ± 4% and preincubation with Ca^{2+} stimulated activity by 340 ± 67% (n = 4).

SDS, an anionic amphiphile, stimulates the Na^+–Ca^{2+} exchange of native sarcolemmal vesicles [8]. In our studies with reconstituted

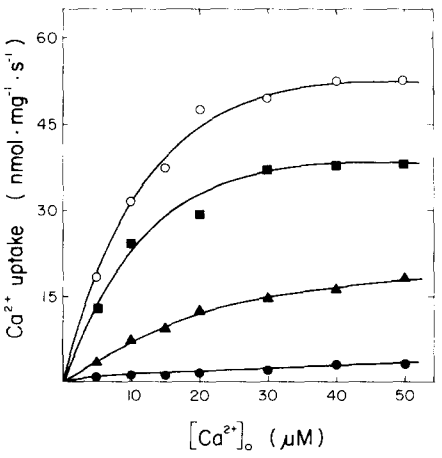

Fig. 6. Initial rate of Na^+–Ca^{2+} exchange as a function of $[Ca^{2+}]_o$. Data are from a representative experiment. Ca^{2+} concentrations are corrected for an endogenous Ca^{2+} level of 2.5 μM in the uptake solutions. Vesicles reconstituted with the following lipid mixtures: ○, asolectin; ■, PC/PS/cholesterol (3 : 5 : 2); ▲, PC/PS/cholesterol (5 : 3 : 2); ●, PC/PS/cholesterol (7 : 1 : 2).

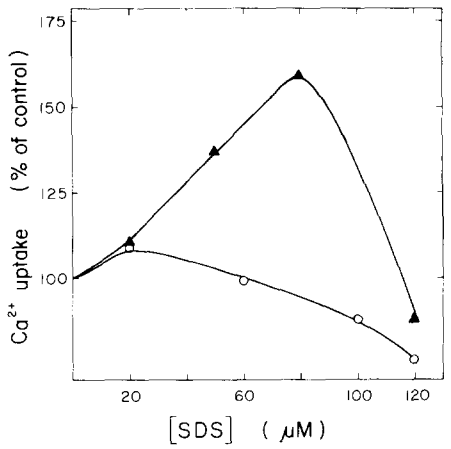

Fig. 7. Effect of SDS on initial rate of Na_i^+-dependent Ca^{2+} uptake in (○) asolectin and (▲) PC/PS/cholesterol (5 : 3 : 2) reconstituted vesicles. The vesicles were exposed to SDS only during the period of Ca^{2+} uptake (1.5 s).

vesicles, addition of SDS to the uptake medium produced varying results (Fig. 7). With asolectin reconstituted vesicles, only a very small stimulation could be elicited at low concentration of SDS. In PC/PS/cholesterol (5 : 3 : 2) vesicles, which have suboptimal Na^+–Ca^{2+} exchange activity, there was a much more substantial response to SDS. In both cases, high SDS concentrations caused an apparent inhibition of Na^+–Ca^{2+} exchange activity which was probably due to increased vesicle leakiness.

Na^+–Ca^{2+} exchange activity is strongly dependent on pH [5], as shown in Fig. 8. Compared to the uptake of Ca^{2+} at pH 7.4, there was an inhibition of exchange at low pH and a stimulation at high pH levels. All the different types of vesicle (native sarcolemmal vesicles, asolectin, PC/PS/cholesterol (5 : 3 : 2) and PC/cardiolipin/cholesterol (5 : 3 : 2)) exhibited similar responses to altered pH, despite wide variations in lipid composition.

Mild pretreatment with proteinases stimulates the Na^+–Ca^{2+} exchange of native sarcolemmal vesicles [6]. Fig. 9 shows the effect of chymotrypsin pretreatment on the Na^+–Ca^{2+} exchange of reconstituted vesicles. In all cases, maximum stimulation was observed after pretreatment with 20 μg/ml of chymotrypsin. The stimulation was inversely proportional to the initial level of Na^+–Ca^{2+} exchange activity. Vesicles reconstituted with asolectin showed a minimum response, whereas vesicles reconstituted with a low PS content (PC/PS/cholesterol (7 : 1 : 2)) showed an enormous response to chymotrypsin (800% stimulation). Similar responses to papain treatment were also observed (not shown).

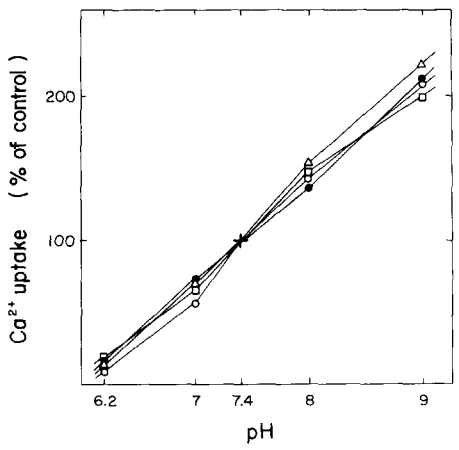

Fig. 8. Influence of extravesicular pH on initial rate of Na_i^+-dependent Ca^{2+} uptake. Na^+-loaded vesicles at pH 7.4 were diluted into Ca^{2+} uptake media at varying pH values. ●, Native vesicles; ○, asolectin; △, PC/PS/cholesterol (5 : 3 : 2); □, PC/cardiolipin/cholesterol (5 : 3 : 2).

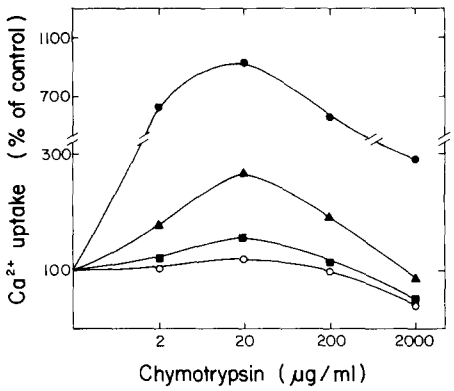

Fig. 9. Chymotrypsin-stimulated Na^+–Ca^{2+} exchange. The reconstituted vesicles were incubated with chymotrypsin (2–2000 μg/ml) for 20 min at 37 °C. At the end of the incubation period the action of chymotrypsin was stopped by the addition of 50 μM PMSF, and the Ca^{2+} uptake was measured. The representative experiment shows initial rate of Na_i^+-dependent Ca^{2+} uptake expressed as percent of control. Vesicles reconstituted with the following lipid mixtures: ○, asolectin; ■, PC/PS/cholesterol (3 : 5 : 2); ▲, PC/PS/cholesterol (5 : 3 : 2); ●, PC/PS/cholesterol (7 : 1 : 2). Note change in ordinate scale.

Discussion

We have previously detailed the interaction of anionic amphiphiles with the Na^+–Ca^{2+} exchanger of cardiac sarcolemmal vesicles. For example, production of phosphatidic acid in the sarcolemmal membrane through the use of phospholipase D markedly stimulates Na^+–Ca^{2+} exchange activity [12]. Likewise, addition of anionic amphiphiles such as SDS or unsaturated fatty acids [8,9] enhances exchange activity. We have been able to model the results as follows: For a membrane lipid component to stimulate Na^+–Ca^{2+} exchange, a negative charge at the membrane interface is required. The stimulation is

further enhanced if the anionic amphiphile also causes some disorder toward the interior of the lipid bilayer [10].

The studies upon which this model is based were all performed using native sarcolemmal vesicles. To investigate further exchanger–lipid interactions and to test our earlier hypotheses, we undertook solubilization-reconstitution experiments. Thus, we would be able to control the lipid environment of the Na^+–Ca^{2+} exchange mechanism. Others have also reported on the solubilization and reconstitution of the Na^+–Ca^{2+} exchanger, but, in general, the lipid mixture used for reconstitution has been asolectin.

We find that in reconstituted vesicles a substantial amount of anionic phospholipid is required for optimal exchange activity (Fig. 1 and Table I). Only specific anionic phospholipids, however, are able to provide the optimal environment for exchange activity. Vesicles reconstituted with PS, cardiolipin or phosphatidic acid demonstrate high activity, whereas vesicles containing PG or PI as the anionic lipid have low exchange activity. In each case, 50% anionic phospholipid by weight produced the best results. PS, cardiolipin and phosphatidic acid can apparently interact with the exchanger in a way that PG and PI cannot. For both PS and phosphatidic acid, an anionic charge is at the hydrophilic terminus of the molecule. Cardiolipin, likewise, has relatively well exposed anionic sites. In contrast, the negative charge on both PG and PI is shielded by a terminal neutral moiety which may prevent interaction with the exchanger. We speculate that these differences are crucial in explaining the relative efficacies of the anionic phospholipids.

In addition to anionic phospholipids, cholesterol is required for high rates of Na^+–Ca^{2+} exchange. Cholesterol has multiple effects on biological membranes, and it is difficult to determine the mechanism whereby cholesterol modulates enzyme activity [26]. Cholesterol may affect fluidity, phospholipid spacing, membrane thickness, or may interact directly with proteins, as has been proposed for band 3 protein [27], erythrocyte glycophorin [28] and ($Na^+ + K^+$)-ATPase [29]. Cholesterol has also been found to increase transport activity in vesicles reconstituted with the acetylcholine receptor [30]. The optimal amount of cholesterol for Na^+–Ca^{2+} exchange was 20% by weight (about 35 mol%). It should be noted that this quantity is similar to endogenous levels of sarcolemmal cholesterol [31].

The validity of our techniques for studying the influence of lipids on Na^+–Ca^{2+} exchange is supported by several control experiments. Low activity in vesicles reconstituted with suboptimal lipid mixtures was not due to loss of the exchanger protein. If these vesicles were solubilized a second time and reconstituted using a more optimal lipid composition, exchange activity would reappear. In addition, SDS-polyacrylamide gel electrophoresis demonstrated identical protein patterns in reconstituted vesicles of widely different lipid composition. Also, our results are not dependent upon the use of one particular solubilization/reconstitution procedure. Similar results to those obtained using the Triton X-100/Biobeads method were also obtained using the cholate-dilution method. Intravesicular volume and phospholipid/protein ratios in the reconstituted vesicles were independent of the lipid composition in the solubilization mixture (Table III). Our analyses include one approximation. Endogenous sarcolemmal phospholipids will be present during membrane solubilization and will be reconstituted along with the exogenous phospholipids. These lipids will account for about 5% of the total lipids in the solubilization mixture and have been ignored in our interpretations.

It is unlikely that different Na^+–Ca^{2+} exchange activities in the different reconstituted vesicles were due to variations in passive permeabilities. The rate of passive Ca^{2+} efflux from reconstituted vesicles was low in several cases (Fig. 5). In addition, even vesicles with very low initial rates of Na^+–Ca^{2+} exchange (e.g., PC/PS (5 : 5)) have the capacity to accumulate substantial Ca^{2+} loads with sufficiently long Ca^{2+} uptake periods.

We cannot explain the high Na^+–Ca^{2+} exchange activity which results when asolectin is used as the reconstitution lipid. There appears to be nothing unique about the phospholipid composition of asolectin (Table I). An unknown factor in asolectin apparently provides the environment necessary for a rapid rate of exchange. The neutral lipid components (which includes sterols) of both asolectin and native sarcolemma enhance the activity provided by the phospholipid component

alone (Table II), consistent with our results (above) with cholesterol.

The affinity of the Na^+–Ca^{2+} exchanger for Ca^{2+} is relatively high ($K_m(Ca^{2+}) < 10\ \mu M$) when reconstitution is done with lipids which result in high activity, e.g., asolectin or PC/PS/cholesterol (3 : 5 : 2) (Fig. 6). Interventions which stimulate the Na^+–Ca^{2+} exchange of native sarcolemmal vesicles also cause an increase in the Ca^{2+} affinity. Examples are proteinase treatment [6], phospholipase D treatment [12], exposure to SDS [8], elevated pH [5], and intravesicular Ca^{2+} [7]. The data suggest that the Na^+–Ca^{2+} exchanger is in an activated state when reconstitution is performed with an optimal lipid mixture. Thus, proteinase, SDS and intravesicular Ca^{2+} have little effect on Na^+–Ca^{2+} exchange activity in vesicles reconstituted with asolectin or PC/PS/cholesterol (3 : 5 : 2). In contrast, large stimulatory effects are observed when sarcolemma is reconstituted into PC/PS/cholesterol mixtures with low PS content. The one exception to this pattern is response to altered pH. The response to pH is identical in native vesicles and in vesicles reconstituted with a variety of lipid compositions. The result implies that the effect of protons on Na^+–Ca^{2+} exchange is different from that of the other interventions. Effects of proteinase treatment, intravesicular Ca^{2+} and anionic lipid components, on the other hand, may all be due to a common mechanism.

Modification of Na^+–Ca^{2+} exchange activity by altered pH could be due to one or both of two factors: a change in the ionization state of either protein amino acid residues or membrane lipid components. The second possibility seemed plausible because Na^+–Ca^{2+} exchange activity is sensitive to charged lipid components. For example, PE (present in large amounts in both sarcolemma and asolectin) will become negatively charged as pH is raised. To test this possibility, we used a lipid mixture for reconstitution which would not change ionization state as pH was varied. Thus, we found that the pH dependence of Na^+–Ca^{2+} exchange in vesicles reconstituted with PC/cardiolipin/cholesterol (5 : 3 : 2) was identical to that of both native vesicles and asolectin reconstituted vesicles (Fig. 8). We conclude that pH modulates Na^+–Ca^{2+} exchange activity by changing the ionization state of amino acid side chains on the exchanger protein. As noted previously [5], the pH dependence suggests a histidine residue may be involved.

Hale et al. [17] have used pronase treatment of solubilized sarcolemma as a step in the partial purification of the Na^+–Ca^{2+} exchanger. They report that stimulation of exchange activity produced by proteolysis of native sarcolemma is no longer evident after reconstitution. The reason may be that they were reconstituting into asolectin vesicles. Since asolectin would by itself induce an activated state, any stimulation caused by a prior proteinase step would be masked.

We have described the dependence of sarcolemmal Na^+–Ca^{2+} exchange activity on the membrane lipid environment. The results were obtained in reconstituted vesicles and are possibly not directly applicable to native membranes. Nevertheless, kinetic properties of the exchanger are generally similar in native and artificial membranes, and it is likely that lipid requirements are also related. We are pursuing further studies using cholesterol analogues and synthetic phospholipids to understand better the relevant protein–lipid interactions.

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