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SODIUM-CALCIUM EXCHANGE AND SIDEDNESS OF ISOLATED CARDIAC SARCOLEMMAL VESICLES

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Summary

The sidedness of isolated rabbit cardiac sarcolemmal vesicles was studied by observing the effects of several permeability-increasing agents on measurements of the amount of sialic acid released by neuraminidase, specific ouabain binding, and K^+-phosphatase and ($Na^+ + K^+$)-ATPase activities. The results suggest that the vesicles are sealed and are about 80% right-side-out. Na^+-Ca^{2+} exchange exhibited by these vesicles could be attributed to the sarcolemma rather than to some contaminating organelle. Ca^{2+} uptake was stimulated by preloading the vesicles with NaCl (and not KCl). Increasing the external [Na^+] induced a rapid Ca^{2+} loss, which could not be mimicked by K^+, Li^+, Rb^+ or choline$^+$. The Na_i^+-dependent Ca^{2+} uptake was inhibited by certain cations: $Cd^{2+} > La^{3+} > Y^{3+} > Mn^{2+} > Co^{2+} > Mg^{2+}$. The Na_i^+-dependent Ca^{2+} influx was enhanced by an inside positive membrane potential and inhibited by an inside negative membrane potential. Potentials were induced by a K^+-valinomycin system.

Introduction

The external [Ca^{2+}] is a critical determinant of the force of cardiac muscle contraction [1,2]. Recent physiological and biochemical experiments [2—4,29, 30] have indicated that Ca^{2+} bound to sarcolemmal membrane phospholipids

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Abbreviations: SDS, sodium dodecyl sulphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)benzene.

is closely related to the strength of cardiac muscle contraction. Although there may be differences among various cardiac tissues [30], the most straightforward interpretation of these findings is that Ca^{2+} enters the cell during systole in proportion to the sarcolemmal-bound Ca^{2+} and thus contributes to tension development. However, voltage-clamp experiments have indicated that the maximum amount of Ca^{2+} which might enter the cell as the 'slow inward' current is probably only 10—20% of that required to support tension development [5,6]. This has prompted many investigators to place the source of the majority of contractile-dependent Ca^{2+} inside the membrane potential barrier (e.g., sarcoplasmic reticulum via a Ca^{2+}-induced mechanism [7] or mitochondria [8]). This source can be placed outside the membrane potential barrier if the Ca^{2+} enters in electroneutral fashion as suggested by Langer et al. [9] (as a 2 : 1 Na^+-Ca^{2+} exchange which could not be detected by voltage-clamp studies). Mullins [10] has recently pointed out that a higher coupling ratio (e.g., 4 : 1) would in fact make Ca^{2+} entry by this mechanism appear as an outward current (opposite to that produced by Ca^{2+} entering through Ca^{2+} channels) which would be difficult to evaluate electrophysiologically. Physiological evidence for Na^+-Ca^{2+} exchange in cardiac muscle tissue has come from several laboratories [11—18]. Langer et al. [7] suggest that Na^+-Ca^{2+} exchange is used as a mechanism for Ca^{2+} entry into cardiac cells and invoked it to explain several inotropic interventions (glycoside, rate staircase, low Na^+ perfusion). Recently, Reeves and Sutko [19] and Pitts [20] have demonstrated the existence of Na^+-Ca^{2+} exchange in a preparation of cardiac sarcolemma.

The biochemical studies of Ca^{2+} binding which we have performed [3,4,29,30] have used the sarcolemmal preparation of Bers [21]. This preparation is of relatively high purity (with respect to sarcolemmal marker enzymes) and has been fairly well characterized with regard to its Ca^{2+}-binding properties and composition. The evaluation of these Ca^{2+}-binding studies and the Na^+-Ca^{2+} exchange experiments described here (and by others [19,20]) may depend on the sidedness and permeability characteristics of the sarcolemmal vesicles used. The present study demonstrates the sidedness, permeability and some basic properties of the Na^+-Ca^{2+} exchange system exhibited by the vesicles used.

Methods

Sarcolemmal isolation. Sarcolemmal vesicles were isolated from the ventricles of adult male New Zealand rabbits by using the method of Bers [21] with an additional step using a DNAase incubation to increase purity and yield. This step is described in more detail elsewhere [29]. This procedure involves Polytron homogenization, a brief treatment with 0.3 M KCl/25 mM pyrophosphate, treatment with DNAase I and differential and sucrose density gradient centrifugation. The sarcolemmal vesicles obtained are enriched about 20-fold (with respect to the homogenate) in the sarcolemmal markers K^+-stimulated *p*-nitrophenylphosphatase, (Na^+ + K^+)-ATPase, ouabain binding, sialic acid and cholesterol. Contamination by mitochondria was determined by succinate dehydrogenase specific activity, which was less than 1/10 of that in the homogenate. The major peak of (Ca^{2+} + Mg^{2+})-ATPase (used as a sarcoplasmic reticular marker) was well separated from the sarcolemmal fraction on the sucrose

density gradients. The sarcolemmal band was collected at a density of 1.11 g/ml (27%, w/w, sucrose) and sarcoplasmic reticulum and mitochondrial bands appeared at higher densities, well separated from the sarcolemma [21].

Sidedness experiments. The strategy used to approach the question of sidedness entails treating the vesicles with various permeability-increasing agents which ought to make intravesicular sites not normally exposed to the bathing medium more accessible.

Permeability perturbations. The conditions under which sodium dodecyl sulphate (SDS), sodium deoxycholate and alamethicin were used were based on those described by Besch et al. [22,23] and were found to have similar optima in our preparation. Vesicles (approx. 1 mg protein/ml) were incubated with SDS (0.3 mg/ml) or deoxycholate (1.2 mg/ml) for 20 min at 20°C. The treated vesicles were then used directly in neuraminidase experiments, diluted 15-fold into ouabain-binding medium or diluted 50-fold into enzyme-assay media. Vesicles were treated with alamethicin (0.75 mg alamethicin/mg protein) by incubation for 10 min at 37°C. The treated vesicles were used directly in neuraminidase experiments or diluted 10-fold into enzyme-assay media. Appropriate controls were run to account for the 33% ethanol in the alamethicin stock solution. Some vesicles were treated with phospholipase C (approx. 4 units/mg protein) for 5 min at 37°C. This procedure removed more than 50% of the phospholipid phosphate measured as described elsewhere [29]. These vesicles were used directly in neuraminidase experiments.

Neuraminidase experiments. Sarcolemmal vesicles were treated with neuraminidase (0.25—0.5 units/mg protein) at pH 5.1 for 30 min at 37°C. The amount of sialic acid released by neuraminidase was assayed directly by using the method of Warren [24] or the vesicles were spun down and acid-hydrolyzed so that the amount of sialic acid remaining could be measured. Even in the presence of detergents only about 75% of the sialic acid capable of being released by acid hydrolysis could be released by neuraminidase treatment.

Ouabain binding. Sarcolemmal vesicles were incubated in a medium containing 100 mM NaCl, 3.5 mM $MgCl_2$, 50 mM Tris, 50 nM—500 mM ouabain, 20 μCi/ml [^{3}H]ouabain with or without 3 mM ATP at pH 7.4 for 10 min at 37°C. Samples were filtered on Millipore filters (0.45 μm), filters were rinsed with 2 ml H_2O, dried and counted in a PPO/POPOP/toluene scintillation cocktail. Specific ouabain binding was taken as the increase in binding when ATP was present.

Other assays. K^+-*p*-nitrophenylphosphatase (K^+-phosphatase) and ($Na^+ + K^+$)-ATPase assays were performed as previously described [21]. Protein was determined by using the method of Lowry et al. [25]. Ca^{2+}-binding experiments were carried out as previously described [3,4]. Sarcolemmal vesicles (approx. 50 μg/ml) were incubated with $^{45}Ca^{2+}$, 5 mM Tris and 0.01—1.0 mM $CaCl_2$ at pH 7.4 for 10 min at 26°C. Aliquots were filtered, rinsed with 1 ml H_2O, dried and counted by liquid scintillation methods. In the presence of 1 mM $MgCl_2$, the Ca^{2+} binding was reduced by only 10—15%.

Na^+-Ca^{2+} exchange. The basic method employed for the study of Na^+-Ca^{2+} exchange was to preincubate or load sarcolemmal vesicles (approx. 3 mg/ml) with NaCl (140 mM) for 30 min at 37°C and then to dilute 25—50-fold into an Na^+-free uptake medium containing $^{45}Ca^{2+}$ (2.5 μCi/ml), 5 mM Tris at pH

7.4, 0.010—1 mM $CaCl_2$ and usually 140 mM KCl or 280 mM sucrose at 37°C for various lengths of time. Aliquots were then applied to Millipore filters (0.45 μm) under suction. The filters were then rinsed twice with 2 ml of a solution containing either 140 mM KCl or 280 mM sucrose and 0.5 mM $LaCl_3$ (to reduce external surface binding), dried and counted. Blanks for the Na^+-Ca^{2+} exchange experiments were vesicles which were either KCl-loaded or not loaded at all, but otherwise, treated as above. Subtraction of these 'binding blanks' from the Na^+-loaded values corrected the uptake or exchange values for Ca^{2+} bound to the external vesicular surface not displaced by the La^{3+} washes. To measure Ca^{2+} influx, the reaction was terminated by the addition of an equal volume of 140 mM KCl/1 mM $LaCl_3$, rather than by filtration as above, and the sample then immediately filtered and rinsed. Both methods yielded comparable results, but stopping KCl/$LaCl_3$ additions allowed uptakes at much shorter times (2 s) to be measured.

Intravesicular volume. The vesicle volume per mg protein was measured as the space which was accessible to [^{3}H]H_2O, but not to [^{14}C]sucrose. Vesicles (approx. 2 mg/ml) were incubated with 1.3 μCi/ml [^{3}H]H_2O in 5 mM Tris (pH 7.4) for 30 min and for 2 min more with 1.3 μCi/ml [^{14}C]sucrose. Samples were then filtered and the filters were counted in Aquasol in two channels in a liquid scintillation counter. Appropriate blanks and controls were run and the contributions of ^{3}H and ^{14}C could be readily resolved.

Materials. Neuraminidase was obtained from Worthington (Freehold, NJ). Phospholipase C was obtained from Boehringer-Mannheim (Indianapolis, IN). $^{45}Ca^{2+}$, $^{22}Na^+$, [^{3}H]ouabain, [^{14}C]sucrose and [^{3}H]H_2O were obtained from New England Nuclear (Boston, MA). A23187 was a generous gift from Dr. R.L. Hamill (Lily Res. Labs, Indianapolis, IN) and alamethicin was kindly furnished by Dr. J.E. Grady (Upjohn Co., Kalamazoo, MI).

Results

Sidedness experiments

The sialic acid released by neuraminidase, the ouabain binding and the K^+-phosphatase activity of sarcolemmal vesicles are likely to be due to the fraction of vesicles in the right-side-out orientation, since the sites involved are believed to face the extracellular space (see Fig. 1). If the vesicles are made permeable, the substances which interact with these sites obtain access to these sites in inside-out vesicles as well as right-side-out vesicles. Thus, the total sialic acid content and ouabain, K^+ and *p*-nitrophenylphosphate sites could be unmasked. Table I shows the effects of deoxycholate, SDS, alamethicin and phospholipase C pretreatments on the sialic acid released by neuraminidase, [^{3}H]-ouabain binding, K^+-phosphatase and (Na^+ + K^+)-ATPase.

It can be seen that phospholipase C, deoxycholate and alamethicin all increase the quantity of sialic acid released by neuraminidase by the same amount. The fact that these agents probably increase permeability by different means suggests that this effect is not artifactual. Alamethicin may have detergent-like effects at the concentration used here [26] and this may explain why alamethicin increased the accessibility of sialic acid to neuraminidase which would not be expected to enter through an alamethicin channel. Deoxycholate

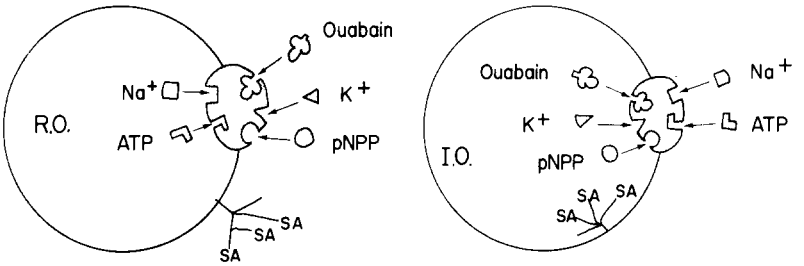

Fig. 1. Sidedness of sarcolemmal vesicles. A schematic diagram showing right-side-out (R.O.) and inside-out (I.O.) sarcolemmal vesicles. The stippled area represents the $(Na^+ + K^+)$-ATPase enzyme. In the right-side-out orientation, sialic acid (SA) and sites which bind ouabain, K^+ and *p*-nitrophenylphosphatase (pNPP) face the extravesicular medium, while Na^+ and ATP sites face the vesicle interior. In inside-out vesicles the orientations are reversed.

and SDS both increased the ouabain binding by the same amount. This amount is also the same as the increase in sialic acid released by neuraminidase discussed above. In addition, the K^+-phosphatase activity was increased by similar amounts by deoxycholate and alamethicin pretreatments. All of these results would suggest that approx. 78% of the sarcolemmal vesicles are right-side-out in orientation. Unfortunately, the results with $(Na^+ + K^+)$-ATPase are not consistent with this if it is assumed that the inaccessibility of ATP (or Na^+) to internal sites limits $(Na^+ + K^+)$-ATPase activity in right-side-out vesicles (see Fig. 1). To be consistent with the above results the treated : control ratio (Table I) would have been expected to be 4—5. The values obtained suggest that only about 40% of the vesicles are right-side-out and sealed. Looking at the previous data another way, it suggests that 20% of the vesicles are inside-

TABLE I

SIDEDNESS OF SARCOLEMMAL VESICLES

Sarcolemmal vesicles were either untreated (control) or treated with phospholipase C, deoxycholate, SDS or alamethicin as described in Methods. The third column indicates the ratio of treated:control for the measured parameters. The last column shows the implied % right-side-out vesicles assuming that the rate-limiting sites for neuraminidase, ouabain binding and K^+-phosphatase normally face the extracellular space and those for $(Na^+ + K^+)$-ATPase normally face the intracellular space. Mean control values for sialic acid removal, specific ouabain binding, K^+-phosphatase and $(Na^+ + K^+)$-ATPase are, respectively, 76 nmol/mg protein, 87 nmol/mg protein, 8.0 μmol/mg protein per h and 20.2 μmol/mg protein per h.

Parameter measured	Treatment (*n*)	Treated:control	% right-side-out
Sialic acid released by neuraminidase	Phospholipase C (3)	1.30 ± 0.05	77
	Deoxycholate (2)	1.26 ± 0.02	79
	Alamethicin (1)	1.22	82
[^{3}H]Ouabain binding	Deoxycholate (5)	1.31 ± 0.07	76
	SDS (1)	1.26	79
K^+-phosphatase	Deoxycholate (4)	1.18 ± 0.04	85
	Alamethicin (3)	1.38 ± 0.06	72
	SDS (2)	1.26 ± 0.00	79
$(Na^+ + K^+)$-ATPase	Deoxycholate (4)	1.58 ± 0.05	38
	Alamethicin (2)	1.64 ± 0.13	39
	SDS (1)	1.70	41

out and sealed. Thus, it might be that 40% of the vesicles are freely permeable.

It seems unlikely for several reasons that such a large fraction of the vesicles are freely permeable. Ca^{2+} binding to the sarcolemmal vesicles is increased 1.9-fold by addition of the divalent cation ionophore, A23187. Unless there is marked asymmetry with respect to the phospholipids which are important in Ca^{2+} binding, this result suggests that virtually all of the vesicles are impermeable to Ca^{2+}. We have seen only a small degree of asymmetry with respect to amino phospholipids [29]. Also previous experiments [3] have shown that [^{14}C]sucrose does not get into these sarcolemmal vesicles. In addition, the vesicle water space measured in the present study is, if anything, slightly larger than that which might have been estimated from the size of the vesicles in electron micrographs (see below). If sucrose entered the vesicles a smaller value would have been obtained. It should also be pointed out that ($Na^+ + K^+$)-ATPase activity was also close to 100% inhibited by 2 mM ouabain (with or without detergent pretreatment) which would be consistent with the vesicles being mostly righ-side-out. No completely satisfactory explanation for the lack of a larger increase in ($Na^+ + K^+$)-ATPase is proposed, although the interpretation of this result is not as straightforward as that of the others in Table I (since sites on both sides of the membrane are involved). That is, the results with sialic acid removal and ouabain binding are quite straightforward and in agreement (and membrane enzyme inactivation or uncoupling is not likely to affect the results). The K^+-phosphatase results are in agreement with these and lead to the same conclusion. We have found, however, that ($Na^+ + K^+$)-ATPase activity is much more labile than K^+-phosphatase activity under a variety of conditions (Bers, D. and Philipson, K., unpublished results). The data of Besch et al. [22] also suggest this to be the case, particularly for deoxycholate and SDS treatments of sarcolemmal vesicles. If, for example, ($Na^+ + K^+$)-ATPase is about 50% inhibited the results would be in agreement with the other parameters.

Intravesicular volume

The intravesicular volume (or space accessible to H_2O but not sucrose) was measured as 18.8 ± 2.3 μl/mg protein. If several assumptions are made a representative vesicle diameter (and surface area to volume ratio) can be obtained. The assumptions are that the vesicles are spherical with half the phospholipids facing inside and half facing outside, that a phospholipid molecules has a surface area of 65 Å^2 and that there are 1.19 μmol phospholipid/mg protein [29]. The diameter thus obtained (0.48 μm) is in reasonable agreement with electron-microscopic measurements which show that most of the vesicles are between 0.2 and 0.5 μm [21]. Another calculation assuming that the vesicles are spherical with an 80 Å thick membrane, half of which is protein and of density 1.11 g/ml gives a diameter of 0.51 μm. The intravesicular volume (measured by [^{14}C]sucrose and [^{3}H]H_2O) responds to osmotic gradients as would be predicted and is abolished by detergent treatment (data not shown).

Na^+-Ca^{2+} exchange

Fig. 2 shows the time course of Na_i^+-dependent Ca^{2+} uptake. Na^+-loaded vesicles were diluted 50-fold into an isotonic KCl-Tris medium containing

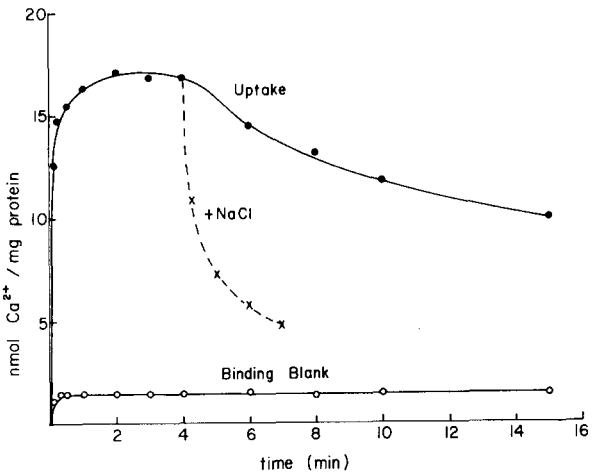

Fig. 2. Time course of Na_i^+-dependent Ca^{2+} uptake. Sarcolemmal vesicles were preloaded in 140 mM NaCl (•) or KCl (○) for 30 min at 37°C. They were then diluted 50-fold into a medium containing 280 mM sucrose, 50 μM $CaCl_2$, 10 mM Tris at pH 7.4 (with appropriate amounts of either NaCl or KCl to compensate for that in the different preload solutions). Aliquots were filtered at various times and the filters were quickly rinsed with two aliquots of 2 ml of 280 mM sucrose + 0.5 mM $LaCl_3$. A parallel experiment in which the $[Na^+]$ was increased to 50 mM at t = 4 min is also shown (X). The binding blank after this intervention is only slightly reduced from that shown for the control.

50 μM $CaCl_2$ at zero time. The binding blank represents the Ca^{2+} bound to the outside of the vesicle which is not removed by the $LaCl_3$/KCl wash. The binding blanks for this particular experiment were K^+-loaded vesicles treated identically. Experiments using vesicles which were not preloaded at all gave values the same or slightly higher than those preloaded with KCl suggesting that there is no K_i^+-stimulated Ca^{2+} uptake. In the rapid uptake experiments, KCl preloads were used so that the ionic composition was more comparable to the NaCl-loaded vesicles at short times. There is no significant difference in the values obtained by diluting the loaded vesicles into a medium containing 140 mM KCl or 280 mM sucrose. However, dilution into 140 mM NaCl almost abolished the Ca^{2+} uptake (see Fig. 5). These results indicate again that K^+ probably does not participate in the exchange and that the net Ca^{2+} uptake is much reduced in the presence of high external Na^+ concentration.

Returning to Fig. 2, it is apparent that the binding blank reaches a stable maximum relatively rapidly. The uptake of Ca^{2+} increases and reaches a plateau (after the binding does) and is maintained for typically 2—3 min after which time the net uptake declines over time. The upper curve in Fig. 3 shows an experiment similar to Fig. 2 where Ca^{2+} uptake reaches a maximum, plateaus and subsequently declines. The Ca^{2+} uptake measured at very short times (e.g., less than or equal to 0.08 min) is linear with respect to time and as such indicates that measuring uptake at, for example, 0.06 min may be a good estimate of the unidirectional influx of Ca^{2+}. The lower curve in Fig. 3 represents such influx measurements carried out by adding $^{45}Ca^{2+}$ to vesicles which had been accumulating cold Ca^{2+} for the lengths of time indicated. At these times, $^{45}Ca^{2+}$ was added and the uptake for 0.06 min was measured. It can be seen that the influx measured in this manner decreased steadily after the begin-

ning of the uptake. This decrease in influx parallels the decrease in net uptake over time, suggesting that a decrease in influx is at least partly responsible for the decline in uptake.

Also shown in Fig. 2 is the effect of increasing the extravesicular [Na^+] from 3 to 50 mM. This caused a rapid loss of intravesicular Ca^{2+} which had previously been taken up. Similar experiments (with appropriate controls and binding blanks) were performed for a series of monovalent cations. In these experiments, Na^+-loaded vesicles were allowed to take up Ca^{2+} for 2 min and then monovalent cations were added (70 mM final concentration while [Ca^{2+}] was a constant 50 μM) and the Ca^{2+} remaining in the vesicles 1 min later was measured. NaCl caused the vesicles to lose 82% of their Ca^{2+} wherease KCl, LiCl, RbCl and choline chloride produced only 1 to 16% release. This demonstrates that the Na^+-stimulated Ca^{2+} loss is quite selective for Na^+ over the monovalent cations tested.

The relative inhibitory effects of several divalent and trivalent cations on Na^+-stimulated Ca^{2+} uptake were also measured. The inhibition sequence and percent inhibition of Ca^{2+} uptake by these cations (50 μM Ca^{2+} and inhibitor) was $Cd^{2+} > La^{3+} > Y^{3+} > Mn^{2+} > Mg^{2+}$ (84, 66, 53, 32, 17, −2%). No significant inhibition by Mg^{2+} was seen when Mg^{2+} and Ca^{2+} concentrations were increased to 0.5 mM.

It is important to determined whether or not this Na^+-Ca^{2+} exchange activity is really representative of the sarcolemma itself or of contamination from some other organelle. Table II shows that the Na^+-Ca^{2+} activity copurifies with the sarcolemmal marker enzyme, K^+-phosphatase. It should be noted that fractions 6 and 7, the putative sarcoplasmic reticular and mitochondrial fractions, respectively (as measured by (Ca^{2+} + Mg^{2+})-ATPase, and succinate dehydrogenase), contain relatively small amounts of this Na^+-Ca^{2+} exchange activity. This suggests that this Na^+-Ca^{2+} exchange activity is indeed sarcolemmal. Mitochondria also exhibit Na^+-induced Ca^{2+} release [27,28] which works only in the Ca^{2+}-efflux mode and is stimulated by Li^+. In addition, the mitochon-

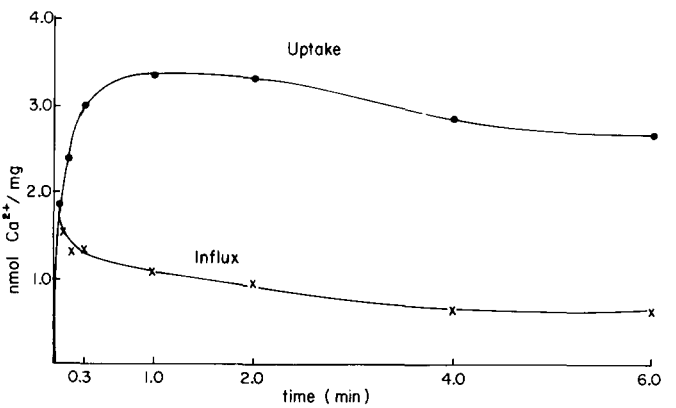

Fig. 3. Time course of Na_i^+-dependent Ca^{2+} uptake and influx. Na^+- or K^+-loaded vesicles were diluted 50-fold into a Ca^{2+}-uptake medium with (•) or without (X) $^{45}Ca^{2+}$. The uptake (•) was measured by adding an aliquot of 140 mM KCl + 1.0 mM $LaCl_3$ at the times indicated, filtering immediately and rinsing the filters twice with 2 ml of 140 mM KCl + 1.0 mM $LaCl_3$. The influx (X) was measured by adding the $^{45}Ca^{2+}$ at the times indicated and measuring the initial uptake by stopping as above, 0.06 min later. Binding blank values have already been subtracted.

drial Ca^{2+}-influx system is not Na^+ dependent, is energy dependent and has membrane potential dependence opposite to that shown here (see below). These differences along with the copurification results in Table II indicate that the sarcolemmal Na^+-Ca^{2+} exchange is distinct from the mitochondrial system.

In the pH range 6.6—8.2, the Na_i^+-dependent Ca^{2+} uptake showed a maximum at pH 7.4. Variations in temperature over the range 20—37°C showed a very small dependence of Na_i^+-dependent Ca^{2+} uptake on temperature. Vesicles could be stored at 4°C overnight without a great loss of activity, while freezing was more detrimental. While several of the experiments described in the present study have Ca^{2+} uptake values of less than 10 nmol/mg, more recent experiments have shown Na_i^+-dependent Ca^{2+} uptake values in the range 25—35 nmol/mg protein with the same qualitative results.

Fig. 4 shows the dependence of Na_i^+-dependent Ca^{2+} uptake (at 2 min) and influx (0.06 min) on Ca^{2+} concentration. Both uptake and influx are half-maximally stimulated by approx. 30 μM Ca^{2+}. This gives us an apparent K_m and also suggests that some information obtained from plateau measurements may be correlated to initial influx values.

Experiments were performed in which the Ca^{2+} ionophore, A23187 (3 μM), was added to vesicles which had taken up Ca^{2+} (40 μM) for 1 min. A23187 caused the vesicles to lose more than 80% of the Ca^{2+} which was retained in controls (3 and 9 min later). This suggests that Ca^{2+} is being taken up against a concentration gradient. In addition, when SDS is added at the plateau of Ca^{2+} uptake, Ca^{2+} is rapidly lost (Fig. 5).

Vesicles were loaded for various lengths of time with $^{22}Na^+$, filtered and rinsed with two 2 ml aliquots of 280 mM sucrose, 0.5 mM $LaCl_3$. The $^{22}Na^+$ level increased only very slowly after 15 min up to 60 min. A similar time course is obtained when Na^+-dependent Ca^{2+} uptake is measured as a funtion of Na^+-preload time.

The Na^+ loss produced by adding 50 μM Ca^{2+} to $^{22}Na^+$-loaded vesicles (70 mM NaCl in loading solution) is about 10—20 nmol/mg protein. This is about 1.5—3 times the Ca^{2+} uptake obtained under similar conditions. Unfortunately, reliable measurements of the initial rate of $^{22}Na^+$ efflux could not be obtained,

TABLE II

COPURIFICATION OF Na^+-Ca^{2+} EXCHANGE WITH K^+-PHOSPHATASE

Samples were preincubated with 140 mM NaCl (or not, as a binding blank) and then allowed to take up Ca^{2+} in a medium containing 280 mM sucrose, 2.8 mM NaCl, 50 μM $CaCl_2$ and 10 mM Tris at pH 7.4 for 3 min at 37°C before filtering. The fractions are homogenate and sucrose gradient fractions (2, 3, 4, 6 and 7). Fraction 2 represents the sarcolemmal fraction; fraction 6, the sarcoplasmic reticulum; fraction 7, the mitochondria.

Fraction	Na^+-dependent Ca^{2+} uptake (nmol/mg protein)	K^+-phosphatase (μmol/mg protein per h)
Homogenate	0.22	0.35
2	5.64	4.62
3	4.56	2.85
4	4.08	2.47
6	1.41	1.14
7	0.23	0.24

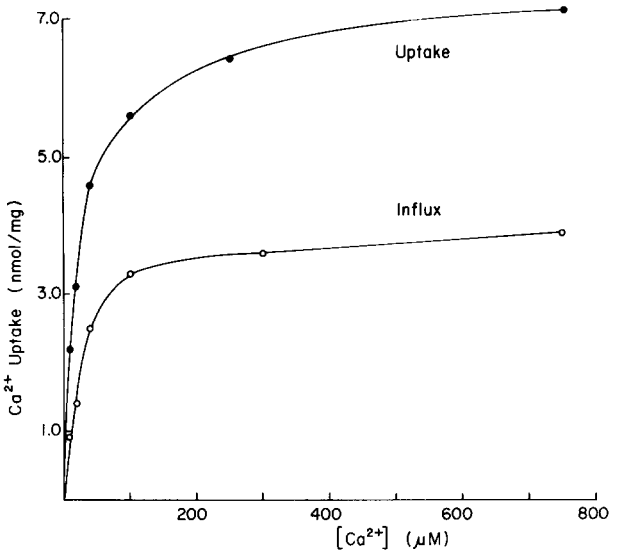

Fig. 4. Na_i^+-dependent Ca^{2+} uptake and influx and Ca^{2+} concentration. The uptake (●) and influx (○) of Ca^{2+} is shown as a funtion of extravesicular $[Ca^{2+}]$. The uptake values were obtained at the plateau of uptake (t = 2 min), and the initial influx was obtained during the first 0.06 min of uptake. The procedures used were otherwise the same as in Fig. 3 and binding blanks have been subtracted.

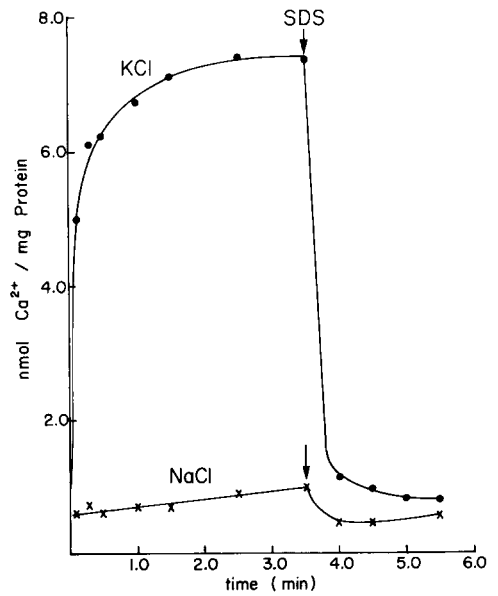

Fig. 5. Na_i^+-dependent Ca^{2+} uptake in KCl and NaCl. NaCl-preloaded or unloaded vesicles were diluted 50-fold into either 140 mM KCl (●) or 140 mM NaCl (X) with 50 μM $CaCl_2$ and 10 mM Tris at pH 7.4 The binding blank for the KCl medium was stable at about 1 nmol/mg protein and for the NaCl medium it was stable at about 0.7 nmol/mg protein. Sodium dodecyl sulphate (SDS) was added after uptake had proceeded for 3.5 min (final concentration 0.3 mg/ml). This addition did not decrease the binding blank significantly.

TABLE III

EFFECT OF MEMBRANE POTENTIAL ON Na^+-DEPENDENT Ca^{2+} INFLUX

Vesicles were loaded in 140 mM KCl (binding blanks), 70 mM NaCl + 70 mM sucrose or 70 mM NaCl + 70 mM KCl. After 50-fold dilution the concentrations of all species except valinomycin and intravesicular Na^+ were the same for controls, binding blanks and experimentals in each group. The loaded vesicles were diluted into media containing different $[K^+]$ and ± valinomycin (15 μg/ml). The expected membrane potential (E_m) (inside with respect to outside) is indicated along with the ideal $[K^+]_i$, $[K^+]_o$ and the Na_i^+-dependent Ca^{2+} influx as % of the non-valinomycin-treated controls. Influx was measured at 0.06 min as described in Methods.

Expected sign of E_m	Expected $[K^+]_i$	$[K^+]_o$	Ca^{2+} influx (% control)
+	0	140	146
0	70	70	92
−	70	2.8	56

so these experiments do not imply a particular stoichiometry for Na^+-Ca^{2+} exchange.

If the coupling is not 2 : 1 (Na^+ : Ca^{2+}) the exchange ought to be sensitive to transmembrane potential. Table III shows the effects of various expected membrane potentials (induced by K^+-valinomycin) on Na^+-dependent Ca^{2+} influx. It can be seen that an inside positive potential stimulated and an inside negative potential inhibited Na^+-dependent Ca^{2+} influx. This suggests that more than two Na^+ move out for each Ca^{2+} which enters or that the exchange is somehow stimulated by an inside positive potential.

Discussion

Vesicle sidedness

The results from the sidedness experiments indicate that the sarcolemmal vesicles are approx. 80% right-side-out. Although the (Na^+ + K^+)-ATPase result could not readily be reconciled with this result, the remarkable consistency of the predicted value from experiments where ouabain binding, K^+-phosphatase and sialic acid removal by neuraminidase (using various permeability perturbations) were measured makes the result convincing. It would be very useful to have some specific ultrastructural corroboration, but our initial attempts have not been successful. Besch et al. [22] performed some experiments similar to those in the present study and estimated that about 75% of their vesicles were inside-out. This is of particular interest, since they use a similar homogenization procedure, but a medium of very low osmolarity. The results in the present study imply that the sarcolemmal vesicles are mostly in their normal orientation which may make interpretation of transport or binding phenomena somewhat more directly applicable to intact cells.

Intravesicular volume

The intravesicular volume obtained (18.8 μl/mg protein) is in reasonable agreement with electron-microscopic results (see Results), but is 3.5—4.0 times the value obtained by Reeves and Sutko [19] using similar techniques.

The volume obtained in the present study is still small and contributes in part to the difficulty of measuring Na^+-Ca^{2+} exchange. If the intravesicular free $[Ca^{2+}]$ is 1 mM, the free Ca^{2+} makes up less than 10% of the total vesicular Ca^{2+} (due to binding). Therefore, great caution should be taken that appropriate binding blanks accompany the exchange experiments.

Na^+-Ca^{2+} exchange

The sarcolemmal vesicles used in the present study exhibit properties of Na^+-Ca^{2+} exchange. This activity copurified with the sarcolemmal marker enzyme, K^+-phosphatase, indicating that the exchange is indeed sarcolemmal. Preloading the vesicles with NaCl allowed them to take up Ca^{2+} rapidly (upon dilution into a Ca^{2+}-containing low $[Na^+]$ medium). If the $[Na^+]_o$ was then increased the Ca^{2+} taken up was rapidly lost. Preloading the vesicles with KCl did not lead to Ca^{2+} uptake nor were K^+, Li^+, Rb^+, or choline able to induce Ca^{2+} efflux from vesicles which had taken up Ca^{2+}. These results indicate that the Na^+-Ca^{2+} exchange can work in both directions and also that, the exchange moiety is selective for Na^+ over other monovalent cations.

It is of interest that the apparent K_m value for Ca^{2+} uptake (stimulated by Na_i^+) is 30 μM. This is similar to the value obtained by Reeves and Sutko [19] (18 μM) and is also similar to the apparent K_m value for high-affinity Ca^{2+}-binding sites determined by Bers and Langer [3] for neonatal rat sarcolemma (prepared essentially by using the same methods used in the present study). The sequence of effectiveness of inhibition of Na_i^+-dependent Ca^{2+} uptake by divalent and trivalent cations found in the present study was $Cd^{2+} > La^{3+} > Y^{3+} > Mn^{2+} > Co^{2+} > Mg^{2+}$. This is also similar to the sequence of inhibition of tension development obtained by Bers and Langer [3] in neonatal rat papillary muscles ($Cd^{2+} > Y^{3+} > La^{3+} > Mn^{2+} \sim Co^{2+} > Mg^{2+}$). In that study this sequence was correlated to the sequence of effectiveness of the same ions at displacing Ca^{2+} bound to isolated cardiac sarcolemma of the neonatal rat ($Y^{3+} > La^{3+} > Cd^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$). They attributed the relatively greater tension-reducing ability of Cd^{2+} to potential intracellular and diffusional effects. The present paper shows Cd^{2+} to be by far the most potent inhibitor of Na_i^+-dependent Ca^{2+} uptake (of the ions tested). If sarcolemmal Na^+-Ca^{2+} exchange is a physiologically important mechanism of Ca^{2+} entry in functioning myocardial tissue, the greater tension-reducing ability of Cd^{2+} may also be partially due to its inhibition of this system. However, it should also be noted that the order of effectiveness of the trivalent cations (La^{3+} and Y^{3+}) in the inhibition of Na^+-Ca^{2+} exchange is the opposite of that found for tension depression and Ca^{2+} displacement.

It is of interest to extrapolate the amount of Ca^{2+} influx which might be produced by the measured Na_i^+-dependent Ca^{2+} influx in a whole tissue. We can attempt this by taking a typical value of Na_i^+-dependent Ca^{2+} influx at a high $[Ca^{2+}]_o$ of about 2 nmol/mg protein per s. Dividing by the sarcolemmal purification factor (20 mg homogenate protein/mg sarcolemmal protein) and multiplying by the quantity of mg homogenate protein/g wet heart, a value of 13 μmol/kg wet heart per s is obtained. The latter value is in the general range of the amount of Ca^{2+} which must be delivered to the myofilaments

for tension development per beat (5—35 μmol/kg wet weight control tension from 0—90% maximum [6]). However, it should be pointed out that these are Na^+-loaded vesicles and Na_i^+-dependent Ca^{2+} influx has not been demonstrated under physiological conditions (e.g., Na_o^+ inhibits Na_i^+-dependent Ca^{2+} influx half-maximally at 30 mM Na_o^+ for $[Ca^{2+}]_o$ of 50 μM).

The results of the experiments where K^+ and valinomycin were used to control the membrane potential indicate that the Na^+-Ca^{2+} exchange is potential sensitive. The simplest interpretation of these results is that the exchange is electrogenic such that more than two Na^+ exchange for each Ca^{2+}. The results also suggest that cell membrane depolarization increases the Na_i^+-dependent Ca^{2+} influx. Reeves (personal communication) has seen a similar potential dependence of Na^+-Ca^{2+} exchange in rabbit sarcolemmal vesicles. Pitts [21] has recently estimated the stoichiometry of Na^+-Ca^{2+} exchange in canine cardiac sarcolemmal vesicles to be 3 Na^+ : Ca^{2+}. Horackova and Vassort [18] have shown evidence for an electrogenic Na^+-Ca^{2+} exchange (more than 2 Na^+ : Ca^{2+}) involved in the regulation of contractility in frog ventricle. The biochemical results are thus consistent with the physiological results. If such an electrogenic Na^+-Ca^{2+} exchange system were functional during cardiac systole, Ca^{2+} entry would produce an outward membrane current which would be difficult to resolve electrophysiologically and might also cause underestimation of the slow inward current. It seems possible, in view of the preceding discussion, that an electrogenic Na^+-Ca^{2+} exchange mechanism might contribute to Ca^{2+} influx and the subsequent tension development in the cardiac muscle excitation-contraction coupling sequence.

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