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# Symmetry properties of the $Na^+$-$Ca^{2+}$ exchange mechanism in cardiac sarcolemmal vesicles

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**The $Na^+$-$Ca^{2+}$ exchange mechanism in cardiac sarcolemmal vesicles can catalyze the exchange of $Ca^{2+}$ on either side of the sarcolemmal membrane for $Na^+$ on the opposing side. Little is known regarding the relative affinities of $Na^+$ and $Ca^{2+}$ for exchanger binding sites on the intra- and extracellular membrane surfaces. We have previously reported (Philipson, K.D. and Nishimoto, A.Y. (1982) J. Biol. Chem. 257, 5111–5117) a method for measuring the $Na^+$-$Ca^{2+}$ exchange of only the inside-out vesicles in a mixed population of sarcolemmal vesicles (predominantly right-side-out). We concluded that the apparent $K_m(Ca^{2+})$ for $Na_i^+$-dependent $Ca^{2+}$ uptake was similar for inside-out and right-side-out vesicles. In the present study, we examine in detail $Na_o^+$-dependent $Ca^{2+}$ efflux from both the inside-out and the total population of vesicles. To load vesicles with $Ca^{2+}$ prior to measurement of $Ca^{2+}$ efflux, four methods are used: 1, $Na^+$-$Ca^{2+}$ exchange; 2, passive $Ca^{2+}$ diffusion; 3, ATP-dependent $Ca^{2+}$ uptake; 4, exchange of $Ca^{2+}$ for $Na^+$ which has been actively transported into vesicles by the $Na^+$ pump. The first two methods load all sarcolemmal vesicles with $Ca^{2+}$, while the latter two methods selectively load inside-out vesicles with $Ca^{2+}$. We are able to conclude that the dependence of $Ca^{2+}$ efflux on the external $Na^+$ concentration is similar in inside-out and right-side-out vesicles. Thus the apparent $K_m(Na^+)$ values ($\approx$ 30 mM) of the $Na^+$-$Ca^{2+}$ exchanger are similar on the two surfaces of the sarcolemmal membrane. In other experiments, external $Na^+$ inhibited the $Na_i^+$-dependent $Ca^{2+}$ uptake of the total population of vesicles much more potently than that of the inside-out vesicles. Apparently $Na^+$ can compete for the $Ca^{2+}$ binding site more effectively on the external surface of right-side-out than on the external surface of inside-out vesicles. Thus, although affinities for $Na^+$ or $Ca^{2+}$ (in the absence of the other ion) appear symmetrical, the interactions between $Na^+$ and $Ca^{2+}$ at the two sides of the exchanger are not the same. The $Na^+$-$Ca^{2+}$ exchanger is not a completely symmetrical transport protein.**

## Introduction

A highly active $Na^+$-$Ca^{2+}$ exchange system is present in the cardiac sarcolemmal membrane and has been implicated in the regulation of myocardial contractility (see Refs. 1–3 for reviews). The

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

exchanger is electrogenic [4–7] and probably catalyzes the countertransport of three sodium ions on one side of the membrane for one $Ca^{2+}$ ion on the opposing side [8,9]. The dependence of vesicular $Na^+$-$Ca^{2+}$ exchange on $Na^+$ and $Ca^{2+}$ levels and the competitive interactions between $Na^+$ and $Ca^{2+}$ have been studied in detail (e.g., Refs. 7, 10–14).

One assumption implicit in the analysis of data on the kinetics of $Na^+$-$Ca^{2+}$ exchange is the homogeneity of vesicles. However, sarcolemmal preparations contain both inside-out and right-side-out vesicles, and both types of vesicle will participate in $Na^+$-$Ca^{2+}$ exchange reactions. For example, if the entire population of vesicles is first preloaded with $Na^+$ by passive diffusion, then both the inside-out and the right-side-out vesicles will be able to take up $Ca^{2+}$ by $Na_i^+$-dependent $Ca^{2+}$ influx. If the oppositely oriented vesicles have different exchange properties, complex kinetic patterns might be discerned and misinterpreted. This could occur, for example, if $Na^+$ or $Ca^{2+}$ interacted differently with the two surfaces of the exchange mechanism.

We have previously addressed this problem by comparing the $Na_i^+$-dependent $Ca^{2+}$ uptake of inside-out vesicles with that of the entire population of vesicles [11]. These experiments were accomplished by selectively preloading different subpopulations of sarcolemmal vesicles with $Na^+$ prior to the $Na_i^+$-dependent $Ca^{2+}$ uptake reaction. Inside-out sarcolemmal vesicles were preloaded with $Na^+$ by ATP-dependent $Na^+$ pumping. $Ca^{2+}$ taken up in exchange for this $Na^+$ could then be measured. The $Na^+$-$Ca^{2+}$ exchange of the entire population of vesicles was measured after preloading with $Na^+$ by passive diffusion. We inferred that the $Ca^{2+}$ binding sites on the two sides of the exchanger had identical properties (i.e., dependence on $[Ca^{2+}]$, pH, membrane potential). The data also suggested that the $Na^+$ binding sites might have asymmetric properties but this was not examined in detail.

In the present report, we extend these studies and examine the competition between $Na^+$ and $Ca^{2+}$ for transport sites on opposing sides of the exchange mechanism. In addition, using improved techniques for measuring $Na_o^+$-dependent $Ca^{2+}$ efflux, we reexamine the properties of the exchanger $Na^+$ binding sites. The results enable us to model some aspects of sarcolemmal $Na^+$-$Ca^{2+}$ exchange.

## Materials and Methods

*Sarcolemmal isolation*

Highly purified sarcolemmal vesicles were isolated from canine ventricles as described by Frank et al. [15].

*$Na_i^+$-dependent $Ca^{2+}$ uptake*

Our general techniques for accurate measurement of $Na^+$-$Ca^{2+}$ exchange are described in a recent review [16]. In this study, we used two techniques to preload vesicles with $Na^+$ in preparation for the $Na_i^+$-dependent $Ca^{2+}$ uptake reaction. The methods are modifications of those previously used in this laboratory [11,17]. All steps were carried out at 37°C.

(i) The digitoxigenin-sensitive, ATP-dependent $Na^+$ pump was used to selectively preload inside-out sarcolemmal vesicles with $Na^+$. To activate $Na^+$ transport, KCl (140 mM)-loaded vesicles (0.006 ml; approx. 3 mg protein/ ml) were diluted to 0.034 ml of solution so that final concentrations were 25 mM KCl, 20 mM NaCl, 5 mM $NaN_3$, 74 mM choline chloride, 3 mM $MgCl_2$, 2.4 mM Tris-ATP, 2.4 mM creatine phosphate, 7 mM Mops (pH 7.4 at 37°C). This was done in the presence and absence of digitoxigenin (100 $\mu$M). After 1.0 min of active $Na^+$ uptake, 0.025 ml of the sample was used directly to measure the $Na_i^+$-dependent $Ca^{2+}$ uptake of the inside-out vesicles as described below. In some experiments EGTA (20 $\mu$M) was also present during this $Na^+$-loading procedure with no apparent effect on results.

(ii) To preload the total population of vesicles with $Na^+$ by passive diffusion, KCl-loaded vesicles (0.006 ml) were first diluted (0.030 ml final volume) in a medium so that concentrations were 28 mM KCl, 50 mM NaCl, 6.0 mM $NaN_3$, 56 mM choline chloride, 100 $\mu$M digitoxigenin, 10 mM Mops (pH 7.4 at 37°C). KCl (56 mM) replaced the NaCl and $NaN_3$ for blanks. After 20 min (a time sufficient for Na equilibration), 0.004 ml of solution containing $MgCl_2$, Tris-ATP and creatine phosphate was added to give final concentrations in the 0.034 ml of 3.0, 2.4 and 2.4 mM, respectively. After 1.0 min, 0.025 ml of the sample was used directly to measure $Na_i^+$-dependent $Ca^{2+}$ uptake (see below). The reason for this protocol was to passively preload vesicles with $Na^+$ in a medium which closely re-

sembled that used to preload by active $Na^+$ pumping (see above). Thus, in both cases, the vesicles were exposed to equal concentrations of $Mg^{2+}$, ATP and creatine phosphate for 1 min. For the case of passive $Na^+$ loading, digitoxigenin was always present to prevent any active $Na^+$ uptake. Again, the presence of 20 $\mu$M EGTA during this procedure did not significantly affect the outcome of results.

$Na_i^+$-dependent $Ca^{2+}$ uptake was initiated by rapidly diluting 0.025 ml of $Na^+$-loaded vesicles to 0.50 ml of $Ca^{2+}$ uptake medium. After 2.0 s, the reaction was quenched by the automatic addition of 0.03 ml of 140 mM KCl, 2 mM $LaCl_3$. The $Ca^{2+}$ uptake medium contained 140 mM KCl, 0.4 $\mu$M valinomycin, 50 $\mu$M digitoxigenin, 0.6 $\mu$Ci $^{45}Ca^{2+}$, variable $^{40}CaCl_2$, variable NaCl, and 10 mM Mops (pH 7.4 at 37°C). When the vesicles had been preloaded with $Na^+$ by active $Na^+$ uptake, blanks were obtained using samples in which digitoxigenin had been included during the $Na^+$ pump procedure ((i) above). For vesicles passively preloaded with $Na^+$, blanks were obtained by using the vesicles which had been exposed to $K^+$ instead of $Na^+$ (see above). These blanks corrected for any $Na^+$ gradient-independent $Ca^{2+}$ uptake and superficially bound $Ca^{2+}$. In the case where vesicles had been preloaded with $Na^+$ by active transport, the blank also corrected for $Ca^{2+}$ taken up in exchange for $Na^+$ which had not actively been pumped into inside-out vesicles. Vesicles were harvested by Millipore filtration (0.45 $\mu$m) and washed with $2 \times 3$ ml of 140 mM KCl, 0.1 mM $LaCl_3$.

*$Na_o^+$-dependent $Ca^{2+}$ efflux*

The strategy was to first preload sarcolemmal vesicles with $Ca^{2+}$, add EGTA to inhibit further $Ca^{2+}$ influx, and then monitor the influence of added extravesicular $Na^+$ on $Ca^{2+}$ efflux. We used four different methods to load vesicles with $Ca^{2+}$. The first two of these methods load the entire population of sarcolemmal vesicles with $Ca^{2+}$, while the latter two methods preload only inside-out vesicles with $Ca^{2+}$ as follows:

*(i) $Na^+$-$Ca^{2+}$ exchange.* $Na^+$ (140 mM)-loaded vesicles (0.003 ml) were diluted to 0.50 ml containing 140 mM KCl (or NaCl for blanks), 0.030 mM $CaCl_2$, 0.6 $\mu$Ci $^{45}CaCl_2$, and 10 mM Mops (pH 7.4 at 37°C). After 1.0 min of $Ca^{2+}$ loading by $Na_i^+$-dependent $Ca^{2+}$ uptake, $Ca^{2+}$ efflux was initiated by the addition of 0.50 ml of medium containing 140 mM KCl, 0.20 mM EGTA, variable NaCl, and 10 mM Mops (pH 7.4 at 37°C). Efflux was terminated by the addition of 0.03 ml of 140 ml KCl, 7.5 mM $LaCl_3$.

*(ii) Passive $Ca^{2+}$ equilibration.* $K^+$ (140 mM)-loaded vesicles were preincubated overnight at 4°C with 1.5 mM $CaCl_2$ and 5 $\mu$Ci/ml $^{45}CaCl_2$. After warming the vesicles to 37°C, $Ca^{2+}$ efflux was initiated by diluting 0.01 ml of the $Ca^{2+}$-loaded vesicles to 0.50 ml of 140 mM KCl, 0.25 mM EGTA, variable NaCl, and 10 mM Mops (pH 7.4 at 37°C). Efflux was terminated by the addition of 0.03 ml of 140 mM KCl, 6 mM $LaCl_3$.

*(iii) $Na^+$ pump / $Na^+$-$Ca^{2+}$ exchange.* $Na^+$ was first actively pumped into inside-out vesicles and then $Ca^{2+}$ was taken up in the exchange for this component of internal $Na^+$. This was similar to the method described earlier in this paper with the modification that concentrations during the $Na^+$ transport step were 25 mM KCl, 16 mM NaCl, 4.5 mM $NaN_3$, 78 mM choline chloride, with or without 0.1 mM digitoxigenin, 0.75 mM $MgCl_2$, 0.6 mM Tris-ATP, 2.4 mM creatine phosphate, 7 mM Mops (pH 7.4 at 37°C). After 1.0 min of $Na^+$ pumping, 0.025 ml were diluted to 0.50 ml in 140 mM KCl, 0.05 mM digitoxigenin, 0.020 mM $CaCl_2$, 0.6 $\mu$Ci $^{45}CaCl_2$, and 10 mM Mops (pH 7.4 at 37°C). After 0.5 min of $Ca^{2+}$ uptake in this medium, $Ca^{2+}$ efflux was initiated by the addition of 0.70 ml of 140 mM KCl, 0.2 mM EGTA, variable NaCl, and 10 mM Mops (pH 7.4 at 37°C). $Ca^{2+}$ efflux was terminated by the addition of 0.03 ml of 140 mM KCl, 10 mM $LaCl_3$.

*(iv) ATP-dependent $Ca^{2+}$ uptake.* $K^+$ (140 mM)-loaded vesicles (0.005 ml) were diluted to 0.25 ml in 140 mM KCl, 0.005 mM $CaCl_2$, 0.3 $\mu$Ci $^{45}CaCl_2$, 10 mM Mops (pH 7.4 at 37°C) in the presence and absence (for blanks) of 1.5 mM $MgCl_2$ and 1.2 mM Tris-ATP. After 3.0 min of active $Ca^{2+}$ uptake, $Ca^{2+}$ efflux was initiated by the addition of 0.25 ml of 140 mM KCl, 0.5 mM EGTA, variable NaCl, and 10 mM Mops (pH 7.4 at 37°C). Efflux was terminated by the addition of 0.03 ml of 140 mM KCl, 15 mM $LaCl_3$. For the experiments carried out at 23°C (Fig. 5), the initial $Ca^{2+}$ uptake period was 10.0 min instead of 3.0 min.

The advantage of the two methods involving $Na^+$-$Ca^{2+}$ exchange is that specifically only the sarcolemmal vesicles in the preparation will become $Ca^{2+}$ loaded. Sarcoplasmic reticular impurities may become $Ca^{2+}$ loaded after ATP-dependent $Ca^{2+}$ uptake. With passive $Ca^{2+}$ equilibration, all intact vesicles, regardless of the organelle of origin, will be $Ca^{2+}$-loaded. These apparent disadvantages of the $Ca^{2+}$ pump and passive $Ca^{2+}$ equilibration methods, however, are obviated by the fact that the observed reaction, $Na_o^+$-dependent $Ca^{2+}$ efflux, will occur only with sarcolemmal vesicles. That is, even if some non-sarcolemmal vesicles have become loaded with $Ca^{2+}$, they will not contribute to $Na_o^+$-dependent $Ca^{2+}$ efflux. (The inner mitochondrial membrane has an $Na^+$-dependent $Ca^{2+}$ efflux mechanism [18]. However, this transport pathway has very low activity compared with that in the sarcolemmal membrane. It is rather unlikely that a small mitochondrial contamination would interfere with any measurements reported here.)

In all cases, osmolarity was maintained by lowering the [KCl] when the [NaCl] was increased. Vesicles were harvested by Millipore filtration and washed with $2 \times 3$ ml of 140 mM KCl, 0.1 mM $LaCl_3$. In each $Ca^{2+}$ efflux experiment, samples were run in triplicate or quadruplicate. Conditions which varied from those described are elaborated in figure or table legends. Data are expressed as means $\pm$ S.E.

## Results

*$Na_i^+$-dependent $Ca^{2+}$ uptake*

We first examine the competition between $Na^+$ and $Ca^{2+}$ for binding sites on the two surfaces of the exchange mechanism. Extravesicular $Na^+$ will inhibit $Na_i^+$-dependent $Ca^{2+}$ uptake by competing with $Ca_o^{2+}$ for binding sites on the extravesicular surface of the exchange mechanism [13]. Fig. 1 shows that extravesicular $Na^+$ is a much more effective inhibitor of the $Na_i^+$-dependent $Ca^{2+}$ uptake of the total population of sarcolemmal vesicles ($\approx 60/40$, right-side out/ inside out [15]) than of the inside-out subpopulation of vesicles.

The experiment shown in Fig. 1 was performed at $[Ca^{2+}] = 20\ \mu M$. In another set of experiments, we used conditions more likely to prevail at the intracellular surface in vivo. As shown in Table I, the $Na^+ - Ca^{2+}$ exchange at 4 $\mu$M $Ca^{2+}$ is inhibited only slightly by 10 mM $Na^+$ in inside-out vesicles but by a much larger amount in the entire population of sarcolemmal vesicles.

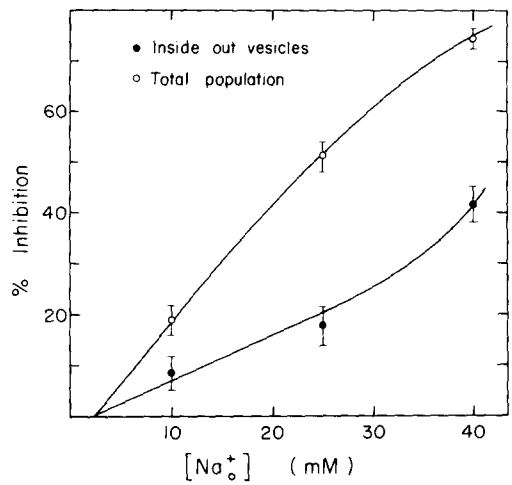


Fig. 1. Inhibition by extravesicular $Na^+$ of $Na_i^+$-dependent $Ca^{2+}$ uptake in inside-out vesicles (●) and the total population of sarcolemmal vesicles (○). When no $Na^+$ was included in the $Ca^{2+}$ uptake medium, the $[Na^+]$ was 2.4 mM from the addition of the vesicles themselves. This explains why the drawn lines do not pass through zero on the abscissa. The $Ca^{2+}$ uptake reaction was carried out for 2.0 s at $[Ca^{2+}] = 20\ \mu M$. $Na_i^+$-dependent $Ca^{2+}$ uptake was $5.1 \pm 0.9$ and $6.0 \pm 1.1$ nmol/mg protein per s for the inside-out and total population of vesicles, respectively. $n = 8$.

One possible source of artifact in these experiments was that, as extravesicular $Na^+$ was increased, the extravesicular $K^+$ was lowered to maintain osmolarity. Valinomycin was present in the medium to maintain an inside-positive membrane potential for optimal $Na_i^+$-dependent $Ca^{2+}$ uptake [5]. Possibly the magnitude of the membrane potential varied differently with altered $[K_o^+]$ in the inside-out and total population cases. To check this possibility, experiments were carried out in the absence of valinomycin. In this situation the membrane potential is determined by the electrogenicity of $Na^+$-$Ca^{2+}$ exchange itself and not by the $K^+$ gradient [5]. In two experiments, patterns similar to those shown in Fig. 1 were obtained. This indicates that the differential inhibitions by $Na_o^+$ were not secondary to effects on membrane potential.

TABLE I

INHIBITION BY EXTRAVESICULAR $Na^+$ OF $Na_i^+$-DEPENDENT $Ca^{2+}$ UPTAKE AT PHYSIOLOGICAL LEVELS OF $Na^+$ AND $Ca^{2+}$

$[Ca^{2+}] = 4$ μM (including 2 μM endogenous $Ca^{2+}$ in solutions). Time for $Ca^{2+}$ uptake was 2.0 s. $n = 3$.

| Vesicles | $Na_i^+$-dependent $Ca^{2+}$ uptake (nmol/mg/s) | | Inhibition (%) |
|---|---|---|---|
| | $[Na_o^+] =$ 2.9 mM | $[Na_o^+] =$ 10 mM | |
| Inside-out | 2.3 ± 0.1 | 2.1 ± 0.2 | 8.7 |
| Total population | 2.4 ± 0.3 | 1.4 ± 0.2 | 41.7 |

When the vesicles are loaded with $Na^+$ by the ATP-dependent $Na^+$ pump, the internal free $[Na^+]$ is not known. Thus, it was possible that the differential inhibition of the $Na_i^+$-dependent $Ca^{2+}$ uptake of the inside-out and total populations of vesicles (Fig. 1) might be a consequence of possible differences in the initial $Na^+$ load. Competition between $Na^+$ and $Ca^{2+}$ at the extravesicular surface would not obviously be affected by the internal $Na^+$ level, but nevertheless, we investigated this consideration. Sarcolemmal vesicles were first passively equilibrated with 140 mM NaCl, with 56 mM NaCl, 84 mM KCl, or with 56 mM NaCl, 84 mM LiCl. $Na_i^+$-dependent $Ca^{2+}$ uptake was then measured in the presence and absence of extravesicular $Na^+$. At $[Na_o^+] = 10$ mM, the $Ca^{2+}$ uptake was inhibited by 24.3 ± 2.1, 22.7 ± 2.8, and 24.0 ± 1.0% for the $Na^+$, $Na^+ + K^+$, and $Na^+ + Li^+$ loaded vesicles, respectively. At $[Na_o^+] = 25$ mM, the respective inhibitions were 55.3 ± 0.9, 68.3 ± 2.1, and 58.3 ± 1.7% for the three different $Na_i^+$-load conditions ($n = 3$). Thus, reduction of the internal $Na^+$ load by 60% does not affect the inhibition of $Na_i^+$-dependent $Ca^{2+}$ uptake by external $Na^+$. We have also previously shown that the apparent $K_m(Ca^{2+})$ for $Na_i^+$-dependent $Ca^{2+}$ uptake is independent of internal $[Na^+]$ (Fig. 1 in Ref. 11).

*$Na_o^+$-dependent $Ca^{2+}$ efflux*

Fig. 2 shows the $Ca^{2+}$ efflux from sarcolemmal vesicles loaded with $Ca^{2+}$ by four different methods. Inside-out vesicles were loaded with $Ca^{2+}$ by ATP-dependent $Ca^{2+}$ uptake or the $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange method, and the total population of vesicles was $Ca^{2+}$-loaded by either passive diffusion or $Na^+$-$Ca^{2+}$ (see Materials and Methods). In these initial experiments only low external $Na^+$ concentrations were used so that the $Ca^{2+}$ content of the vesicles did not change by a large fraction during the efflux experiment, and approximate initial rates were measured. The results with vesicles preloaded with $Ca^{2+}$ by $Na^+$-$Ca^{2+}$ exchange (closed circles) or $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange (open circles) are similar to those we have previously reported (Fig. 6 in Ref. 11).

We noted (Fig. 2) that the magnitude of $Na_o^+$-dependent $Ca^{2+}$ efflux was greater when the $Ca^{2+}$ preload had been obtained by passive diffusion rather than by $Na^+$-$Ca^{2+}$ exchange although the entire population of sarcolemmal vesicles should

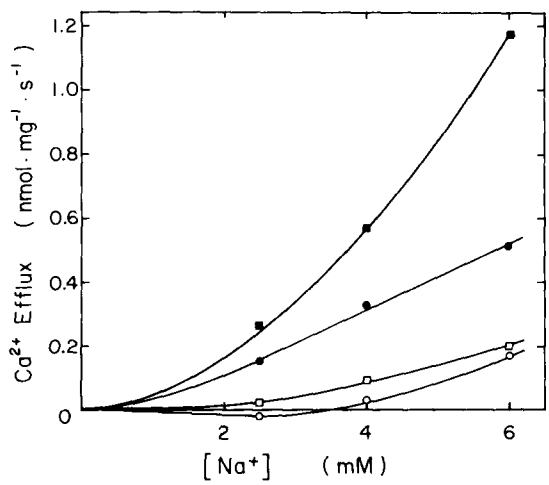


Fig. 2. $Na_o^+$-dependent $Ca^{2+}$ efflux. Sarcolemmal vesicles were preloaded with $Ca^{2+}$ by passive diffusion (■), $Na^+$-$Ca^{2+}$ exchange (●), ATP-dependent $Ca^{2+}$ uptake (□), or $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange (○). See Materials and Methods for details. The initial $Ca^{2+}$ loads at the initiation of the $Ca^{2+}$ efflux reaction were 38.9 ± 2.1, 45.0 ± 6.1, 24.0 ± 1.7, and 19.6 ± 2.2 nmol/mg protein, respectively. The efflux period was 18 s except for the case where vesicles were $Ca^{2+}$-preloaded by passive diffusion. Since the $Ca^{2+}$ efflux was especially large in this case, a shorter efflux period was chosen (10 s). Data specifically represent only the $Na_o^+$-dependent component of $Ca^{2+}$ efflux. When the vesicles were $Ca^{2+}$-loaded by $Na^+$-$Ca^{2+}$ exchange or $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange, the actual $Na_o^+$ concentration was 0.4 mM when no $Na^+$ was included in the $Ca^{2+}$ efflux media. $n = 4$–6. S.E. bars are not shown for clarity but averaged 21.4 ± 4.3% of the mean values.

TABLE II

INHIBITION OF $Na_o^+$-DEPENDENT $Ca^{2+}$ EFFLUX BY INTRAVESICULAR $Na^+$

See Materials and Methods. In all cases 10 mM Mops (pH 7.4, 37°C) and $^{45}Ca^{2+}$ were also present during the overnight equilibration procedure. Initial $Ca^{2+}$ loads prior to initiation of $Ca^{2+}$ efflux were $35.8 \pm 2.5$ and $40.0 \pm 3.3$ nmol/mg protein in the absence and presence of $Na^+$, respectively. $n = 4$.

| Passive $Ca^{2+}$ loading conditions | $[Na^+]$ (mM) in efflux medium | $Ca^{2+}$ content (nmol/mg) after 10 s efflux | $Na_o^+$-dependent $Ca^{2+}$ efflux (nmol/mg) |
|---|---|---|---|
| 140 mM KCl, 1.5 mM $CaCl_2$ | 1 | $26.7 \pm 1.9$ | |
| | 6 | $18.4 \pm 1.5$ | $8.3 \pm 0.4$ |
| 91 mM KCl, 49 mM NaCl, 1.5 mM $CaCl_2$ | 1 | $31.2 \pm 1.7$ | |
| | 6 | $26.6 \pm 2.4$ | $4.6 \pm 0.8$ |

contribute to the $Ca^{2+}$ efflux in both cases. Likewise, a consistently larger $Ca^{2+}$ efflux was obtained with $Ca^{2+}$ pump-loaded vesicles in comparison with $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange-loaded vesicles although, in both cases, $Ca^{2+}$-loaded inside-out sarcolemmal vesicles should be contributing to the reaction. A possible explanation is that, when vesicles are $Ca^{2+}$ loaded by either the $Na^+$-$Ca^{2+}$ exchange or $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange methods, the vesicles will also contain internal $Na^+$ during the $Ca^{2+}$ efflux reaction. Internal $Na^+$ could compete with $Ca^{2+}$ for sites on the intravesicular surface of the exchanger and thus inhibit $Na_o^+$-dependent $Ca^{2+}$ efflux.

Two experiments tested this possibility. In the first experiment (Table II), vesicles were passively loaded with $Ca^{2+}$ overnight in the presence and absence of $Na^+$. Normally no $Na^+$ would be present during this step. The vesicles equilibrate with both the $Ca^{2+}$ and $Na^+$ (if present). The presence of the internal $Na^+$ inhibited $Na_o^+$-dependent $Ca^{2+}$ efflux by 45% (Table II). In the second set of experiments, we tested the effect of manipulations designed to lower intravesicular $Na^+$ prior to the $Ca^{2+}$ efflux reaction. Vesicles were loaded with $Ca^{2+}$ by $Na^+$-$Ca^{2+}$ exchange starting with $Na^+$-loaded vesicles (see Materials and Methods). Substantial $Na^+$ remains within the vesicles at the end of the 1.0 min $Ca^{2+}$ uptake period. To lower this intravesicular $Na^+$, monensin (a monovalent cation ionophore) was added for the last 0.5 min of the $Ca^{2+}$ uptake procedure. The monensin would cause a loss of intravesicular $Na^+$. As shown in Table III, a reduction in intravesicular $Na^+$ (induced by monensin) stimulated the subsequent $Na_o^+$-dependent $Ca^{2+}$ efflux by 51%.

These experiments demonstrated that internal $Na^+$ could interfere with $Ca^{2+}$-efflux measurements. Thus, further studies on $Na_o^+$-dependent $Ca^{2+}$ efflux were done using vesicles preloaded with $Ca^{2+}$ by passive equilibration (total population of vesicles) or by ATP-dependent $Ca^{2+}$ up-

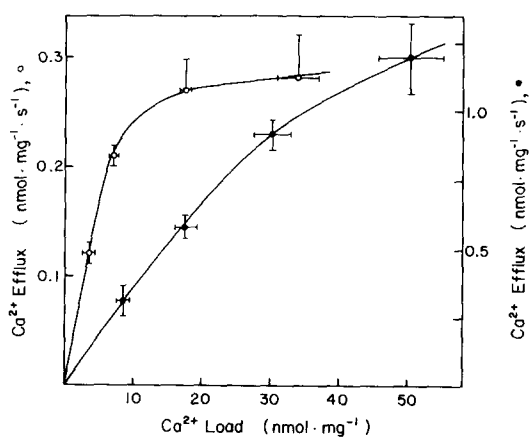


Fig. 3. $Na_o^+$-dependent $Ca^{2+}$ efflux as a function of $Ca^{2+}$ load. Vesicles were loaded with $Ca^{2+}$ by ATP-dependent $Ca^{2+}$ uptake (○, inside-out vesicles; $n = 5$) or by passive equilibration (●, total population of vesicles, $n = 3$). With ATP-dependent $Ca^{2+}$ uptake, the four data points were obtained by allowing $Ca^{2+}$ pumping to proceed for 0.25, 0.5, 1.5 and 5.0 min, respectively. Vesicles were preequilibrated with 0.2, 0.5, 1.0 and 1.5 mM $Ca^{2+}$ to achieve different loads by passive diffusion. See Materials and Methods for details. $[Na_o^+] = 6.0$ mM and only the $Na^+$-dependent component of $Ca^{2+}$ efflux is shown. Efflux periods were 18 s (after $Ca^{2+}$ pump loading) and 10 s (after passive $Ca^{2+}$ loading).

TABLE III

STIMULATION OF $Na_o^+$-DEPENDENT $Ca^{2+}$ EFFLUX BY REMOVAL OF INTRAVESICULAR $Na^+$

See Materials and Methods for loading procedures. Initial $Ca^{2+}$ loads prior to initiation of $Ca^{2+}$ efflux were 49.6 and 41.9 nmol/mg of protein for the control and plus monensin cases, respectively. $n = 6$.

| $Ca^{2+}$ loading conditions | $[Na^+]$ (mM) in efflux medium | $Ca^{2+}$ content (nmol/mg) after 18 s efflux | $Na_o^+$-dependent $Ca^{2+}$ efflux (nmol/mg) |
|---|---|---|---|
| $Na_i^+$-dependent $Ca^{2+}$ uptake (1 min) | 0.4 | 44.0 ± 3.5 | |
| | 6.0 | 34.7 ± 2.9 | 9.3 ± 1.6 |
| $Na_i^+$-dependent $Ca^{2+}$ uptake (1 min) with monensin (10 μM) present for last 0.5 min | 0.4 | 34.7 ± 4.1 | |
| | 6.0 | 20.7 ± 2.5 | 14.0 ± 2.1 |

take (inside-out vesicles). These load procedures could be accomplished without the use of any internal $Na^+$. A set of control experiments examined the dependence of $Na_o^+$-dependent $Ca^{2+}$ efflux on the size of the $Ca^{2+}$ load. Vesicles were loaded to different degrees by two methods: (a) overnight equilibration with different concentrations of $Ca^{2+}$ or (b) ATP-dependent $Ca^{2+}$ uptake for different periods of time. Fig. 3 shows that the magnitude of $Na_o^+$-dependent $Ca^{2+}$ efflux varied with the size of the $Ca^{2+}$ load. The $Na_o^+$-induced $Ca^{2+}$ efflux from inside-out vesicles saturated with increasing $Ca^{2+}$ load more readily than the $Ca^{2+}$ efflux from the total population of vesicles. This is consistent with only a fraction (≈ 40%) of the sealed vesicles being inside-out. For example, a $Ca^{2+}$ load of 30 nmol/mg of sarcolemmal protein after ATP-dependent $Ca^{2+}$ pumping would be equivalent to 75 nmol/mg of inside-out vesicle protein if 40% of the sealed vesicles are inside out. Thus the real $Ca^{2+}$ load per vesicle may be much larger than the measured apparent $Ca^{2+}$ load when only a subfraction of vesicles is taking up $Ca^{2+}$. The results shown in Fig. 3 also indicated that experiments on the $Na_o^+$-dependence of $Ca^{2+}$ efflux could give distorted data if the vesicular $Ca^{2+}$ content changed significantly during measurements. Efflux rate would continuously decline as the $Ca^{2+}$ load fell. The following $Ca^{2+}$ efflux experiments thus used low $Na^+$ concentrations and short efflux periods to minimize this problem.

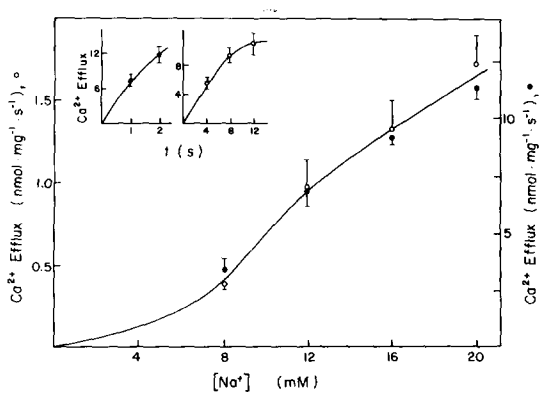


Fig. 4. $Na_o^+$-dependent $Ca^{2+}$ efflux. Sarcolemmal vesicles were preloaded with $Ca^{2+}$ by ATP-dependent $Ca^{2+}$ uptake (○, inside-out vesicles) or by passive equilibration (●, total population of vesicles). Initial $Ca^{2+}$ loads were 24.7 ± 5.3 and 39.3 ± 1.6 nmol/mg protein, and efflux periods were 4.0 and 1.0 s, respectively. Insets show time-dependencies of $Na_o^+$-dependent $Ca^{2+}$ efflux (in nmol/mg) at $[Na^+] = 16$ mM. $n = 3$.

Fig. 4 demonstrates the dependence on $[Na_o^+]$ of $Ca^{2+}$ efflux from both inside-out and the total population of sarcolemmal vesicles. There is little difference between the $Na^+$ concentration dependencies in the two cases. The efflux periods were 4.0 and 1.0 s, respectively. The insets (Fig. 4) show that the $Na_o^+$-dependent effluxes were approximately linear at these times. The relatively large standard error bars reflect variations in the absolute magnitude of $Ca^{2+}$ efflux in different preparations rather than variations in the shape of the curve in different experiments.

The maximal $[Na_o^+]$ used for the experiments shown in Fig. 4 was 20 mM. Possibly, differences in the response of inside-out and right-side-out vesicles become apparent only at higher $Na^+$ levels. To examine this possibility, we carried out $Ca^{2+}$ efflux experiments at 23°C (instead of 37°C) at $Na_o^+$ concentrations up to 60 mM. The lower

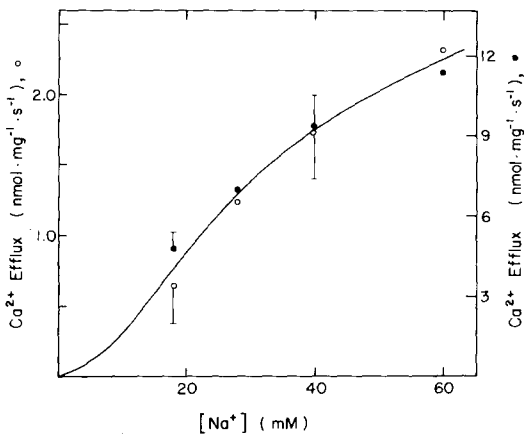


Fig. 5. $Na_o^+$-dependent $Ca^{2+}$ efflux at 23°C. Sarcolemmal vesicles were preloaded with $Ca^{2+}$ by ATP dependent $Ca^{2+}$ uptake (○, inside-out vesicles) or by passive equilibration (●, total population of vesicles). Initial $Ca^{2+}$ loads were $15.3 \pm 2.1$ and $44.0 \pm 3.8$ nmol/mg, and efflux periods were 3.0 and 1.0 s, respectively. $n = 5$.

temperature was required to slow the $Ca^{2+}$ efflux so that large depletion of the internal $Ca^{2+}$ load would not occur during measurements. As shown in Fig. 5, the dependencies of $Ca^{2+}$ efflux on $[Na_o^+]$ from the inside-out and total populations of vesicles are again similar.

The absolute magnitude of $Na_o^+$-dependent $Ca^{2+}$ efflux from vesicles loaded by passive equilibration is about 5–7-fold greater than that from vesicles loaded by ATP-dependent $Ca^{2+}$ uptake (Figs. 2, 3 and 4). If the $Na_o^+$-dependent $Ca^{2+}$ efflux activities of inside-out and right-side-out vesicles were equal, the data would suggest that a relatively small fraction of the vesicles ($< 20\%$) was inside out. However, there is no reason to assume that differently oriented vesicles will demonstrate equal activities, and these data do not allow quantitation of vesicle sidedness. Other investigations [15] indicate that the vesicle preparation consists of 45% right-side-out, 31% inside-out, and 24% leaky vesicles.

## Discussion

The $Na^+$-$Ca^{2+}$ exchange mechanism can catalyze the countertransport of $Ca^{2+}$ on either side of the sarcolemmal membrane for $Na^+$ on the opposite side. The exchange mechanism must therefore have both $Na^+$ and $Ca^{2+}$ binding sites on both intra- and extracellular membrane surfaces. Sarcolemmal vesicle preparations contain a mixture of inside-out and right-side-out vesicles [15], making investigation of the two surfaces of the exchange mechanism difficult. We have approached this problem by using techniques which selectively preload different subpopulations of vesicles with either $Na^+$ or $Ca^{2+}$. The $Na^+$-$Ca^{2+}$ exchange properties of these subpopulations can then be determined. Using this approach, we have previously concluded that the apparent $K_m(Ca^{2+})$ of the exchanger was similar on the two membrane surfaces [11]. These measurements have now been extended.

In the first set of experiments (Fig. 1), extravesicular $Na^+$ was a much more potent inhibitor of $Na_i^+$-dependent $Ca^{2+}$ uptake in the total population of sarcolemmal vesicles (inside-out plus right-side-out vesicles) than in the inside-out vesicles. Approx. 60% of the sealed vesicles in our preparation are right-side-out [15], and these vesicles will dominate the exchange properties of the entire population. We conclude that external $Na^+$ competes with $Ca^{2+}$ more effectively at the external surface of right-side-out than of inside-out vesicles. This demonstrates that the $Na^+$-$Ca^{2+}$ exchange mechanism is not a symmetric transporter. $Na^+$ and $Ca^{2+}$ compete for transport sites in a different manner at the two surfaces.

This result has physiological applications. The external surface of an inside-out vesicle is the intracellular surface of an intact myocardial cell. The data indicate that cellular $Ca^{2+}$ efflux through the exchanger will not be strongly inhibited by a small rise in intracellular $Na^+$. Table I (top line) shows that an intracellular $[Na^+]$ of 10 mM would have a minimal inhibitory influence on cellular $Ca^{2+}$ efflux (though the $Na^+$ could also influence intracellular $Ca^{2+}$ by stimulating $Ca^{2+}$ influx). If the experiment had been performed only using the entire population of vesicles (Table I, bottom line), the erroneous conclusion might have been made that intracellular $Na^+$ is a more potent inhibitor of cellular $Ca^{2+}$ efflux than is actually the case.

Reeves and Sutko [13] have also examined the inhibition of $Na_i^+$-dependent $Ca^{2+}$ uptake by extravesicular $Na^+$. They found a nonlinear Hill plot for the competitive inhibition by $Na_0^+$ and concluded that the $Ca^{2+}$ binding site could bind either

1 $Ca^{2+}$ or 1–2 $Na^+$ ions. However, Reeves and Sutko [13] used a mixed population of sarcolemmal vesicles in their experiments. We show (Fig. 1 and Table I) that the $Na_i^+$-dependent $Ca^{2+}$ uptakes of inside-out and right-side-out vesicles respond differently to external $Na^+$. This nonhomogeneity of vesicle response could by itself distort the kinetic analysis. Thus, some aspects of modeling $Na^+$-$Ca^{2+}$ exchange data may require information about the differently oriented vesicles.

The differences in response of $Na^+$-$Ca^{2+}$ exchange to $Na_o^+$ (Fig. 1) are not due to an effect of ATP. Caroni and Carafoli [18] have reported that the activity of sarcolemmal $Na^+$-$Ca^{2+}$ exchange could be regulated by phosphorylation reactions. However, we have been unable to find any effect of ATP on vesicular $Na^+$-$Ca^{2+}$ exchange under any conditions. In measuring the $Na^+$-$Ca^{2+}$ exchange of inside-out vesicles, some ATP is always present, since it is used to initially preload the vesicles with $Na^+$. To ensure against artifacts induced by the ATP, the vesicles which were preloaded with $Na^+$ by passive diffusion (for measurement of $Na^+$-$Ca^{2+}$ exchange in the entire population of vesicles) were also exposed to ATP (see Materials and Methods). Control experiments indicated that the preexposure to ATP had no effect on the subsequent $Na^+$-$Ca^{2+}$ exchange. The ATP also did not affect experiments by promoting ATP-dependent $Ca^{2+}$ uptake during $Na^+$-$Ca^{2+}$ exchange measurements. $Na^+$-$Ca^{2+}$ exchange is a much faster transport system that ATP-dependent $Ca^{2+}$ uptake and little $Ca^{2+}$ pumping would be expected in 2.0 s. Additionally, control experiments indicated that the presence of vanadate, an inhibitor of the ATP-dependent $Ca^{2+}$ pump, had no effect on our measurements.

To study $Na_o^+$-dependent $Ca^{2+}$ efflux, we used four methods to preload vesicles with $Ca^{2+}$ (Fig. 2). The efflux after $Ca^{2+}$ loading by the $Na^+$-$Ca^{2+}$ exchange (total population of vesicles) and $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange (inside-out vesicles) methods is similar to that which we have described previously [11]. As before, no significant $Ca^{2+}$ efflux from the inside-out sarcolemmal vesicles after $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange loading can be observed until the $Na_o^+$ level is raised above 4 mM. By comparing the $Ca^{2+}$ effluxes after loading by the two $Na^+$-$Ca^{2+}$ exchange methods (Fig. 2), it might be concluded (as we suggested previously [11]) that the dependence of $Ca^{2+}$ efflux on extravesicular $Na^+$ was different for the inside-out and right-side-out vesicles. However, these vesicles will have intravesicular $Na^+$ which will modify the pattern of $Ca^{2+}$ efflux. As discussed above in conjunction with the $Na_i^+$-dependent $Ca^{2+}$ uptake experiments, $Na^+$ competes most effectively with $Ca^{2+}$ at the external surface of right-side-out vesicles which is equivalent to the internal surface of inside-out vesicles. Therefore, it would be predicted that internal $Na^+$ would be an especially potent inhibitor of $Ca^{2+}$ efflux from inside-out vesicles. This could explain the observed low level of $Na_o^+$-dependent $Ca^{2+}$ efflux after loading by the $Na^+$ pump/$Na^+$-$Ca^{2+}$ exchange method. When we compare the $Ca^{2+}$ efflux patterns after loading by passive equilibration (total population of vesicles) and ATP-dependent $Ca^{2+}$ uptake (inside-out vesicles) (Fig. 2), we note a difference in the magnitude of $Ca^{2+}$ effluxes but it is no longer obvious whether there is a difference in the shape of the curves. For further experiments on the dependence of $Ca^{2+}$ efflux on $[Na^+]$, we chose to use $Ca^{2+}$ loading by passive equilibration or ATP-dependent $Ca^{2+}$ uptake to avoid the problems caused by intravesicular $Na^+$.

Figs. 4 and 5 show that the $Ca^{2+}$ effluxes from vesicles loaded by either passive equilibration or ATP-dependent $Ca^{2+}$ uptake have similar dependencies on $[Na^+]$. We infer that the apparent $K_m(Na^+)$ for $Na_o^+$-dependent $Ca^{2+}$ efflux is similar for inside-out and right-side-out vesicles. Kadoma et al. [12] have measured the $[Na^+]$ dependence of $Ca^{2+}$ efflux from sarcolemmal vesicles loaded by three different methods. In contrast to the present study (Fig. 4), they find that $Ca^{2+}$ efflux from vesicles $Ca^{2+}$-loaded by passive equilibration is less sensitive to external $Na^+$ than in vesicles loaded by ATP-dependent $Ca^{2+}$ uptake. The reason for this difference is unclear but Kadoma at al. [12] only report the results of one experiment using passively loaded vesicles, and the experiments using ATP-dependent $Ca^{2+}$ uptake were with vesicles from a separate sarcolemmal preparation (Table I in Ref. 12).

The experiments comparing the exchanger binding sites at the two membrane surfaces were carried out with sites exposed to identical ionic

conditions. In vivo, the two sarcolemmal surfaces are exposed to different ionic environments. The competitive interactions of $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and $H^+$ will determine the binding constants which apply in the physiologic state. The results presented here help define the symmetry of the exchanger molecule itself and, in general, cannot be directly extrapolated to the in vivo situation.

In summary, we have previously reported that the $Na^+$-$Ca^{2+}$ exchange mechanism has similar apparent affinities for $Ca^{2+}$ on both surfaces of the sarcolemmal membrane [11]. The present study finds that the $Na^+$ sites are also symmetrical; similar $Na^+$ concentration dependencies for inside-out and right-side-out vesicles are detected. Asymmetry is observed, however, in the interaction between $Na^+$ and $Ca^{2+}$ sites. Inhibition of the binding of $Ca^{2+}$ by $Na^+$ occurs most noticeably at the extracellular membrane surface. It is conceptually difficult to envision the structural significance of the conclusions. A speculation is that on one surface of the exchanger the same amino acid residues bind either a sodium or a calcium ion. At the other surface, only some of the amino acid residues involved in $Na^+$ binding are also involved in $Ca^{2+}$ binding. With this model, it is possible to have symmetric $K_m$ values for $Na^+$ and $Ca^{2+}$ although competitive interactions might be asymmetric. The conclusions were inferred from experiments using a mixed population of vesicles. Unequivocal definition of the two surfaces of the $Na^+$-$Ca^{2+}$ exchange mechanism requires physical separation of inside-out and right-side-out sarcolemmal vesicles or the development of inhibitors which affect $Na^+$-$Ca^{2+}$ exchange at only one membrane surface.

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