We have measured Na⁺-Ca²⁺ exchange in the inside-out vesicles of highly purified cardiac sarcolemma from dog ventricles. This was accomplished in a mixed population of sarcolemmal vesicles by first loading the inside-out vesicles with Na⁺ through the action of the glycoside-sensitive, ATP-dependent Na⁺ pump. Due to the asymmetric nature of active Na⁺ transport, this will only occur in inside-out vesicles. We then examine the exchange of extravesicular Ca²⁺ for the digitoxigenin-sensitive component of internal Na⁺. We confirm that the Na⁺-Ca²⁺ exchange mechanism and the ATP-dependent Na⁺ pump are present on the same vesicles and that Na⁺-Ca²⁺ exchange has a sarcolemmal origin. Na⁺-Ca²⁺ exchange of inside-out sarcolemmal vesicles is compared with Na⁺-Ca²⁺ exchange in the total population of sarcolemmal vesicles. This is measured as the exchange of external Ca²⁺ for Na⁺ taken up by passive diffusion (which will occur in both inside-out and right side-out vesicles). Evidence is presented which implies that Na⁺-Ca²⁺ exchange in the total population is due primarily to right side-out sarcolemmal vesicles. We find that Na⁺-dependent Ca²⁺ uptake in the inside-out vesicles and the total population of vesicles has similar values for $K_M$ (Ca²⁺), identical responses to altered pH, and similar responses to valinomycin-induced membrane potentials. These data strongly imply that the characteristics of the Ca²⁺ binding sites on the two sides of the exchange mechanism are symmetrical. Na⁺-dependent Ca²⁺ efflux experiments suggest that the Na⁺ binding sites of the Na⁺-Ca²⁺ exchange mechanism may be different on the opposite sides of the sarcolemmal membrane. The dependence of Ca²⁺ efflux on [Na⁺] in inside-out vesicles implies that in vivo a small shift in intracellular Na⁺ levels will have marked effects on Ca²⁺ influx.

A Na⁺-Ca²⁺ exchange system operating across the sarcolemmal membrane of cardiac muscle cells has been implicated in the regulation of myocardial contractility and ionic exchange (e.g., Refs. 1-4). The first direct identification of sarcolemmal Na⁺-Ca²⁺ exchange was made recently by Reeves and Sutko (5) using a vesicular preparation. Using this biochemical approach, several properties of the Na⁺-Ca²⁺ exchange mechanism have now been reported. This highly active transport system appears to be specifically located on the sarcolemmal membrane (5-8) and is electrogenic with bidirectional; both Na⁺-dependent Ca²⁺ efflux (12, 13) and Na⁺-dependent Ca²⁺ uptake have been characterized. A complex dependence of vesicular Na⁺-Ca²⁺ exchange on pH has also been described (14).

One problem inherent in the above studies is the homogeneity of vesicular preparations of cardiac sarcolemma with respect to sidedness. Isolated sarcolemma contains both right side-out (extracellular surface outward) and inside-out (cytoplasmic surface facing outward) vesicles (15-18). Do the measured properties of Na⁺-Ca²⁺ exchange in a sarcolemmal preparation primarily reflect activity of vesicles of only one orientation? Are the affinities for Na⁺ and Ca²⁺ at the intra- and extracellular binding sites of the exchange mechanism similar? In this report, we directly address these questions by taking advantage of the presence of the ATP-dependent, glycoside-sensitive Na⁺ pump in the same sarcolemmal vesicles which possess Na⁺-Ca²⁺ exchange activity. The Na⁺ pump is asymmetrically situated in the sarcolemma to actively extrude Na⁺ from intact cells. In an isolated preparation, ATP-dependent Na⁺ uptake will occur only in inside-out sarcolemmal vesicles. By studying the exchange of extravesicular Ca²⁺ for Na⁺ made intravesicular through the action of the Na⁺ pump, we are able to determine the properties of Na⁺-Ca²⁺ exchange in inside-out cardiac sarcolemmal vesicles. These properties are then compared with the properties of Na⁺-Ca²⁺ exchange in the total population of sarcolemmal vesicles (inside-out plus right side-out). We are able to compare the dependencies of the initial rates of Na⁺-dependent Ca²⁺ uptake on Ca²⁺ concentration, pH, and membrane potential. In addition, the Na⁺-dependent Ca²⁺ effluxes of the inside-out and total populations of sarcolemmal vesicles are investigated.

**MATERIALS AND METHODS**

**Sarcolemmal Isolation.** Sarcolemmal vesicles were isolated from trimmed dog ventricles by modifying previous procedures (13). Minced ventricles (50 to 100 g) were homogenized in a Waring Blender (2 x 10 s, high speed) at 4 °C in 250 mM sucrose, 300 mM KCl, 25 mM sodium pyrophosphate, 1 mM dithiothreitol, and 20 mM Tris/maleate (pH 7.6, 22 °C). The homogenate was then centrifuged at 177,000 × g for 20 min. The supernatant was discarded, and the pellet was washed in the same medium and centrifuged again. This pellet was suspended with a glass-Teflon homogenizer in 250 mM sucrose, 1 mM dithiothreitol, and 20 mM Tris/maleate (pH 7.6, 22 °C). This resuspension was layered on the bottom of discontinuous sucrose gradients (3 ml/gradient) containing 34, 32, 29, 26, and 11% (4, 4, 8, 7, and 4 ml, respectively) sucrose (w/w). These gradients were spun overnight (~16 h) at 122,000 × g in a Beckman SW 27 rotor. A white band centered at the top of the 26% sucrose was collected as the sarcolemmal fraction. The sarcolemma was spun down (177,000 × g, 75 min) and resuspended in either 140 mM NaCl or KCl, 5 mM Tris/maleate (pH 7.4, 97 °C) and stored in liquid nitrogen. Some enzymatic

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properties of the sarcolemma are described under "Results."

**ATP-Dependent Na" Pumping—**In some experiments, inside-out sarcolemmal vesicles were loaded with Na" through the inward-directed activity of the ATP-dependent, glycoside-sensitive Na" pump. K" (140 mM)-loaded vesicles (3 to 6 mg/ml) were first preincubated for 20 min at 37° in the absence or presence of 50 µM digitoxigenin (dissolved in ethanol). An appropriate quantity of ethanol was added to the samples which were then incubated for 20 min at 37°. These K"-loaded vesicles (6 µl) were then added to a reaction mixture (34 µl final volume) in which final concentrations were: KCl (25 mM), NaCl (25 mM), choline chloride (74 mM), Tris/ATP (2.4 mM), Tris/creatine phosphate (2.4 mM), MgCl₂ (4.8 mM), Tris/malate (3.0 mM, pH 7.4, 37°C). The mixture also contained digitoxigenin (50 µM) when digitoxigenin-treated vesicles were used to initiate the reaction. Na" pumping was allowed to proceed for 1 min at 37° at which time active Na" pumping was blocked (through dilution and the presence of digitoxigenin) and the Na"-Ca²⁺ exchange reaction was simultaneously initiated (through dilution in the presence of Ca²⁺, see below). Control experiments determined that 1 min produced maximal Na" loading via the Na" pump (as detected by Na"-selective exchange). Creatine phosphate acted in the ATP-dependent Na" pumping reaction medium as an ATP-regenerating system with endogenous sarcolemmal creatine kinase consistent with the measurements of other laboratories (10, 12, 13). To prepare the total population of vesicles (inside-out plus right side-out) for Na"-Ca²⁺ exchange in a comparable medium, initially Na" (140 mM)-loaded vesicles (6 µl) were equilibrated with NaCl, choline chloride, and digitoxigenin (to give 30 µl) for 30 min at 37°. ATP, creatine phosphate, and MgCl₂ were then added in a small volume (4 µl) and this mixture (34 µl final volume) was incubated for 1 min at 37°. The final concentrations were: NaCl (50 mM), choline chloride (74 mM), Tris/ATP (2.4 mM), Tris/creatine phosphate (2.4 mM), MgCl₂ (4.8 mM), digitoxigenin (50 µM), Tris/malate (3.0 mM, pH 7.4, 37°C). The absence of K" and the presence of ATP, creatine phosphate, and MgCl₂ were then added in a small volume (4 µl) and this mixture (34 µl final volume) was incubated for 1 min at 37°. ATP, creatine phosphate, and MgCl₂ were then added in a small volume (4 µl) and this mixture (34 µl final volume) was incubated for 1 min at 37°. The final concentrations were: NaCl (50 mM), choline chloride (74 mM), Tris/ATP (2.4 mM), Tris/creatine phosphate (2.4 mM), MgCl₂ (4.8 mM), digitoxigenin (50 µM), Tris/malate (3.0 mM, pH 7.4, 37°C). The absence of K" and the presence of digitoxigenin ensures that no active Na" pumping occurs and that Na" on the opposite side of the membrane. Control experiments (13) using agents such as La⁴⁺, EGTA, and digitoxigenin revealed the intravesicular nature of Ca²⁺ taken up by Na"-Ca²⁺ exchange.

**ATP-Dependent Ca²⁺ Pumping—**Activity of the sarcolemmal Ca²⁺ pump was determined by the addition of K"-loaded sarcolemmal vesicles (5 µl) to 245 µl of either 140 mM KCl or NaCl, 2.4 mM Tris/ATP, 2.4 mM MgCl₂, 5 µM labeled CaCl₂, 6 mM Tris/malate (pH 7.4, 37°C). The reactions were stopped by the addition of La³⁺ and the vesicles were collected by Millipore filtration as described above. ATP-dependent Ca²⁺ uptake by sarcolemmal vesicles does not occur in the NaCl medium (due to the presence of sarcolemmal Na"-Ca²⁺ exchange) (7, 8, 22) and the Ca²⁺ uptake in NaCl was subtracted as a blank from the uptake in KCl. This property distinguishes the sarcolemmal and sarcoplasmic reticular Ca²⁺ pumps. The ATP-dependent Ca²⁺ uptake in NaCl was about 10% of that in KCl.

For Ca²⁺ uptake experiments, media were treated with Chelex 100 (Bio-Rad). Ca²⁺ contamination was about 2 µM as determined using a Ca²⁺-selective electrode (Orion Research Inc., Cambridge, MA).

All data are presented as the mean ± standard error of the mean. Error bars in figures also represent standard errors.

**RESULTS**

**Properties of Cardiac Sarcolemma—**Most of the data presented here were obtained with a highly enriched sarcolemmal preparation from dog ventricles with the following characteristics: K"-p-nitrophenylphosphatase activity was 29.6 ± 2.3 µmol of p-nitrophenol liberated/mg of protein/h. The purification factor for this sarcolemmal marker enzyme was 78.5 ± 7.8 and its yield was 15.3 ± 1.8%. The purification factor was obtained by dividing sarcolemmal fraction enzyme specific activity by crude homogenate total activity. Sarcolemmal (Na" + K")-ATPase activity (unstimulated by alamethin) was 41.3 ± 5.1 µmol of P, liberated/mg of protein/h, (Na" + K")-ATPase purification factor was 53.7 ± 6.6 and its yield was 12.0 ± 1.8%. Sarcolemmal (Na" + K")-ATPase activity could be stimulated 166 ± 27% by including alamethin (12.5 µg/ml; donated by Dr. J. E. Grady, The Upjohn Co.) in the assay reaction medium. This suggests that a majority of the sarcolemmal vesicles are in a right side-out orientation (16). n = 6 for all the above values. Fluoride-stimulated adenylyl cyclase activity was 1900 ± 100 pmol/mg of protein/min (n = 3). Less than 0.5% of the inner mitochondrial membrane marker succinate dehydrogenase was recovered in the sarcolemmal fraction. As discussed below, an ATP-dependent Ca²⁺ pump, attributable to sarcolemmal vesicles (see "Materials and Methods"), was present in the preparation. Although small amounts of contaminating membranes may exist in our sarcolemmal preparation, the enzymatic activities of interest in this study (Na"-Ca²⁺ exchange; ATP-dependent Na" pumping) are restricted to the sarcolemmal membrane (to the best of current knowledge). Thus, there is no a priori reason to suspect any artifacts in our measurements due to membrane impurities.

**Experimental Protocol and Its Rationale—**We compare characteristics of Na"-Ca²⁺ exchange in inside-out sarcolemmal vesicles with characteristics found in the total population of sarcolemmal vesicles. Na"-Ca²⁺ exchange in inside-out vesicles is measured by using the ATP-dependent Na" pump to specifically Na" load inside-out vesicles (due to the asymmetric nature of active Na" pumping). Na"-dependent Ca²⁺ uptake (i.e. Na"-Ca²⁺ exchange) in inside-out vesicles can then be examined in detail. As shown in Table I, if either Na", K", Mg", or ATP was omitted from the active Na"-loading reaction medium, no Na" pumping occurred and the presence of digitoxigenin in this medium no longer affected the ensuing reaction.
Na\textsuperscript+–Ca\textsuperscript2+ exchange reaction. This substantiates that prior active Na\textsuperscript+ pumping (with substrate dependencies similar to that of (Na\textsuperscript+ + K\textsuperscript−)-ATPase) must occur to observe Na\textsuperscript+–dependent Ca\textsuperscript2+ uptake which can be attributed to inside-out vesicles. Control experiments indicated that digitoxigenin (50 \textmu M), a membrane-permeable glycoside, maximally inhibited Na\textsuperscript+ pumping more rapidly than any of our measurements. This was in contrast to the less membrane-permeable glycoside, ouabain, which required preincubation to reach its inhibitory site (on the inner surface of inside-out sarcolemmal vesicles).

We were interested in comparing Na\textsuperscript+–Ca\textsuperscript2+ exchange measured (as described above) in inside-out sarcolemmal vesicles with Na\textsuperscript+–Ca\textsuperscript2+ exchange as measured in the total population of sarcolemmal vesicles. The latter measurement is made by diluting vesicles loaded with Na\textsuperscript+ by passive diffusion (which will occur in both inside-out and right-side-out vesicles) into a Na\textsuperscript+–free medium containing labeled Ca\textsuperscript2+. To make the comparison between Na\textsuperscript+–Ca\textsuperscript2+ exchange in inside-out vesicles and the total population of vesicles meaningful, the experimental protocol was arranged to make conditions between the two different measurements as comparable as possible. Thus (see "Materials and Methods"), before Na\textsuperscript+–Ca\textsuperscript2+ exchange, the vesicles are incubated with ATP, creatine phosphate, and Mg\textsuperscript2+ for 1 min for both inside-out vesicle experiments and for total population experiments. A 20-fold dilution is performed before Ca\textsuperscript2+ transport is activated and the final concentrations of these substances are 120 \textmu M (ATP), 120 \textmu M (creatine phosphate) and 240 \textmu M (MgCl\textsubscript2). Control experiments indicated that these quantities had no effect on Na\textsuperscript+–Ca\textsuperscript2+ exchange. In a short report, Reinlih et al. (23) found that, under very specific conditions, low levels of ATP (10 \textmu M) stimulated Na\textsuperscript+–Ca\textsuperscript2+ exchange. We have been unable to verify this finding. Digitoxigenin (50 \textmu M) was present during the Na\textsuperscript+–Ca\textsuperscript2+ exchange reaction but did not affect this process (102 ± 3\% of control, n = 3).

The inside-out vesicles of cardiac sarcolemma are also capable of ATP-dependent Ca\textsuperscript2+ uptake through a (Ca\textsuperscript2+, Mg\textsuperscript2+)-ATPase mechanism (7, 8, 22) and it was necessary to determine whether this activity would interfere with any of our measurements. ATP-dependent Ca\textsuperscript2+ uptake activity in the sarcolemmal preparation was 4.3 ± 0.2 nmol of Ca\textsuperscript2+ /mg of protein/min (n = 3) and this activity was unaffected by 50 \textmu M digitoxigenin (103 ± 2\% of control activity, n = 3). Active Ca\textsuperscript2+ pumping could potentially interfere with our experiments at two different times. During the ATP-dependent Na\textsuperscript+–loading procedure, there is no added Ca\textsuperscript2+ present but conceivably unlabeled Ca\textsuperscript2+ contamination (~2 \textmu M) could participate in a Ca\textsuperscript2+ pump reaction. This Ca\textsuperscript2+ could then contribute to subsequent 45Ca\textsuperscript2+ uptake through Ca\textsuperscript2+–Ca\textsuperscript2+ exchange (13). However, this should occur to an equal extent in the blanks (in which the only difference is the presence of digitoxigenin) and should not affect the difference observed after blank subtraction. Since ATP is still present (although diluted to 120 \textmu M) during the Na\textsuperscript+–Ca\textsuperscript2+ exchange reaction which follows the active Na\textsuperscript+ loading, the active Ca\textsuperscript2+ pump could possibly induce artifacts at this stage. This is unlikely for three reasons: 1) again, appropriate blanks should subtract out any contribution to Ca\textsuperscript2+ uptake due to ATP-dependent Ca\textsuperscript2+ pumping. 2) We find that inclusion of Na\textsubscript{3}VO\textsubscript{4} (200 \textmu M), a powerful inhibitor of sarcolemmal ATP-dependent Ca\textsuperscript2+ pumping (22), in the Na\textsuperscript+–Ca\textsuperscript2+ exchange medium does not affect the Ca\textsuperscript2+ uptake which we attribute to inside-out vesicle Na\textsuperscript+–Ca\textsuperscript2+ exchange. 3) The initial rate of ATP-dependent Ca\textsuperscript2+ uptake in cardiac sarcolemma is much slower (7, 8) than that of Na\textsuperscript+–Ca\textsuperscript2+ exchange and is unlikely to be substantial during the 2.5 s used for most of the Ca\textsuperscript2+ uptake measurements in this study.

The disadvantage of our technique to measure Na\textsuperscript+–Ca\textsuperscript2+ exchange in inside-out vesicles is that the initial internal Na\textsuperscript+ level is unknown. This is because the Na\textsuperscript+ load is achieved by ATP-dependent Na\textsuperscript+ transport and it is uncertain how this level will compare with a Na\textsuperscript+ load achieved by passive equilibration. The magnitude of Na\textsuperscript+–dependent Ca\textsuperscript2+ uptake will depend on the internal Na\textsuperscript+ level. However, other characteristics of Na\textsuperscript+–dependent Ca\textsuperscript2+ uptake are independent of this parameter. This is demonstrated in Fig. 1A which shows the initial rate of Na\textsuperscript+–dependent Ca\textsuperscript2+ uptake as a function of external Ca\textsuperscript2+ concentration at three different internal Na\textsuperscript+ levels. The Na\textsuperscript+ loads (28, 56, or 140 mM) were achieved by passive diffusion. Although the magnitude of Ca\textsuperscript2+ uptake is dependent upon Na\textsuperscript+, the dependence of Ca\textsuperscript2+ uptake on Ca\textsuperscript2+ concentration is unaltered. This is shown in Fig. 1B in which the Na\textsuperscript+–dependent Ca\textsuperscript2+ uptake data are normalized (to Ca\textsuperscript2+ uptake at [Na\textsuperscript+] = 140 mM) and displayed as an Eadie-Hofstee plot. (Normalization does not affect K\textsubscript{m} values obtained from an Eadie-Hofstee plot.) The data can be fit with a single line (K\textsubscript{m}(Ca\textsuperscript{2+}) = 28 \textmu M) independent of Na\textsuperscript+.

Thus, although we will be unable to compare the absolute magnitudes of Na\textsuperscript+–Ca\textsuperscript2+ exchange in inside-out vesicles and in the total population of sarcolemmal vesicles (due to differences in initial Na\textsuperscript+ loads and to uncertainties in the fraction of vesicles which are inside out), we will still be able to make valid comparisons between other properties of this exchange.

**Na\textsuperscript+–dependent Ca\textsuperscript2+ Uptake—**Fig. 2 shows the time course

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**Table I**

Characteristics of digitoxigenin-sensitive, ATP-dependent Na\textsuperscript+ uptake (as detected by Na\textsuperscript+–Ca\textsuperscript2+ exchange)

See "Materials and Methods" for details.

<table>
<thead>
<tr>
<th>Medium</th>
<th>n</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Minus Na\textsuperscript+</td>
<td>5</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Minus K\textsuperscript+</td>
<td>2</td>
<td>-5.5</td>
</tr>
<tr>
<td>Minus Mg\textsuperscript2+</td>
<td>3</td>
<td>2.7 ± 1.7</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>3</td>
<td>-3.0 ± 3.1</td>
</tr>
</tbody>
</table>

\*Choline chloride replaced the NaCl.
\*Na\textsuperscript+-loaded vesicles replaced K\textsuperscript+-loaded vesicles.
of Na+-dependent Ca2+ uptake for sarcolemmal vesicles loaded with Na+ via the action of the ATP-dependent Na+ pump (inside-out vesicles). Also shown is the time course for the total population of vesicles which were loaded with Na+ by passive diffusion under otherwise similar conditions. The shape of the time course curves are similar for both situations. Approximate linearity is maintained until about 6 s. As described above, the absolute magnitudes of Ca2+ uptake cannot be compared since intravesicular Na+ levels may not be comparable.

The effects of membrane potentials (induced by the presence of valinomycin (0.4 μM) in the Ca2+ uptake medium) on the Na+-dependent Ca2+ influx of inside-out sarcolemmal vesicles was examined (measured after 2.5 s of Ca2+ uptake). Inside-positive potentials stimulate Na+-dependent Ca2+ uptake at both 10 and 100 μM Ca2-. At 10 μM Ca2+, uptake increased from 0.64 ± 0.03 to 1.23 ± 0.07 nmol of Ca2+/mg of protein/s (23% stimulation). At 100 μM Ca2+, the Ca2+ uptake increased from 2.3 ± 0.4 to 4.4 ± 0.3 nmol/mg of protein/s (91% stimulation) (n = 3). This is similar to previous data (10, 14) obtained using the entire population of sarcolemmal vesicles where it was found that valinomycin-induced stimulation was independent of Na+ concentration. We have discussed the use of valinomycin to study the electrogenicity of Na+-Ca2+ exchange in detail elsewhere (10).

The dependence on Ca2+ concentration of the initial rate of Na+-dependent Ca2+ uptake in both the inside-out vesicles and the total population of sarcolemmal vesicles is shown in Fig. 3. Little difference between the shape of the two curves is evident. The relatively large error bars are due to variation in absolute magnitude of Ca2+ uptake in different experiments rather than to variation in the shapes of the curves. These data are plotted as an Eadie-Hofstee plot in Fig. 4. The Km(Ca2+) values are 33 and 28 μM for inside-out vesicles and the total population of vesicles, respectively. These values are in the same range as those reported by others for total populations of cardiac sarcolemmal vesicles (9, 14, 15, 18, 24) but contrast with the value (1.5 μM) reported by Caroni et al. (11). As seen in Fig. 3, the absolute magnitude of Na+-dependent Ca2+ uptake is similar for the inside-out and total populations of vesicles. We reiterate that such a comparison is not meaningful since the [Na+] is not known for the inside-out vesicles and Ca2+ uptake will depend on the internal Na+ load (Fig. 1). The initial internal Na+ is 50 mM for the total population experiment (chosen to make Ca2+ uptakes similar for the two types of experiment). In experiments in which Na+-dependent Ca2+ uptake is compared between inside-out vesicles and a total population preloaded with 120 mM Na+, the Ca2+ uptake is 2.2 times larger (n = 2) for the total population of vesicles. Qualitative calculations (based on (Na++K+)-ATPase activity, 3 Na+ transported/ATP, estimated intravesicular volumes) imply that the internal [Na+] of the inside-out vesicles could reach high levels as a result of active Na+ pumping.

The response to changes in pH of Na+-Ca2+ exchange in inside-out vesicles and the total population of vesicles is seen in Fig. 5. Na+-dependent Ca2+ uptake is markedly depressed at low pH and stimulated at high pH for both situations. The results for the two experiments are almost superimposable. In these experiments, the vesicles are maintained at pH 7.4 until initiation of the Na+-Ca2+ exchange reaction. Only the pH of the external Ca2+ uptake medium is altered. We have previously (14) completed a detailed examination of the effects of pH on Na+-Ca2+ exchange in the total population of cardiac sarcolemmal vesicles. Results comparable to those in Fig. 5 were obtained.

To this point, we have not detected any difference in the
Na\(^+\)-dependent Ca\(^{2+}\) transport properties of inside-out vesicles and the total population of sarcolemmal vesicles. However, we have found two agents which distinguish between these two populations: polymixin B and digitonin. As shown in Table II, both these agents (at low concentrations) inhibit Na\(^+\)-Ca\(^{2+}\) exchange to a substantially greater extent (2- to 3-fold) in the inside-out vesicles as compared to the total population of vesicles. The polymixin B (8 \(\mu\)M) and the digitonin (0.002%) are present only during the 2.5 min of Ca\(^{2+}\) uptake portion of the experiment; the agents are not present during the initial Na\(^+\) loading of the vesicles. The implications of these results will be discussed below.

Na\(^+\)-dependent Ca\(^{2+}\) Eflux—Na\(^+\)-dependent Ca\(^{2+}\) efflux is another mode of sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchange (12, 13). To study Na\(^+\)-dependent Ca\(^{2+}\) efflux, inside-out vesicles and the total population of vesicles were first loaded with Ca\(^{2+}\) in exchange for Na\(^+\) internalized by active pumping or passive diffusion respectively (as described above). Further Ca\(^{2+}\) uptake was then blocked by the addition of a KCl, EGTA efflux medium containing various concentrations of Na\(^+\). Na\(^+\)-dependent Ca\(^{2+}\) efflux was then quantitated by Millipore filtration after 0.2 min of efflux. These techniques for studying Ca\(^{2+}\) efflux have been described in detail elsewhere (13).

Fig. 6 displays the Ca\(^{2+}\) efflux as a function of external Na\(^+\) concentration for inside-out vesicles (for two different levels of initial Ca\(^{2+}\) loading) and for the total population of vesicles. In both cases, Ca\(^{2+}\) efflux increases as external Na\(^+\) increases. For the inside-out vesicles, however, a higher Na\(^+\) concentration is needed to initiate Ca\(^{2+}\) efflux. At 4 mM Na\(^+\), no Na\(^+\)-dependent Ca\(^{2+}\) efflux is observed in the inside-out vesicles, while significant efflux has already begun to occur in the total population of vesicles. The steepest portion of the Ca\(^{2+}\)-efflux curve (Fig. 6) is from 8 to 12 mM Na\(^+\) for the inside-out vesicles and from 4 to 8 mM Na\(^+\) for the entire population of sarcolemmal vesicles. Passive Ca\(^{2+}\) efflux (efflux in the absence of added external Na\(^+\)) was similar for both situations and accounted for a loss of less than 10% of the initial Ca\(^{2+}\) load during the time period (0.2 min) of an efflux experiment.

In the inside-out vesicle experiments, only a fraction of the total population of vesicles becomes loaded with Ca\(^{2+}\); In these experiments, the actual Ca\(^{2+}\) content per mg of inside-out vesicle protein is therefore much greater than what we ascertain (since we measure total preparation protein). We investigated whether the observed difference (Fig. 6) in the pattern of Na\(^+\)-dependent Ca\(^{2+}\) efflux between inside-out vesicles and the total population of vesicles was due to a difference in initial Ca\(^{2+}\) content. Ca\(^{2+}\) loading for inside-out vesicles was performed in Ca\(^{2+}\) uptake media containing either 2.5 or 10 \(\mu\)M Ca\(^{2+}\) and the mean Ca\(^{2+}\) loads at initiation of the efflux experiments were 2.2 or 7.5 nmol/mg of protein, respectively. As shown in Fig. 6, this difference in the initial Ca\(^{2+}\) content of the inside-out sarcolemmal vesicles had no discernible effect on the Na\(^+\)-dependent Ca\(^{2+}\) efflux as a function of Na\(^+\) concentration. Thus, the properties of Na\(^+\)-dependent Ca\(^{2+}\) efflux appear independent of initial Ca\(^{2+}\) load just as some characteristics of Na\(^+\)-dependent Ca\(^{2+}\) uptake are independent of initial Na\(^+\) load (see above; Fig. 1). The mean initial Ca\(^{2+}\) load for the efflux experiments using the total population of vesicles was 5.7 nmol/mg of protein.

<table>
<thead>
<tr>
<th>Inhibition of Na(^+)-Ca(^{2+}) exchange</th>
<th>Inside-out</th>
<th>Total population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymixin B (8 (\mu)M)</td>
<td>3</td>
<td>37.9 ± 2.2</td>
</tr>
<tr>
<td>Digitonin (0.002%)</td>
<td>4</td>
<td>32.2 ± 6.8</td>
</tr>
</tbody>
</table>

Time for Ca\(^{2+}\) uptake (and exposure to polymixin and digitonin) was 2.5 s. Valinomycin (0.4 \(\mu\)M) was included in the Ca\(^{2+}\) uptake medium. [Ca\(^{2+}\)] = 10 \(\mu\)M. See "Materials and Methods" for other details.

![Graph showing Ca\(^{2+}\) Uptake (%)](http://www.jbc.org/)

**Fig. 6. Na\(^+\)-dependent Ca\(^{2+}\) efflux.** Inside-out vesicles or the total population of sarcolemmal vesicles were first loaded with Ca\(^{2+}\) by Na\(^+\)-dependent Ca\(^{2+}\) uptake (see "Materials and Methods") for 1.0 min in 0.5 ml of Ca\(^{2+}\) uptake medium. At 1 min, 0.5 ml of efflux medium was added. The efflux medium contained 140 mM KCl, 1 mM EGTA, 5 mM Tris/maleate (pH 7.4, 37 °C), and various concentrations of Na\(^+\). The quantity of KCl was decreased to maintain osmolality. After 0.2 min of efflux, 0.5 ml of 140 mM KCl, 5 mM LaCl\(_3\) were added to block Ca\(^{2+}\) fluxes and the mixture was rapidly filtered. The Ca\(^{2+}\) concentration in the Ca\(^{2+}\) uptake medium (before EGTA addition) was 2.5 or 10 \(\mu\)M for the inside-out vesicles (○, respectively) and 10 \(\mu\)M for the total population of vesicles (C). Ca\(^{2+}\) efflux in Na\(^+\)-free medium (passive efflux) was subtracted from the efflux in the presence of Na\(^+\) to determine the Na\(^+\)-dependent Ca\(^{2+}\) efflux. The data are presented as the percent of the Ca\(^{2+}\) load lost after 0.2 min of Na\(^+\)-dependent Ca\(^{2+}\) efflux as compared with the Ca\(^{2+}\) load after 0.2 min of efflux in a Na\(^+\)-free efflux medium. The initial Ca\(^{2+}\) loads were 2.2 (n = 2), 7.5 ± 1.2 (n = 3) (inside-out vesicles loaded in 2.5 and 10 \(\mu\)M Ca\(^{2+}\), respectively), and 5.7 ± 1.0 (n = 3) (total population) nmol/mg.
**DISCUSSION**

**Na⁺-Ca²⁺ Exchange in Cardiac Sarcolemmal Vesicles**

Na⁺-Ca²⁺ exchange in cardiac sarcolemmal vesicles has been investigated fairly extensively (5, 6, 9–15). These studies have all been done with preparations which contain both inside-out and right side-out vesicles. If vesicles of different sidedness have different Na⁺-Ca²⁺ exchange properties, the interpretation of these studies becomes ambiguous. Using highly purified sarcolemmal vesicles, we have compared Na⁺-Ca²⁺ exchange in inside-out vesicles and in the total population of vesicles. Na⁺-Ca²⁺ exchange in inside-out vesicles is observed by first loading vesicles with Na⁺ through the action of the digitoxigenin-sensitive, ATP-dependent Na⁺ pump. Due to the asymmetric nature of active Na⁺ pumping, this will occur only in inside-out vesicles. Extravesicular Ca²⁺ taken up in exchange for the digitoxigenin-sensitive component of internal Na⁺ can only be due to Na⁺-Ca²⁺ exchange in inside-out vesicles. These experiments demonstrate that the glycoside-sensitive Na⁺ pump and Na⁺-Ca²⁺ exchange activity are present on the same vesicles and confirm (6) that Na⁺-Ca²⁺ exchange is a sarcolemmal activity. Na⁺-Ca²⁺ exchange in the total population of vesicles is measured as the exchange of external Ca²⁺ for Na⁺ in sarcolemmal vesicles which had been preloaded with Na⁺ by passive diffusion (which will occur in all sarcolemmal vesicles). Care is taken to ensure that the ionic conditions are similar for both types of determination. Appropriate controls guarantee that Na⁺-Ca²⁺ exchange measurements represent Ca²⁺ transported across sarcolemmal membranes in response to Na⁺ on the opposite side of the membrane.

The disadvantage of our technique for measuring Na⁺-Ca²⁺ exchange in inside-out vesicles is that the initial intravesicular Na⁺ level is unknown. This is not the case when the entire population is used for Na⁺-Ca²⁺ exchange since passive equilibration can be used for Na⁺ loading. The magnitude of Ca²⁺ uptake will depend on the internal Na⁺ level. Control experiments (Fig. 1), however, demonstrate that other properties of Na⁺-Ca²⁺ exchange (e.g. K_m(Ca²⁺)) are independent of Na⁺. Thus, although the absolute magnitude of Na⁺-Ca²⁺ exchange cannot be compared, a valid comparison can still be made of other characteristics of initial rates of Na⁺-Ca²⁺ exchange in the inside-out and total populations. The magnitude of Ca²⁺ uptake does not tell us what fraction of the vesicles are oriented inside-out. Nevertheless, the data directly demonstrate that some sealed, inside-out vesicles do exist in our sarcolemmal preparation. The question of the relative proportions of inside-out and right side-out vesicles will be addressed below.

The advantage of our technique is that an unequivocal assignment of a component of Na⁺-Ca²⁺ exchange to inside-out vesicles can be made. The sole assumption is that inward ATP-dependent Na⁺ transport will occur only in inside-out sarcolemmal vesicles. This technique obviates the need to first fractionate inside-out and right side-out vesicles to study interactions on the two sides of the Na⁺-Ca²⁺ exchange mechanism.

Recently, several other investigators have reported isolation of cardiac sarcolemmal fractions with relatively high purity (e.g. 16, 17, 25). These preparations are similar in that a relatively low density sarcolemma (p ≤ 1.11 g/cm³) is obtained. The sidedness of some of these preparations has been investigated using measurements (sialic acid removed by neuraminidase, [3H]ouabain binding, enzyme activities) on intact and disrupted vesicles. Using indirect methods such as this, we (15) concluded that a preparation similar to that used here was 80% right side-out. Jones et al. (16), Van Alstyne et al. (17), Pitts and Okhuysen (18), and Wakabayashi and Goshima (19) have also presented evidence that their sarcolemmal preparations contained predominantly right side-out vesicles. Two preliminary reports on the separation of inside- and right side-out vesicles of cardiac sarcolemma using affinity chromatography have appeared (23, 26).

Our interpretation (see below) of the data is based on the probability that a substantial fraction of the Na⁺-Ca²⁺ exchange observed in the total population is due to right side-out vesicles. This is supported by two arguments. The large body of evidence from our laboratory (15) and from others (16–18, 24) which implies the presence of a large majority of right side-out sarcolemmal vesicles. However, these studies are indirect and, in any case, do not prove that a majority of the sarcolemmal vesicles which demonstrate Na⁺-Ca²⁺ exchange are right side-out. That is, even if a majority of right side-out vesicles is present, what we observe as Na⁺-Ca²⁺ exchange may be dominated by the inside-out vesicles.

A more cogent argument for the involvement of primarily right side-out vesicles in the Na⁺-Ca²⁺ exchange process (after passive loading with Na⁺) is provided by our data obtained with polymixin B and digitonin (Table II). If vesicles are Na⁺ loaded actively (inside-out vesicles only), rather than passively (total population), and all other conditions being equal, there is then greater inhibition of the subsequent Na⁺-Ca²⁺ exchange by both polymixin B and digitonin. We can think of no other interpretation of these data other than there being a different population of sarcolemmal vesicles undergoing Na⁺-Ca²⁺ exchange in the two cases. By far the simplest explanation is that mostly right side-out vesicles participate in the Na⁺-Ca²⁺ exchange of the total population and that the right side-out vesicles are relatively insensitive to a brief (2.5 s) exposure to low concentrations of digitonin and polymixin B. A maximum contribution of inside-out vesicles to the Na⁺-Ca²⁺ exchange of the entire population of vesicles can be estimated at 33% from the digitonin data (Table II). This calculation assumes that the inhibition of Na⁺-Ca²⁺ exchange in the total population by digitonin is only due to the inside-out vesicles which are present and that the right side-out vesicles are not being inhibited. The calculated percentage gives an upper limit and the true percentage is smaller if these assumptions are incorrect. A similar calculation using the polymixin results (Table II) would conclude that 48% of the intact sarcolemmal vesicles are inside-out. However, since our procedure only gives an upper limit (and suggests that the polymixin is also partially inhibiting Na⁺-Ca²⁺ exchange in some right side-out vesicles), the estimate of 33% inside-out vesicles obtained using the digitonin data is more reliable.

**Digitonin binds tightly to membrane cholesterol (27)** (in which cardiac sarcolemma is highly enriched (19)). It is possible that the cholesterol is more susceptible to digitonin at the cytoplasmic surface of the sarcolemma (or the outer surface of inside-out vesicles). Cholesterol-digitonin interactions could then perturb membrane structure and thus inhibit Na⁺-Ca²⁺ exchange more potently in the inside-out vesicles. This observation is consistent with the finding that only cholesterol at the cytoplasmic surface of erythrocytes is susceptible to cholesterol oxidase (28). Polymixin B (a cationic polypeptide antibiotic) binds to membranes containing specific distributions of phospholipids (29). As in other membranes (30), there is likely to be an asymmetric arrangement of phospholipids across the sarcolemma. We can hypothesize that this asymmetry leads to an asymmetric interaction of polymixin with the sarcolemma and to greater inhibition of Na⁺-Ca²⁺ exchange in inside-out vesicles where only the cytoplasmic surface of the sarcolemma is exposed to the antibiotic. The digitonin and the polymixin could be inhibiting Na⁺-Ca²⁺ exchange by either inhibiting Ca²⁺ influx or by
stimulating Ca\(^{2+}\) efflux (by making the vesicles leaky). For the present discussion this is irrelevant. The point is, they act differently on the inside-out versus the total populations of vesicles. Therefore, there must be a difference in these populations.

The sum of the data suggests that Na\(^{+}\)-Ca\(^{2+}\) exchange in the total population is due primarily to right side-out vesicles. Contingent upon unequivocal validation of this assertion, our most striking result is that the properties of the Ca\(^{2+}\) binding sites on the two sides of the Na\(^{+}\)-Ca\(^{2+}\) exchange mechanism are symmetrical. This is indicated by the similar dependence on Ca\(^{2+}\) concentration of Na\(^{+}\)-dependent Ca\(^{2+}\) uptake in the inside-out and total populations of sarcolemmal vesicles (Figs. 3 and 4). Similarity in the characteristics of the active Ca\(^{2+}\) binding sites on the two sides of the membrane is also suggested by the effects of pH on Na\(^{+}\)-dependent Ca\(^{2+}\) uptake (Fig. 5). Since the vesicles are exposed to the altered pH of the external Ca\(^{2+}\) uptake medium for only 2 s in these experiments, the effects of pH are probably exerted only at the external surface of the vesicle. (This assumes there is not a very rapid equilibration of H\(^{+}\) across the membrane.) We have presented evidence (14) that the effects of pH on Na\(^{+}\)-Ca\(^{2+}\) exchange are due to a direct interaction of H\(^{+}\) with the Na\(^{+}\)-Ca\(^{2+}\) exchange mechanism and are not due to effects of pH on vesicle permeability or on some other membrane transport system. (In the conclusions of this prior study (14), we assumed that the effects of pH on inside-out vesicles would be similar to those observed on the entire population. This assumption is verified in the present report.) The identical distinctive responses of the inside-out and total populations of vesicles to pH changes (Fig. 5) is suggestive that the chemical nature of the Ca\(^{2+}\) binding sites is similar on the opposing sides of the membrane. That is, it appears that the protein(s) involved in Na\(^{+}\)-Ca\(^{2+}\) exchange are symmetrical with respect to their interactions with Ca\(^{2+}\). In vivo, the two sides of the membrane are subject to different ionic conditions and regulatory factors and, under these influences, differential properties of the two sides of the exchange mechanism may emerge.

The response of Na\(^{+}\)-Ca\(^{2+}\) exchange in inside-out vesicles to valinomycin-induced membrane potentials is consistent with this process being electrogenic with 3 or more Na\(^{+}\) ions exchanging for each Ca\(^{2+}\). The response is very similar to that seen previously (10, 14) in the entire population of vesicles. The data suggest that the stoichiometry is identical for Na\(^{+}\)-Ca\(^{2+}\) exchange operating in either direction across the sarcolemmal membrane.

The data on sarcolemmal Ca\(^{2+}\) efflux induced by external Na\(^{+}\) (Fig. 6) suggest there may be some subtle difference in the nature of the Na\(^{+}\) binding sites of the Na\(^{+}\)-Ca\(^{2+}\) exchanger on the two sides of the membrane. There appears to be a larger threshold for Na\(^{+}\) before Na\(^{+}\)-dependent Ca\(^{2+}\) efflux becomes apparent for the inside-out sarcolemmal vesicles. It is notable that Na\(^{+}\)-dependent Ca\(^{2+}\) efflux for inside-out vesicles (which corresponds to Na\(^{+}\)-dependent Ca\(^{2+}\) influx in an intact myocardial cell) has an especially steep dependence on Na\(^{+}\) between 8 and 12 mM Na\(^{+}\). This is close to in vivo intracellular Na\(^{+}\) activity (31) and suggests that small changes in intracellular Na\(^{+}\) levels (e.g., in response to digitalis) can have a substantial effect on Ca\(^{2+}\) influx and, hence, contractility.

In summary, we have compared Na\(^{+}\)-Ca\(^{2+}\) exchange in the inside-out and total populations of cardiac sarcolemmal vesicles. The assignment of a component of Na\(^{+}\)-Ca\(^{2+}\) exchange to a subpopulation of inside-out vesicles is straightforward without relying on the asymmetric nature of ATP-dependent Na\(^{+}\) pumping). We present evidence that Na\(^{+}\)-Ca\(^{2+}\) exchange in the total population is due primarily to right side-out vesicles. This evidence is indirect and the unequivocal characterization of Na\(^{+}\)-Ca\(^{2+}\) exchange in right side-out vesicles has not been accomplished. Nevertheless, the data support the conclusion that there is no apparent difference in the interaction of Ca\(^{2+}\) with either side of the exchange mechanism. This would validate the use of the mixture of inside-out and right side-out vesicles present in sarcolemmal preparations for many Na\(^{+}\)-Ca\(^{2+}\) exchange studies. There may be some difference in the Na\(^{+}\) binding sites of the exchange mechanism on the two sides of the sarcolemmas which needs to be investigated further.

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