The effect of membrane potential on the Na"-Ca'" exchange activity of isolated sarcolemmal vesicles from dog ventricles is examined. Na"-Ca'" exchange is monitored as Na"-dependent Ca'" uptake as described by Reeves and Sutko ([1979] Proc. Natl. Acad. Sci. U. S. A. 76, 590-594). Membrane potential is controlled by varying internal and external K+ in the presence of valinomycin. Inside-positive potentials stimulate Na"-dependent Ca'" influx. This stimulation is independent of Ca" concentration. The results indicate that Na"-Ca" exchange by itself can generate a substantial potential (~ -90 mV) in the sarcolemmal vesicles. The data are consistent with an electrogenic Na"-Ca" exchange mechanism in which three or more Na" are exchanged for one Ca". This electrogenic exchange may have important implications in the control of myocardial tension development.

Extracellular Ca'" has an important regulatory role in the contractile responses of cardiac muscle. Some of the effects of the external Ca" may be mediated by a Na"-Ca" exchange mechanism. This hypothesis has been derived from both Ca" flux studies (1) and from functional studies of the antagonistic effects of Na" and Ca" on contractility (2). In a recent review, Mullins (3) has discussed the implications of an electrogenic Na"-Ca" exchange (in which three or more Na" are exchanged for one Ca") in cardiac muscle. If cardiac Na"-Ca" exchange were electrogenic, Ca" movements could be influenced by the membrane potential changes which occur during an action potential or during experimental intervention such as high external potassium or voltage clamp.

A technique for measuring Na"-Ca" exchange in isolated cardiac sarcolemmal vesicles has recently been developed by Reeves and Sutko (4). Using this procedure, Pitts (5) has reported that the Na"-Ca" exchange mechanism mediated the exchange of three Na" for one Ca". By following the movements of a lipophilic cation, Reeves and Sutko (6) have determined recently that Na"-Ca" exchange generates a current in cardiac membrane vesicles. We have previously presented preliminary evidence (7) that Na"-Ca" exchange activity could be affected in the sarcolemmal vesicles by "clamping" the voltage across the membrane by varying the K+ gradient across the membrane. This was done in conjunction with valinomycin. In this report, we further characterize and quantify the effects of imposed membrane potentials on Na"-Ca" exchange activities.

METHODS

Sarcolemmal vesicles were isolated from dog ventricles as described previously (8, 9). This preparation uses low salt extraction, differential and sucrose gradient centrifugation, and DNase (9) to improve purity and yield. Sarcolemmal markers are purified about 30-fold as compared to the initial homogenate. Contamination with mitochondria and sarcoplasmic reticulum is minimal. The vesicles are primarily (~ 80%) in a right-side-out orientation (7).

Na"-Ca" exchange is measured at 37°C as described previously (7). Vesicles loaded with 140 mM NaCl, 10 mM Tris (pH 7.4, 37°C) are diluted 50-fold into a KCl (140 mM), 10 mM Tris medium containing [beta]Ca" and [gamma]Ca" (usually 40 μM). In the presence of the outwardly directed Na" gradient, a rapid uptake of Ca" ensues. At plateau (~2 min) about 35 nmol of Ca"/mg of protein have accumulated (at 40 μM Ca''). Vesicles are collected by filtration through Millipore filters (0.45 μm) and washed with two 3-ml aliquots of 280 mM sucrose containing 1 mM LaCl3, to displace externally bound Ca". In all cases, control experiments are run using KCl-loaded vesicles instead of NaCl-loaded vesicles to correct for any Ca" uptake which is not Na"-dependent and for any remaining externally bound Ca". To measure Ca" uptake at early times, 5 μl of vesicles are suspended on the side of a plastic test tube containing 245 μl of Ca"-containing dilution medium. The reaction is then started by rapid mixing and stopped by the addition of 250 μl of 280 mM sucrose, 1 mM LaCl3. Of this mixture 440 μl are then applied to the Millipore filter.

Valinomycin was dissolved in ethanol and was added to the dilution medium to give a final concentration of 0.36 μM valinomycin and 0.4% ethanol. An equal quantity of ethanol was added to controls.

RESULTS AND DISCUSSION

Characteristics of Valinomycin-stimulated Na"-Ca" Exchange—The experiments to be described below rely on the ability of valinomycin specifically to increase K+ permeability of dog cardiac sarcolemmal vesicles. The potential across the membrane is then determined by the prearranged K+ gradient according to the Nernst equation. Na"-Ca" exchange activity is monitored as Na"-dependent Ca" uptake in the sarcolemmal vesicles from dog heart. That Na"-dependent Ca" uptake is truly a manifestation of Na"-Ca" exchange in the sarcolemmal fraction has been verified in recent reports (4-7). Activity is measured by diluting Na"-loaded vesicles into a medium containing KCl (140 mM) and Ca" ([see "Methods"]). When valinomycin (0.36 μM) is included in the KCl dilution medium, an inside-positive potential is created across the sarcolemmal membranes. This results in a stimulation of the Na"-dependent Ca" uptake (see below). This is energetically consistent with three or more internal Na" exchanging with one external Ca" to create a net outward current.

The effects of valinomycin-induced potentials on Na"-Ca" exchange as a function of Ca" concentration are shown in Fig. 1. In this experiment, the Ca" uptake was measured 0.04 min after the reaction was begun when Na"-dependent Ca" uptake is linear (5, 7). It is thus specifically Na"-dependent Ca" influx which is enhanced. The percentage increase in Ca" influx is independent of the Ca" concentration in the dilution medium.

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We have done several control experiments (data not shown) to test the validity of the results: 1) Stimulating effects of valinomycin are only seen in the presence of K+. If Na+-loaded vesicles were diluted into a sucrose rather than a K+ medium containing Ca2+ (see "Methods"), the Ca2+ uptake which ensues is unaffected by the presence of valinomycin. 2) The stimulating effects of valinomycin are dependent on the ratio \( K_+/K_+ \) as would be expected from the Nernst relationship rather than on the absolute quantities of K+ present. 3) In most experiments the reaction is stopped after 30 s. There is thus little time for the prearranged K+ gradient to redistribute. 4) The concentration of valinomycin (0.36 \( \mu \)M) is sufficient to produce maximum effects. 5) Preliminary experiments with a potential-sensitive fluorescent dye (DiS-C(4)(5)) (10) indicate that valinomycin does indeed rapidly induce a membrane potential in the presence of a K+ gradient in the sarcolemmal vesicles. The use of valinomycin-induced membrane potentials to affect Ca2+ transport has also been discussed by Zimniak and Racker (11).

The stimulating effect of an inside-positive membrane potential on Na+-dependent Ca2+ uptake decreases with increasing time as shown in Fig. 2. By 2.0 min (when Ca2+ uptake has reached its maximal level), the effect is actually to produce a slight inhibition (which does not increase at later times). This is analogous to the results of Reeves and Sutko (6) who monitored the accumulation of the lipophilic cation, tetraphenylphosphonium, during Na+-dependent Ca2+ uptake into cardiac sarcolemmal vesicles. In their system there was an initial rapid accumulation of tetraphenylphosphonium which subsequently decreased. The reasons for the decreasing effect of valinomycin with time are not clear but probably involve some combination of the following factors: 1) The initial K+ gradient will be decreasing as K+ diffuses across the membrane. 2) As Na+-dependent Ca2+ uptake proceeds, the intravesicular store of Na+ will be depleted resulting in decreased Ca2+ influx. This will occur more rapidly in the presence of valinomycin where the initial Na+-Ca2+ exchange was stimulated. 3) Although the Na+-dependent Ca2+ influx is stimulated by an inside-positive membrane potential, the efflux of Ca2+ may also be stimulated. Thus, an enhanced uptake of Ca2+ would be observed only at short time periods. The combination of a decreased Na+ gradient (resulting in decreased Ca2+ influx) and enhanced Ca2+ efflux could result in the small valinomycin-stimulated decrease in net Ca2+ uptake at longer times (Fig. 2).

### Effect of Varying Membrane Potential on Na+-Ca2+ Exchange

In the experiments above, K+ was initially present only in the extravesicular medium when the Na+-Ca2+ exchange reaction was begun. In an attempt to quantitate the effects of membrane potential, experiments were undertaken in which both the intra- and extravesicular concentrations of K+ were controlled in the presence and absence of valinomycin (for details see legend to Fig. 3). The theoretical potential difference across the membrane was then calculated using the Nernst relationship. The effects of a range of membrane potentials (compared to control values obtained in the absence of valinomycin) on Na+-dependent Ca2+ influx are shown in Fig. 3. The Na+-dependent Ca2+ influx increases as the membrane potential becomes more inside-positive. The point at which Ca2+ influx is unaffected by membrane potential, the x axis intercept, occurs at about -60 mV. This indicates that in

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**Figure 1.** Dependence of Na+-dependent Ca2+ influx on membrane potential and Ca2+ concentration. Ca2+ uptake was measured 0.04 min after dilution of the sarcolemmal vesicles. The Na+–loaded vesicles initially contained no K+ and were diluted into 140 mM KCl ± valinomycin (0.36 \( \mu \)M).

**Figure 2.** Time course of stimulation by valinomycin on Na+-dependent Ca2+ uptake. [Ca2+] = 40 \( \mu \)M. Other conditions as in Fig. 1. n = 3. Error bars represent standard error.

**Figure 3.** Dependence of Na+-dependent Ca2+ influx on membrane potential. Ca2+ influx was measured 0.05 min after addition of vesicles to the dilution medium. The sarcolemmal vesicles were initially preincubated (30 min, 37°C) in a medium containing 110 mM NaCl, 30 mM KCl, and 10 mM Tris (pH 7.4, 37°C). The external dilution medium contained 40 \( \mu \)M CaCl2, 10 mM Tris, and KCl (7.5 to 140 mM). If the KCl was less than 140 mM, choline chloride was added to maintain osmolarity. The dilution medium also contained either valinomycin (0.36 \( \mu \)M) in ethanol (0.4% final concentration) or only ethanol in controls. The theoretical potential was calculated using the equation: \[ E_M = 61.5 \log K_+/K_+ \]. n = 3. Error bars represent the standard error.
the absence of valinomycin the potential difference which is created across the sarcosomal membranes by the action of an electrogenic Na⁺-Ca²⁺ exchange is −60 mV. That is, the exchange of three or more internal Na⁺ for one external Ca²⁺ results in the net outward movement of positive charge. This produces an inside-negative potential unless another mechanism for regulating membrane potential exists (e.g. a K⁺ gradient plus valinomycin). It is interesting that when the membrane potential is "clamped" at 0 mV a significant stimulation still occurs. This is consistent with the concept that Na⁺-dependent Ca²⁺ influx by itself creates an inside-negative membrane potential which will then tend to inhibit further Na⁺-Ca²⁺ exchange. Any intervention which makes the membrane potential more positive (inside) will stimulate Na⁺-dependent Ca²⁺ uptake.

In these experiments, the vesicles were initially preincubated in a medium containing 110 mM NaCl and 30 mM KCl. The external dilution medium used to initiate Na⁺-dependent Ca²⁺ influx contained variable amounts of KCl (with choline chloride used to maintain osmolarity) plus 40 μM Ca²⁺. In the absence of valinomycin, the external K⁺ concentration did not affect Na⁺-Ca²⁺ exchange activity. Apparently the permeability of K⁺ is not sufficient (without ionophore) to determine the membrane potential of the vesicles. It is not possible to be certain of the membrane potential which is actually present in a vesicle. For example, the K⁺ concentration could be changing during the course of a measurement. The data of Fig. 3 should thus be considered in a qualitative manner. Nevertheless, the implications of these results are still likely to be valid.

In conclusion, we find that Na⁺-Ca²⁺ exchange is affected by membrane potential in a manner consistent with the electrogenic exchange of three or more Na⁺ for one Ca²⁺. An alternative explanation is that the Na⁺-Ca²⁺ exchange protein itself is affected by the membrane potential. For example, the membrane potential may induce a protein conformational change which influences activity. An electrogenic exchange, however, is also consistent with the data of Pitts (5), who has measured a 3:1 stoichiometry, and Reeves and Sutko (6) who have found that sarcosomal Na⁺-Ca²⁺ exchange produces a current. Evidence from other systems, such as the squid axon, has also implicated an electrogenic mechanism for Na⁺-Ca²⁺ exchange (12). The ability of Na⁺-Ca²⁺ exchange to respond to a changing membrane potential in the intact myocardium may be a significant phenomenon. An electrogenic Na⁺-Ca²⁺ exchange may contribute to Ca²⁺ movements during the action potential (3), during K⁺-induced depolarization (13), or during voltage clamp (2).

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REFERENCES