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Effects of Membrane Potential on Sodium-Dependent Calcium Uptake by Sarcolemma-Enriched Preparations from Canine Ventricle

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Summary. The effect of membrane potential on sodium-dependent calcium uptake by vesicles in an isolated cardiac sarcolemma preparation was examined. Initial time course studies showed that the reaction deviated from initial velocity conditions within minutes. This appeared to be due, in part, to loss of the sodium gradient. Assays carried out to 10 sec revealed a linear component of uptake (2 to 10 sec) and a faster component (complete by 2 sec). The latter was eliminated by loading the preparation with ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA). This maneuver did not affect the slow component, and subsequent studies used preparations containing EGTA. Potassium Nernst potentials (E_K), established by potassium gradients in the presence of valinomycin, were varied from −100 to +30 mV by changing $[K^+]_o$ from 1.18 to 153.7 mM ($[K^+]_i$ = 50 mM). The initial velocity of sodium-dependent calcium uptake was stimulated twofold by changing E_K from −100 to 0 mV and another twofold by raising E_K from 0 to +30 mV. For the total range of E_K and $[K^+]_o$, 32 to 36% of the increase appeared to reflect stimulation by extravesicular potassium. The remainder appeared to be due to membrane potential. The profile of sodium-dependent calcium uptake versus E_K suggested that calcium influx through electrogenic sodium/calcium exchange may be much more affected by the positive region of the cardiac action potential than by the negative region.

Key Words sodium/calcium exchange · excitation-contraction coupling · sarcolemma · membrane potential · sodium · calcium · heart

Introduction

The membrane potential of the cardiac cell resides over time predominantly in two sequential states: (a) one of hyperpolarization (inside of cell negative relative to the extracellular space, i.e., the resting membrane potential); and (b) one of relative depolarization (i.e., the plateau of the action potential). The former is associated with low intracellular free calcium and a relaxed state of the muscle. The latter is associated with a rise in free calcium and the development of tension. It is generally agreed that the transition from the resting membrane potential to the plateau of the action potential triggers calcium influx. Likewise, at some point over the cardiac cycle, calcium must move out of the cell.

Mullins (1979, 1981) suggested that membrane potential could affect calcium entry and exit across the sarcolemma through a system which promotes sodium/calcium exchange (Reuter & Seitz, 1968; Baker et al., 1969; Glitsch et al., 1970; Langer & Serena, 1970; Langer, 1972; Blaustein, 1974; Reuter, 1974). The thermodynamic basis for this suggestion was the assumption that more than two sodiums are coupled to the countertransport of calcium which would mean that the exchanger promotes the movement of net charge. Thus, at equilibrium for the exchange reaction, intracellular free calcium would be linked to membrane potential and the concentrations of external sodium and calcium and internal sodium by the following equation (Blaustein & Hodgkin, 1969):

$$[Ca^{2+}]_i = \frac{[Ca^{2+}]_o[Na^+]_i^r}{[Na^+]_o^r e^{-(r-2)E_m F/RT}} \quad (1)$$

where the subscripts "i" and "o" refer to the intracellular and extracellular concentrations of the cations; E_m is the membrane potential; F, R and T have their usual chemical meaning; and r is the number of sodiums moved per calcium. [Note that the equilibrium is independent of membrane potential for $r = 2$ (i.e., no net charge movement).] A number of studies on free calcium in myocardial cells (Sheu & Fozzard, 1982; Allen et al., 1983) or sodium-dependent calcium movement into or out of vesicles in sarcolemma-enriched preparations from cardiac tissue (Bers et al., 1980; Caroni et al., 1980; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980; Reeves et al., 1980) are consistent with $r > 2$. Furthermore, Pitts (1979) concluded that $r = 3$ based on measurements of sodium and calcium fluxes associated with

Table 1. Test of filtration to stop calcium uptake[a]

Time prior to wash following filtration (sec)	Calcium association with vesicles and filters (nmol/mg protein)	
	I	II
3	53.1	49.2
15	56.7	54.5
30	54.3	49.6
60	51.6	50.4
120	49.9	62.0

[a] Aliquots of the sarcolemma preparation were exposed 14 to 16 hr at 4 to 5°C to a loading medium containing 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 at 37°C). Subsequently, the suspension (10 μl) was diluted 30-fold into a reaction medium at 37°C containing 40 μM $CaCl_2$ with ^{45}Ca, 160 mM LiCl, and 10 mM Tris-Cl, pH 7.4, to begin the reaction. After 30 sec of reaction, the suspension was filtered and the filter was washed twice with 5 ml of an ice-cold KCl/KH_2PO_4 buffer. The time between filtration and washing was varied from 3 to 120 sec. Values for two experiments, corrected for filter blanks, are presented.

a cardiac sarcolemma preparation and Reeves and Hale (1984) concluded that $r = 3$ on the basis of a thermodynamic approach.

The purpose of the present study was to determine the relationship between membrane potential and sodium-dependent calcium movement. Conditions for measurement of the initial velocity of uptake were developed and employed. The reversal potentials (Mullins, 1981) for electrogenic sodium/calcium exchange for the conditions employed were −660 and −392 mV for values of r (Eq. 1) of 3 and 4, respectively. As expected, the results showed that deviations from the reversal potential in a positive (less negative) direction increased the rate of calcium uptake which confirmed the work of others (Bers et al., 1980; Caroni et al., 1980; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980; Reeves et al., 1980). The results, however, also showed a break in the relationship between membrane potential and the initial velocity of sodium-dependent calcium uptake at about 0 mV. The reaction was activated to a greater extent (per unit change in potential) by moving from 0 to +30 mV than it was by moving from −100 to 0 mV.

Materials and Methods

Sarcolemma-Enriched Preparations

The isolation procedure described previously (Van Alstyne et al., 1980) was employed with slight modifications (Frankis & Lindenmayer, 1984) to obtain sarcolemma-enriched preparations from canine ventricle. The preparation is (a) enriched 27- to 40 plus-fold with putative sarcolemma markers, (b) composed of membrane vesicles, and (c) osmotically active. Based on activation of Na^+,K^+-ATPase activity and changes in binding of [^{3}H]ouabain by freezing and thawing, the preparation was concluded to consist of 18 ± 4% leaky vesicles, 14 ± 2% sealed inside-out (I/O) vesicles and 68 ± 4% sealed right-side out (R/O) vesicles (Frankis & Lindenmayer, 1984). Thus, approximately 83% of the sealed vesicles appear to be in the R/O configuration.

Sodium-Stimulated Calcium Uptake: Initial Filtration Procedures

The sarcolemma-enriched preparation was preloaded with sodium by incubating approximately 200 μg protein in 0.5 ml of a loading medium overnight (12 to 15 hr) at 5°C. The loading medium typically consisted of 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 at 37°C). In control experiments, KCl or LiCl was substituted for NaCl (*see below*) on an equimolar basis. Calcium uptake over time was assessed by adding 0.22 ml of the loaded preparation (prewarmed to 37°C) to 6.6 ml of a reaction medium at 37°C which contained 160 mM LiCl, 10 mM Tris-Cl, pH 7.4, and 40 μM $CaCl_2$ with ^{45}Ca (specific activity of ~75 cpm/pmol). The suspension was gently agitated during the course of the reaction by use of a metabolic shaking incubator. The reaction was subsequently terminated by filtration of aliquots of the suspension (Millipore, 0.45 μm). The vesicles/filter were washed twice with 5 ml aliquots of an ice-cold "washing" solution which contained 200 mM KCl and 5 mM KH_2PO_4, pH 7.4. Immediately after washing, the filters were removed and assayed for ^{45}Ca by liquid scintillation spectroscopy. The data were corrected for the amount of calcium associated with the preparation at zero time. Sodium-dependent calcium uptake was equated to calcium uptake by membrane vesicles preloaded with sodium *minus* the uptake by vesicles preloaded with either potassium or lithium. The latter are labeled as "control" in the figure legends.

Initial experiments were carried out to test the effectiveness of filtration as an inhibitor of calcium movement. Aliquots of the preparation, preloaded with sodium, were exposed to a medium containing calcium and ^{45}Ca for 30 sec. Subsequently, the samples were filtered, but washes of the filters were delayed from 3 to 120 sec. The amount of calcium associated with the vesicles/filter was not appreciably altered by the delay in washing (Table 1). Accordingly, it was concluded that filtration was effective in terminating calcium movement. A second experiment examined the effect of repetitive washes of the filter on calcium associated with the vesicles. Aliquots of the vesicular suspension, preloaded with sodium and then exposed to calcium and ^{45}Ca, were either filtered (Experiment A, Table 2) or diluted with 5 ml of the washing solution and then filtered (Experiment B, Table 2). After the first wash of the filter, ^{45}Ca associated with the vesicles/filter did not change upon an additional one to three washes. Variants of this procedure are detailed under Results.

Rapid Quench Studies

Some experiments were carried out with a Dionex Multimixer (Model D-132, Dionex, Sunnyvale, Calif.). This device consists of a syringe block submerged in a thermostated bath (37°C). Reactants, drawn from reservoir syringes into drive syringes, were forced by a pneumatic activator through a multijet mixer around a length of capillary tubing to a second mixer where they were chemically quenched. The quenching solution consisted of

200 mM KCl, 5 mM KH_2PO_4 and 150 μM EGTA[1], pH 7.4, at 37°C. A 0.3-ml sample of the quenched solution was collected and filtered. The syringe block velocity was monitored by the voltage drop across a slide wire potentiometer, and the valve-switching events were monitored with a dual-beam storage oscilloscope. Each experiment was carried out on a minimum of three preparations. Calcium associated with the preparation at zero time was assayed in reaction tubes under identical conditions except that 5 ml of the quenching medium was added *prior* to addition of the membrane preparation.

Sodium-Stimulated Calcium Uptake: Modified Filtration Procedures

The sarcolemma-enriched preparation was preloaded for 15 to 18 hr at 5°C as described above. The loading medium was modified to contain 120 mM NaCl, 50 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.4 at 37°C) and 24.9 μM $CaCl_2$ with ^{45}Ca. Based on a dissociation constant of 0.1355 μM for the Ca-EGTA complex, determined according to the method of Bers (1982), and a dissociation constant of 7.9 mM for the Mg-EGTA complex (Wallick et al., 1973), free calcium was calculated to be 0.73 nM. In separate experiments, NaCl was replaced by 120 mM LiCl in order to assess calcium uptake in the absence of intravesicular sodium. In order to achieve a uniform distribution of EGTA between the extravesicular and intravesicular spaces (Schilling & Lindenmayer, 1984), the glass tubes containing the membrane suspension were immersed in a dry ice/acetone bath to freeze the preparation and then placed in a water bath at 37°C to promote thawing. The freeze/thaw procedure was repeated a total of five times (Schilling & Lindenmayer, 1984). This procedure, did not significantly alter sodium-dependent calcium uptake (Hungerford et al., 1984). The reaction was measured as follows. A 10-μl bead of the loaded preparation was placed on the side of a polypropylene tube (17 × 100 mm, Fisher Scientific Co., Pittsburgh, Pa.) which contained 990 μl of reaction medium. The reaction medium consisted of 120 mM LiCl, 50 mM KCl, 1 mM $MgCl_2$, 0.1 μM valinomycin, 10 mM Tris-Cl (pH 7.4 at 37°C) and $CaCl_2$ with ^{45}Ca. In all experiments the ^{45}Ca in the loading and reaction media had the same specific activity (30 to 35 cpm/pmol). The assays were initiated by vortexing. An electronic metronome was used to time calcium uptake into the vesicle preparation. The assays were terminated 2 to 6 sec later by the rapid addition of 5 ml of an ice-cold stop/wash buffer. This buffer contained 160 mM KCl, 1 mM $CaCl_2$, 200 μM $LaCl_3$, and 10 mM Tris-Cl, pH 7.4 at 4°C (Bartschat & Lindenmayer, 1980). The suspension was then rapidly filtered (Millipore, 0.45 μm). After rinsing the reaction vessel and washing the filter with two 5-ml aliquots of the ice-cold stop/wash buffer, the filter was immediately removed and assayed for ^{45}Ca. ^{45}Ca associated with the vesicles at zero time was determined in assays to which 5 ml of ice-cold stop/wash buffer was added to the reaction medium prior to the membrane suspension. Sodium-dependent calcium uptake was determined as described above.

Metallochromic Indicator Studies

Sodium-dependent calcium uptake was measured at 37°C using an Aminco DW2 UV-VIS spectrophotometer and antipyralazo III, a metallochromic indicator sensitive to changes in free calcium (Scarpa, 1978; Scarpa et al., 1978). Under the conditions of the experiments described below, the change in absorbance as a function of calcium concentration was linear. The sarcolemma-enriched preparation, loaded with 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 at 37°C) and the reaction medium were prewarmed to 37°C. The reactants were mixed in a cuvette to yield a final volume of 2.5 ml which contained 146.1 mM KCl, 13.9 mM NaCl, 45.6 μM antipyralazo III, 10 mM Tris-Cl, pH 7.4 at 37°C, and 2.5 mg protein of the loaded preparation. Following a brief period of incubation (15 to 20 sec), a stable baseline in the absence of calcium was recorded. Subsequently, a small aliquot of 10 mM $CaCl_2$ in 10 mM Tris-Cl (pH 7.4) was added to the cuvette to yield final concentrations of: 145 mM KCl, 13.8 mM NaCl, 45 μM antipyralazo III, 10 mM Tris-Cl, 75 μM $CaCl_2$, and 0.99 mg protein/ml of the loaded preparation. In parallel assays extravesicular sodium was 160 mM and potassium was absent. Based on the differential absorbance spectra of antipyralazo III recorded under similar experimental conditions (i.e., no protein present), the wavelength pair of 598 to 646 nm was used to monitor changes in absorbance. The wavelength chopping frequency was 250 Hz and the time constant was 100 msec.

Table 2. Effect of filter washes on calcium associated with vesicles/filter[a]

Number of washes	Calcium associated with vesicles and filters (nmol/mg protein)			
	Experiment A		Experiment B	
	I	II	I	II
0	261.0	204.0	81.1	82.9
1	57.1	63.2	49.3	48.9
2	56.7	59.8	47.2	47.6
3	59.6	54.5	45.3	47.8
4	56.1	54.5	49.4	47.9

[a] Aliquots of the sarcolemma-enriched preparation were exposed 12 to 15 hr to a loading medium (4 to 5°C) containing 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 at 37°C). The vesicle suspension (10 μl) was diluted 30-fold into a reaction medium at 37°C containing 40 μM $CaCl_2$ with ^{45}Ca, 160 mM LiCl and 10 mM Tris-Cl, pH 7.4. After 5 min, the reaction was terminated by filtration (Experiment A) or by dilution with 5 ml of an ice-cold KCl/KH_2PO_4 buffer followed immediately by filtration (Experiment B). In each case the number of washes of the filter was varied from 0 to 4 and the ^{45}Ca content of the filters was then determined. Values, corrected for filter blanks, for two different membrane preparations are presented.

Materials

Trizma base and EGTA were purchased from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of EGTA were adjusted to pH 7.4 for 37°C with Tris base. ^{45}Ca was obtained from New England Nuclear (Boston, Mass.) and antipyralazo III was obtained from ICN Pharmaceuticals, Inc. (Plainview, N.Y.). Orion 0.1 M standard calcium chloride (Orion Research Incorporated, Cambridge, Mass.) was used as a calcium stock along with an Orion calcium electrode (932001) and an Orion digital pH/mV

[1] The abbreviation used is: EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid.

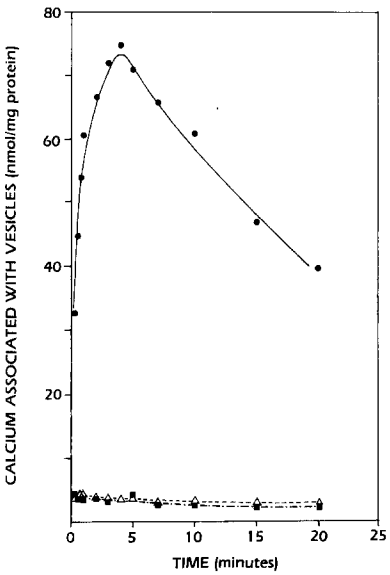

Fig. 1. Calcium uptake by vesicles in the sarcolemma-enriched preparation. Aliquots of the membrane preparation were incubated 12 to 15 hr at 5°C with 10 mM Tris-Cl (pH 7.4 for 37°C), and either 160 mM NaCl (●), LiCl (■), or KCl (△). After prewarming to 37°C, these suspensions were added to a medium containing final concentrations of 154.7 mM LiCl plus 5.3 mM of the salt used in loading, 40 μM $CaCl_2$ with ^{45}Ca and 10 mM Tris-Cl, pH 7.4. The reactions were terminated at the indicated times by filtration

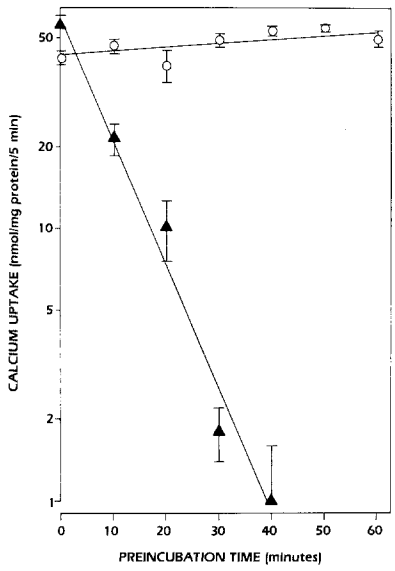

Fig. 2. Effect of preincubation at 37°C on sodium-dependent calcium uptake. Aliquots of the sarcolemma preparation were incubated 12 to 15 hr at 5°C with a medium containing 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 at 37°C). The loaded suspension was then divided into two aliquots. One aliquot (○) was incubated at 37°C in the loading medium. At the times indicated, the suspension was diluted 30-fold into a reaction medium containing final concentrations of 154.7 mM LiCl, and 5.3 mM NaCl, 40 μM $CaCl_2$ with ^{45}Ca and 10 mM Tris-Cl, pH 7.4. The reactions were terminated 5 min later by addition of the stopping solution and filtration. The other aliquot (▲) was diluted 30-fold into the same reaction medium except that no calcium was present. At the times indicated, 40 μM $CaCl_2$ with ^{45}Ca was added. The reactions were terminated 5 min later as above. In parallel experiments, aliquots loaded with 160 mM LiCl were assayed to allow correction for uptake in the absence of sodium. Values on the ordinate reflect sodium-dependent calcium uptake over the 5-min exposure to calcium

meter (Model 701A) to determine free calcium as a function of total calcium and EGTA. All other chemicals were of reagent grade.

Results

Initially, sodium-dependent calcium uptake by the sarcolemma preparation was measured by the protocol originally reported by Reeves and Sutko (1979). As shown by those workers, calcium uptake by sodium-loaded vesicles was found to be much greater than for lithium-loaded or potassium-loaded vesicles over the first few minutes of the assay (Fig. 1). Subsequently, vesicular calcium declined in the former towards values obtained for the lithium- or potassium-loaded vesicles. The decline in vesicular calcium after the uptake (Fig. 1) could have been due to either deterioration of the vesicles or to loss of the sodium gradient over the course of the assay. Two experiments were carried out to test these possibilities. First, vesicles loaded with 160 mM NaCl were prewarmed to 37°C without dilution. At 10-min intervals thereafter, aliquots were diluted into a medium containing calcium and low sodium. The reaction was then terminated 5 min after the dilution. Sodium-dependent calcium uptake over 5 min, following 10- to 60-min preincubations at 37°C, remained the same (Fig. 2). Thus, deterioration of the preparation as a cause for the decline seemed unlikely. Second, vesicles loaded with sodium were diluted into a medium at 37°C containing low sodium but no calcium. Calcium uptake was initiated at varying times thereafter by addition of calcium with ^{45}Ca. After 5 min of exposure to calcium, the reactions were terminated. In this case, sodium-dependent calcium uptake declined exponentially with the time of preincubation ($t_{1/2}$ = 6.6 min). This profile was judged to be consistent with a loss of intravesicular sodium and, therefore, of the sodium gradient over time at 37°C.

The sodium-dependent association of calcium with the sarcolemma-enriched preparation suggested a process which moves calcium into the intravesicular space from the extravesicular medium. An alternate possibility was that the calcium was

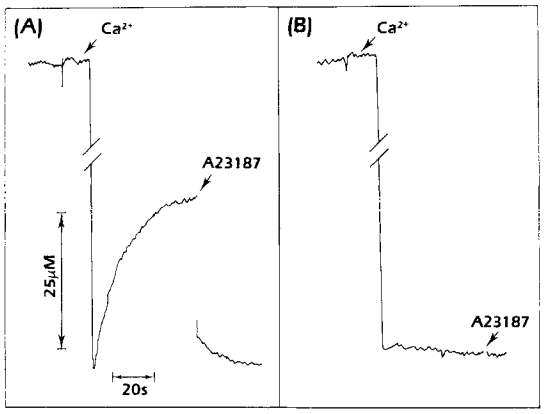

Fig. 3. Sodium-dependent calcium uptake measured by antipyralazo III. Aliquots of the sarcolemma preparation were incubated 15 to 18 hr at 5°C with a medium containing 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 for 37°C). The differential absorbance changes of the metallochromic indicator, antipyralazo III, were measured in reaction media at 37°C which contained final concentrations of 45 μM antipyralazo III, 10 mM Tris-Cl (pH 7.4), 0.99 mg protein/ml and either 145.0 mM KCl and 13.8 mM NaCl (Panel *A*) or 160 mM NaCl (Panel *B*). The differential absorbance was recorded at 598 and 646 nanometers, peak absorbance and isosbestic point, respectively. The reaction was started by the addition of 75 μM $CaCl_2$. The calcium ionophore, A23187 (1 μg/ml), was subsequently added as indicated

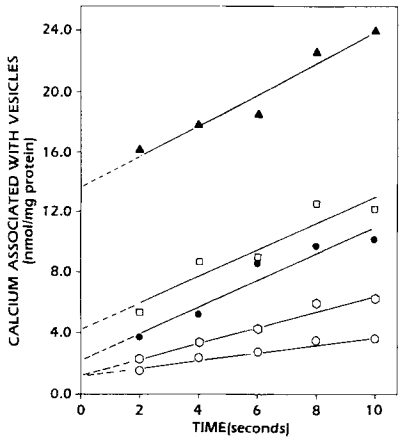

Fig. 4. Effects of extravesicular calcium on sodium-dependent calcium uptake versus time. Aliquots of the sarcolemma preparation were incubated 15 to 18 hr at 5°C with 10 mM Tris-Cl (pH 7.4 for 37°C), and either 160 mM NaCl or 160 mM LiCl (control). After prewarming to 37°C, the loaded preparation was added to reaction media containing final concentrations of 154.1 mM LiCl plus 5.3 mM NaCl or 160 mM LiCl (control). These suspensions were quickly placed in one syringe of the multimix device. A second syringe contained various concentrations of $CaCl_2$ plus ^{45}Ca in 160 mM LiCl. The contents of these two syringes were mixed to start the reaction. Final extravesicular calcium concentrations were 10 (○), 20 (○), 40 (●), 100 (□) and 383 (▲) μM. A third syringe contained a stopping solution with the following concentrations (in mM): 160 KCl, 0.2 $LaCl_3$, 1 $CaCl_2$ and 10 Tris-Cl (pH 7.4). The reaction was terminated by addition of the stopping solution after reaction times of 2 to 10 sec. The syringes were filled with freshly diluted preparation at each new reaction time and four samples were collected. The first two samples were discarded. The remaining two samples were collected, filtered and washed immediately. All samples were filtered within 60 sec of the initial dilution of the loaded vesicle preparation (i.e., added to the first syringe). This constraint was employed to minimize dissipation of the sodium gradient (Fig. 2). Net sodium-dependent calcium uptake (ordinate) is the amount of calcium associated with sodium-loaded vesicles minus the amount of calcium associated with lithium-loaded vesicles. The lines drawn were derived from least-squares analysis of the mean data obtained from three sarcolemma preparations

simply bound to the external surface of sealed vesicles and to both surfaces of leaky vesicles. In order to differentiate these possibilities, we tested the effects of the calcium ionophore, A23187, using antipyralazo III to monitor free calcium in the extravesicular medium. Vesicles were loaded with 160 mM sodium as described in Fig. 1. After dilution into a low sodium medium, the addition of calcium caused an abrupt increase in absorbance (ΔA at 598 to 646 nm) which was followed by a slower decrease (Fig. 3*A*). The latter was consistent with a decrease in free extravesicular calcium due either to movement of calcium into sealed vesicles or to binding to membrane surfaces. Subsequent addition of A23187 increased absorbance signifying an increase in extravesicular free calcium. This result was consistent with the loss of calcium from sealed vesicles. In the absence of a sodium gradient, there was no evidence of time-dependent calcium uptake and subsequent addition of A23187 was without effect on the concentration of free extravesicular calcium (Fig. 3*B*).

Given the nonlinear profiles obtained for the reaction measured over minutes, we next explored the nature of sodium-dependent calcium uptake between 2 and 10 sec using a rapid-quench technique. Calcium uptake appeared to be linear over this time range and the rate of uptake (slope of lines) increased slightly as extravesicular calcium was raised from 10 to 383 μM (Fig. 4). Positive intercepts, however, were found for time zero and the intercepts appeared to increase in a nonsaturable manner with increasing extravesicular calcium. Rapid-quench studies encompassing earlier times suggested that a fast component of uptake between 0 and 2 sec was responsible for the positive intercepts (Fig. 5). The time profile from 0.07 to 5.6 sec required two exponential porcesses for best fit. Similar results were reported by Kadoma et al. (1982) for sodium-dependent calcium efflux from vesicles in a sarcolemma-enriched preparation. It should be noted that a tangential line to the earliest component of the curve yielded a rate greater than 20 nmol/mg/sec which is similar to values reported

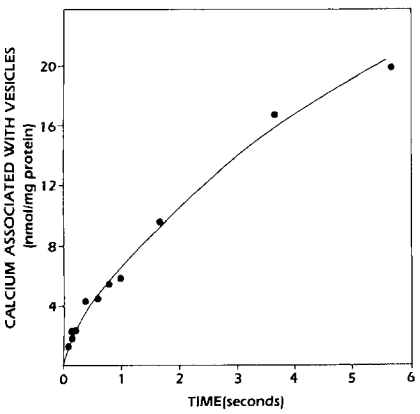

Fig. 5. Sodium-dependent calcium uptake assayed by rapid-quench techniques. Aliquots of the sarcolemma preparation were loaded for 15 to 18 hr at 5°C with 160 mM NaCl and 10 mM Tris-Cl (pH 7.4 for 37°C). Assays were performed as described in the legend of Fig. 4 with the following changes. First, the concentration of calcium with ^{45}Ca in the second syringe was 200 μM which, upon dilution, yielded a final concentration of 100 μM. Second, after prewarming to 37°C, the loaded preparation was diluted into either 160 mM LiCl or 160 mM NaCl. Third, the reaction times were 0.07 to 5.6 sec. The data were corrected for calcium uptake measured in the absence of a sodium gradient. Each point is the mean obtained from four preparations

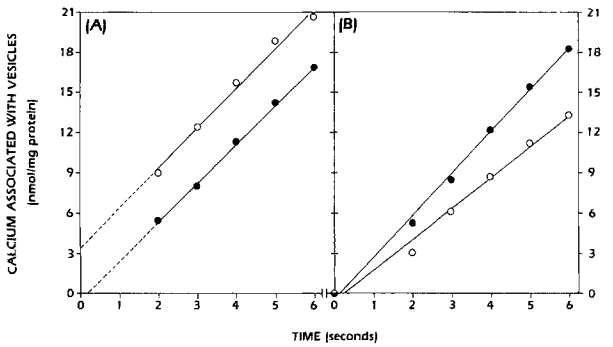

Fig. 6. Effect of EGTA and valinomycin on sodium-dependent calcium uptake. *Panel A:* Aliquots of the sarcolemma preparation were incubated 15 to 18 hr at 5°C with 120 mM NaCl or 120 mM LiCl (control), 50 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-Cl (pH 7.4 for 37°C) in either the absence (○) or presence (●) of 5 mM EGTA and 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}). Following 5 freeze/thaw cycles, the suspension was prewarmed to 37°C and added to reaction media containing final concentrations of 50 mM KCl, 1 mM $MgCl_2$, 0 (○) or 50 μM EGTA (●), 0.099 μM valinomycin, 10 mM Tris-Cl (pH 7.4), and either 118.8 mM LiCl plus 1.2 mM NaCl or 120 mM LiCl (control). Total extravesicular calcium was either 89.0 μM (●) or 39.2 μM (○) to yield equal concentrations of free extravesicular calcium in the presence and absence of EGTA. *Panel B:* Aliquots of the preparation were incubated 15 to 18 hr at 5°C with 120 mM NaCl or LiCl (control), 50 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.4 for 37°C) and 5 mM EGTA plus 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}). Following 5 freeze/thaw cycles, the suspension was prewarmed to 37°C, and added to a reaction medium containing final concentrations of either 118.8 mM LiCl plus 1.2 mM NaCl or 120 mM LiCl (control), 50 mM KCl, 1 mM $MgCl_2$, 50 μM EGTA, 89.9 μM $CaCl_2$ with ^{45}Ca (39.2 μM free Ca^{2+}), 10 mM Tris-Cl (pH 7.4) and either 0.099 μM valinomycin in ethanol (●) or an equivalent volume of ethanol (○). The data (averages, n = 2) in both panels were corrected for calcium movements in the absence of sodium

by Caroni et al. (1980). The data beyond one second yielded a rate of 2.6 nmol/mg/sec. The latter was similar to that shown in Fig. 4 (i.e., for $[Ca^{2+}]_o$ = 100 μM) and to values reported by others for similar experimental conditions (Philipson & Nishimoto, 1980; Kadoma et al., 1982; Reeves & Sutko, 1983).

Kadoma et al. (1982) suggested a number of reasons for the two components of sodium-dependent calcium efflux. One of these was that charge separation by electrogenic sodium/calcium exchange would progressively inhibit the reaction rate over time. Hungerford et al. (1984) found that inclusion of a system to dissipate charge separation (i.e. equal potassium in intravesicular and extravesicular spaces in the presence of valinomycin) stimulated the sodium-dependent calcium influx but did not eliminate the two components shown in Fig. 4.

A second possibility for the nonlinearity was calcium accumulation in the intravesicular space (perhaps adjacent to the inner membrane surface). This was tested by inclusion of a calcium sink, EGTA, in the intravesicular space (Fig. 6*A*). This maneuver generated a linear uptake of calcium in the presence of 39.2 μM extravesicular free calcium which was sodium dependent. Over a number of experiments, this linear profile was found to extrapolate to zero uptake at zero time. Thus, initial velocity conditions were satisfied. These conditions were consistently observed for extravesicular free calcium concentrations up to 109 μM. In some experiments, initial velocity conditions were satisfied for extravesicular free calcium concentrations up to 989 μM while in others the time profile deviated from initial velocities at higher free calcium. Whereas the inclusion of EGTA appeared necessary to establish initial velocity conditions, previous studies reported diverse effects of EGTA on sodium/calcium exchange. In squid axon, intracellular EGTA inhibits sodium-dependent calcium influx (Baker, 1972), although it is without effect on sodium-dependent calcium efflux at constant internal calcium (Baker & McNaughton, 1976). In sarcolemma preparations, EGTA stimulated sodium-dependent calcium uptake at low extravesicular calcium (<20 μM); however, when $[Ca^{2+}]_{o,free}$ = 40 μM, EGTA had no effect on the rate of sodium-dependent Ca uptake (Trosper & Philipson, 1984). The data shown in Fig. 6*A* confirm the latter observation.

Inclusion of equal potassium in the extravesicu-

lar and intravesicular spaces with valinomycin stimulated the initial rate of uptake but did not affect the intercept at zero time (Fig. 6*B*). The latter result was consistent with the movement of net charge by the sodium-dependent reaction. The next experiment was designed to determine the relationship between membrane potential and the initial velocity of sodium-dependent calcium uptake. Membrane potentials, varied from −100 mV (inside negative) to +30 mV (inside positive), were established by loading the preparation with 50 mM potassium and then placing the loaded suspension in media containing various potassium concentrations. Valinomycin was included to make the vesicles highly permeable to potassium such that the membrane potential E_m should have equalled the potassium Nernst potential E_K:

$$E_m = E_K = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}. \quad (2)$$

Previous studies (Bartschat et al., 1980; Schilling et al., 1984), with a voltage-sensitive dye, provided evidence consistent with the development of potentials under these conditions. While the potentials were found to dissipate over minutes (Bartschat et al., 1980), the experiments described here were carried out for only 3 sec. The initial velocity of sodium-dependent calcium uptake increased approximately twofold between −100 and 0 mV and about another twofold between 0 and +30 mV (Fig. 7). While a smooth curve was drawn through the data in Fig. 7, it should be noted that the data between −100 and 0 mV were well described by a linear relationship from which the data between 0 and +30 mV substantially deviated.

We next addressed whether the result of Fig. 7 was due to membrane potential or to potassium *per se* (top ordinate of Fig. 7). Several studies (Philipson & Nishimoto, 1980; Reeves & Sutko, 1980; Hungerford et al., 1984) showed that depolarization with potassium/valinomycin enhances sodium-dependent calcium uptake by sarcolemma preparations, which is consistent with an electrogenic process. Allen and Baker (1983) compared the chemical effects of potassium to changes in potential induced by voltage-clamp maneuvers on sodium/calcium exchange in squid axons. They found that the effects of potassium and potential were not equivalent on Na efflux stimulated by outside calcium but were equivalent to Ca efflux stimulated by external sodium. Thus, attempts were first made to establish membrane potentials with thiocyanate gradients instead of with potassium/valinomycin. In this case equal potassium was present in the extravesicular and intravesicular spaces. The rationale was based on experiments which showed that the sarcolemma preparation is much more permeable to thiocyanate than to chloride, sodium or potassium (Schilling, 1981; Schuil, 1981). Thus, the membrane potential might be approximated by the thiocyanate diffusion potential, E_{SCN}:

$$E_m = E_{SCN} = \frac{RT}{F} \ln \frac{[SCN^-]_i}{[SCN^-]_o}. \quad (3)$$

For conditions where the membrane potential should have ranged from −80 to +15 mV (i.e., if Eq. 3 were valid), relatively little effect was observed on the initial velocity of sodium-dependent calcium uptake (Fig. 8*A*). When potassium gradients and thiocyanate gradients were combined in the absence of valinomycin to give $E_{SCN} = E_K$, a greater effect was observed but addition of valinomycin further enhanced the response (Fig. 8*B*). These data suggested that either thiocyanate gradients were unable to establish significant potentials or that potassium rather than membrane potential was responsible for much of the relationship shown in Fig. 7. Thus, experiments were carried out with chloride salts in the presence of potassium gradients

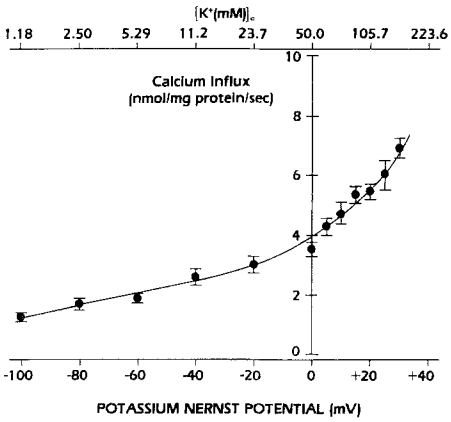

Fig. 7. Effect of membrane potential and/or potassium on the initial velocity of sodium-dependent calcium uptake. Aliquots of the sarcolemma preparation were incubated 15 to 18 hr at 5°C with 120 mM NaCl or LiCl (control), 50 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.4 for 37°C), 5 mM EGTA and 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}). After 5 freeze/thaw cycles the suspension was prewarmed to 37°C and added to reaction media containing final concentrations of 1.2 mM or 0 (control) NaCl, 1 mM $MgCl_2$, 0.099 μM valinomycin, 50 μM EGTA, 89.0 μM $CaCl_2$ with ^{45}Ca (39.2 μM free Ca^{2+}) and 10 mM Tris-Cl (pH 7.4). The reaction media also contained concentrations of KCl (upper abscissa) required to yield potassium Nernst potentials (lower abscissa; text Eq. 2) which ranged from −100 to +30 mV. Sufficient LiCl was included to maintain the ionic strength of the reaction media equal to that of the loading media. The reactions were terminated after 3 sec. The data (means; $n = 3$) were corrected for calcium movements in the absence of sodium

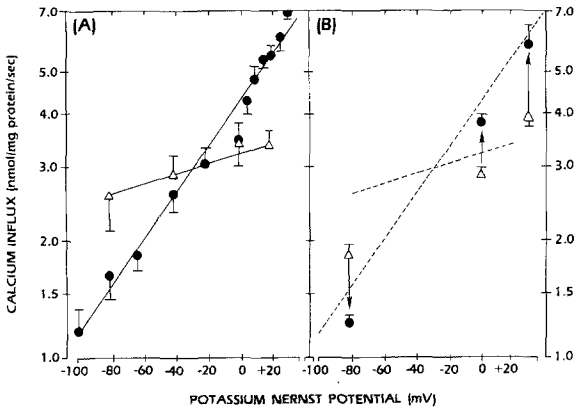

Fig. 8. Effect of thiocyanate and potassium gradients and valinomycin on the initial velocity of sodium-dependent calcium uptake (3-sec assays). *Panel A:* The solid circles are data presented in Fig. 7. The open triangles are the results of assays where the loading procedure was modified to yield membrane suspensions with various diffusion potentials for the thiocyanate anion while maintaining equivalent intravesicular and extravesicular potassium concentrations. For the latter, aliquots of the sarcolemma preparation were loaded for 15 to 18 hr at 5°C in 120 mM NaCl or 120 mM LiCl (control), 10 mM Tris-Cl (pH 7.4 for 37°C), 5 mM EGTA, 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}), 1 mM $MgCl_2$, and either 2.51, 11.19 or 50 mM KSCN. KCl was present, as needed, to maintain potassium at 50 mM. Following 5 freeze/thaw cycles and subsequent prewarming to 37°C, the suspensions were added to reaction media containing final concentrations of either 118.8 mM LiCl plus 1.2 mM NaCl or 120 mM LiCl (control), 1 mM $MgCl_2$, 50 μM EGTA, 89.0 μM $CaCl_2$ with ^{45}Ca (39.2 μM free Ca^{2+}), 10 mM Tris-Cl (pH 7.4), plus sufficient KSCN to yield thiocyanate Nernst potentials of −80, −40, 0 and +20 mV. Thus, thiocyanate in/out ratios were 2.51/49.53, 11.19/49.61, 50/50 and 50/23.65, respectively. KCl was present, as needed, to maintain extravesicular potassium at 50 mM. Lines were derived from regression analysis of the data. *Panel B:* Assays were carried out as described in Panel *A* except that both the thiocyanate and potassium Nernst potentials were equal. The assays were run in the absence (△) and presence (●) of 0.1 μM valinomycin. Values on abscissa are for fully developed Nernst potentials. The results (means ± SE; $n = 3$) in both panels were corrected for calcium movements in the absence of sodium. Broken lines are regression lines from Panel *A*

in the absence and presence of valinomycin. For potassium gradients from 50/1.18 mM (inside/out) to 50/153.7 mM, the initial rate of sodium-dependent calcium uptake was stimulated by 1.6-fold in the absence of valinomycin (Fig. 9*A*). Addition of valinomycin increased the stimulation to fivefold. [It is of interest that zero stimulation by valinomycin occurred at an E_K of −54.1 mV which equates to $[K^+]_o = 6.6$ mM (Fig. 9*B*)]. These results were consistent with some stimulation of the reaction by potassium in a manner that was independent of membrane potential. It should be noted, however, that potassium gradients in the absence of valinomycin

Table 3. Effects of potassium on sodium-dependent calcium uptake[a]

[K^+(mM)]	Rate of sodium-dependent Ca^{2+} uptake (nmol/mg/sec)
2	1.18
5	1.38 ± 0.02
10	1.23 ± 0.11
20	1.37 ± 0.07
50	1.64 ± 0.13
100	1.85 ± 0.05

[a] Aliquots of the sarcolemma preparation were exposed 15 to 18 hr at 5°C to media containing 45 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.4 at 37°C), 5 mM EGTA, 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}), and KCl and LiCl. KCl was varied as shown and sufficient LiCl was added to keep the sum of $K^+ + Li^+$ constant (100 mM). After 5 freeze/thaw cycles, the suspension was prewarmed to 37°C and then added into media containing final concentrations of 0.45 mM NaCl, 1 mM $MgCl_2$, 50 μM EGTA, 150.1 μM $CaCl_2$ with ^{45}Ca (100.2 μM free Ca^{2+}), 0.099 μM valinomycin, 10 mM Tris-Cl (pH 7.4), and KCl (as shown) with sufficient LiCl to maintain $K^+ + Li^+ = 100$ mM. Potassium in the reaction medium was always equal to potassium in the loading medium. The results (means ± SE, $n = 3$; or average, $n = 2$) were corrected for calcium movements in the absence of sodium.

were previously found to cause the development of some membrane potential (i.e., −30 to −40 mV at a maximum) in many but not all of the sarcolemma preparations (Bartschat et al., 1980; Schilling et al., 1984).

The next experiment tested whether a potassium gradient versus the presence of potassium *per se* was required for the stimulation shown in Fig. 7. Sodium-dependent calcium uptake was studied under conditions where $[K^+]_i = [K^+]_o$. The initial rate of uptake was stimulated 1.6-fold (Table 3) over the range of 2 to 100 mM potassium. The stimulation of calcium uptake by potassium in the absence of valinomycin (Fig. 9), therefore, did not require a potassium gradient. Thus, it appeared that 32 to 36% of the stimulation in Fig. 7 was due to the increase of potassium while the remainder was due to membrane potential. Furthermore, it is possible that extravesicular potassium caused the stimulation since intravesicular potassium was held constant (Figs. 7–9).

Discussion

Sodium-dependent calcium movements, promoted by sarcolemma preparations from heart, have generally been ascribed to a sodium/calcium exchange

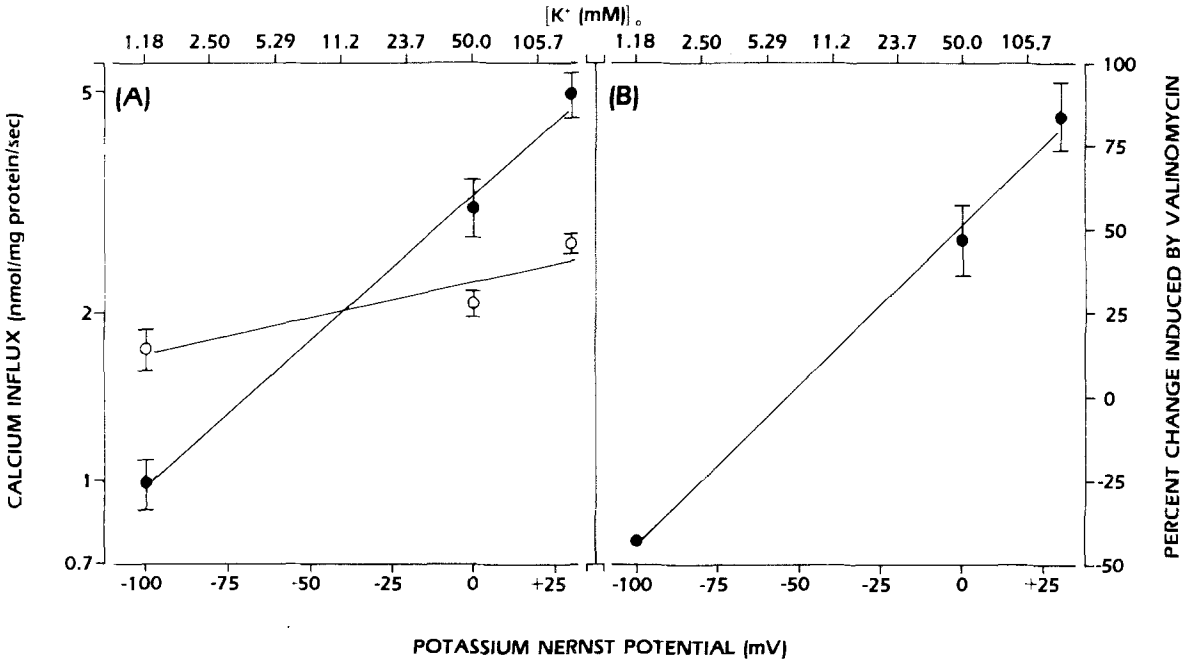

Fig. 9. Effects of potassium gradients and valinomycin on the initial velocity of sodium-dependent calcium uptake (3-sec assays). *Panel A:* Aliquots of the preparation were incubated 15 to 18 hr at 5°C with 120 mM NaCl or LiCl (control), 50 mM KCl, 1 mM $MgCl_2$, 10 mM Tris (pH 7.4 for 37°C), 5 mM EGTA and 24.9 μM $CaCl_2$ with ^{45}Ca (0.73 nM free Ca^{2+}). After 5 freeze/thaw cycles the suspension was prewarmed to 37°C and diluted 100-fold into media containing final concentrations of 118.8 mM LiCl plus 1.2 mM NaCl or 120 mM LiCl (control), 1 mM $MgCl_2$, 50 μM EGTA, 89.0 μM $CaCl_2$ with ^{45}Ca (39.2 μM free calcium) and 10 mM Tris-Cl (pH 7.4). The concentration of KCl was varied in the reaction media (upper abscissa) in the absence [(○) ethanol only] and presence (●) of 0.1 μM valinomycin in ethanol. Potassium Nernst potentials for these conditions are shown on the lower abscissa. LiCl was used to maintain the ionic strength of the extravesicular medium equal to that of the loading medium. The data were corrected for calcium movements in the absence of sodium (lithium-loaded vesicles) to yield sodium-dependent calcium movement (means ± SE, $n = 3$). *Panel B:* Percent change in sodium-dependent calcium uptake versus membrane potential. The data, in Panel *A*, were used to calculate the percent stimulation (0 and +30 mV) and inhibition (−100 mV) of sodium-dependent calcium influx by valinomycin

that moves net charge (Pitts, 1979; Reeves & Sutko, 1979; Bers et al., 1980; Caroni et al., 1980; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980; Reeves et al., 1980; Philipson et al., 1982). As stated above, net charge movement is thought to result from an exchange of more than two sodiums per calcium (Eq. 1, $r > 2$).

The purpose of this study was to characterize the effects of membrane potential on sodium-dependent calcium uptake. This required the use of initial velocity conditions. Examinations of uptake versus time showed that the sarcolemma preparation was unable to maintain initial velocity conditions for extended periods when calcium uptake was activated at 37°C by an outwardly directed sodium gradient. Over longer assay times (i.e., over minutes), the sodium gradient appeared to be lost ($t_{1/2}$ = 6.6 min). This result agreed with studies on ^{22}Na movements (Schilling et al., 1984). The rapid collapse of the sodium gradient was probably due to the large surface area-to-volume ratio of intact vesicles in the preparation since the permeability coefficient for sodium appears to be similar to that of intact myocardial cells. Nonetheless, when assay times were reduced to 10 sec or less, changes in the sodium gradient would contribute minimally to the deviation from initial velocity conditions.

Using rapid quench techniques, a component of sodium-dependent calcium uptake did appear to be linear from 2 to 10 sec. However, there was also the suggestion of a faster component of uptake (complete prior to 2 sec). A subsequent study from 0.07 to 5.6 sec was best fit by two exponential processes which further suggested that there were two components of sodium-dependent calcium uptake. The time profile of uptake could be converted to one component in two ways: (1) By dropping the assay temperature to below 20°C (Hungerford et al., 1984; *also see* Reeves et al., 1981); (2) by inclusion of EGTA in the loading medium and carrying out the reaction at 37°C in the presence of lower extravesicular free calcium (i.e., <109 μM; Fig. 6). Both maneuvers appeared to eliminate the fast component and the latter did so without any apparent effect on

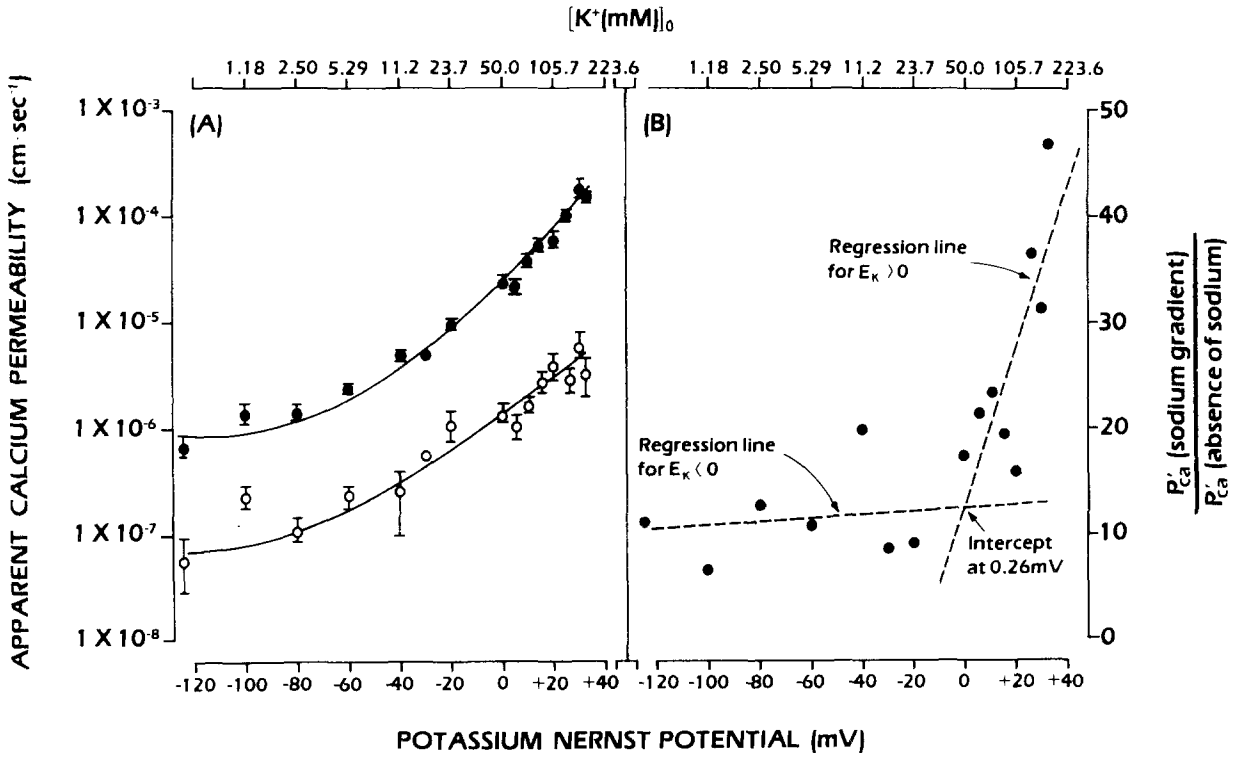

Fig. 10. Effect of membrane potential on the apparent calcium permeability coefficient. Assays were carried out as described in the legend to Fig. 7. Initial velocities of calcium influx (nmol/mg/sec) were converted to units of flux (pmol/cm²/sec) by assuming a vesicular volume of 7.5 μl/mg (Schilling et al., 1984) and an average radius of 50 nm (Van Alstyne et al., 1980). *Panel A:* The apparent calcium permeability coefficient (P'_{Ca}) at each value of E_K was calculated by Eq. (4) (*see* text) for fluxes determined in the presence of a sodium gradient (●) and in the absence of sodium (○). *Panel B:* Ratio of P'_{Ca} for the two conditions

the slow component. This was consistent with the conclusion that the two components were independent.[2]

When the preparation was loaded with EGTA, valinomycin stimulated the initial velocity of sodium-dependent calcium uptake for $[K^+]_i = [K^+]_o$. Since valinomycin would clamp E_K at 0 mV under these conditions (Eq. 2), the stimulation was consistent with the outward movement of net positive charge in a manner that was coupled to the inward movement of calcium. When E_K was varied from −100 to +30 mV, the sodium-dependent component of calcium uptake was enhanced. As stated above, this increase was about twofold between −100 and 0 mV and another twofold between 0 and +30 mV. While 32 to 36% of this increase could be ascribed to elevation of external potassium *per se* (i.e., not to changes in E_K), the remainder appeared to be related to the change in E_K. This profile is similar to that observed for squid axon (Allen & Baker, 1983). The discrepancy between the increase in sodium-dependent calcium uptake per unit change in E_K below and above 0 mV suggested that the mechanism of the reaction changed as E_K approached 0 mV. In an attempt to clarify this suggestion, the data for calcium uptake at various membrane potentials were used to calculate the apparent permeability coefficient for calcium, P'_{Ca}, by the constant field equation for net flux (Goldman, 1943; Hodgkin & Katz, 1949):

$$P'_{Ca} = J_{Ca} \frac{RT}{zFE_K} \frac{(1 - e^{zFE_K/RT})}{[Ca^{2+}]_i e^{zFE_K/RT} - [Ca^{2+}]_o} \quad (4)$$

where J_{Ca} = calcium flux (pmol/cm²/sec) and z = valence of calcium. Plots of P'_{Ca} versus E_K for calcium uptake in (1) the presence of an outwardly directed sodium gradient and in (2) the absence of sodium, revealed increases in P'_{Ca} as E_K was varied from −123 to +32 mV (Fig. 10*A*). The ratio of P'_{Ca} for the two cases, however, stayed essentially con-

[2] Initially, it was thought possible that the two components act in sequence. For example, the fast component could have reflected movement of calcium across the membrane while the slow component might have reflected a redistribution of intravesicular calcium between binding sites and the free state. Since EGTA eliminated the former without affecting the latter, this possibility now seems remote.

stant from −123 to about 0 mV but increased sharply as E_K was made more positive (Fig. 10*B*). Thus, while P'_{Ca} was 10- to 12-fold greater in the presence of a sodium gradient below 0 mV, the sodium gradient did not substantially change the relationship between P'_{Ca} and E_K until the latter was between 0 and +32 mV.

The parallelism between P'_{Ca} and E_K in the presence of a sodium gradient and in the absence of sodium for $E_K < 0$ could be interpreted in several ways. *First,* sodium-dependent calcium uptake did not involve the movement of net charge below 0 mV. This seemed unlikely since valinomycin affected sodium-dependent calcium movement when an outwardly directed, an inwardly directed or no potassium gradient was present (Fig. 9). *Second,* some other cation substituted for sodium in its absence. Potassium would seem to be an unlikely candidate since extravesicular potassium appeared to stimulate the reaction whereas extravesicular sodium is known to inhibit the reaction (Pitts, 1979; Reeves & Sutko, 1979; Bers et al., 1980; Reeves & Sutko, 1983). This left magnesium, Tris and protons as the potential cationic substitutes. The assays were carried out in the absence of concentration gradients for magnesium, Tris and presumably protons. However, this would not necessarily preclude an exchange of calcium for one of these cations since there was an inwardly directed calcium gradient. *Third,* calcium influx, while stimulated by intravesicular sodium, did not result from the coupled exchange of calcium for sodium across the membrane. In addition, internal sodium might have shifted maximum activation to higher potentials. This could explain the deviation from the parallelism at positive potentials. In this regard, Pitts (1979) reported quantitative evidence for the coupling of calcium and sodium movements (i.e., by comparing movements of calcium and sodium) promoted by an isolated sarcolemma preparation. *Fourth,* the parallelism was coincidental. Activation, for example, of a channel and stimulation of an exchange moving net positive charge outward could have similar profiles over a limited range of potentials. Further, activation of the exchange may have increased as E_K became more positive while the channel might approach a maximal level of activation. The latter could also explain the deviation in the parallelism at positive potentials.

Recently, a curve similar to those in Fig. 10*A* was reported for the sarcolemma preparation in the absence of sodium (Schilling & Lindenmayer, 1984). The major difference was that the curve, at comparable values of E_K, was shifted upwards (i.e., to higher values of P'_{Ca}) by about two orders of magnitude over the curve for zero sodium in Fig. 10*A*. This difference may be explained by the heat-lability of voltage-sensitive calcium influx measured in the absence of sodium. Heating the preparation between 30 and 37°C for 15 min substantially depressed the reaction (Schilling & Lindenmayer, 1984). For the assays carried out in the present study, the loaded preparation was heated at 37°C for times greater than 5 min prior to start of the reaction. Preheating the preparation, however, did not affect the initial velocity of sodium-dependent calcium uptake (*unpublished observations*). These considerations favor the conclusion that the parallelism between the curves (Fig. 10*A*) for $E_K < 0$ was coincidental. If so, the pathway involved in sodium-dependent calcium influx was presumably separate and independent of the pathway responsible for calcium influx in the absence of sodium. Some caution must be applied to this conclusion, however, since (1) calcium influx measured at zero sodium in the two studies employed somewhat different conditions (e.g., vastly different extravesicular free calcium concentrations) and (2) the reaction reported by Schilling and Lindenmayer (1984) was inhibited by extravesicular sodium.

The mechanism of the sharp increase in sodium-dependent calcium influx at positive potentials is unknown. Previous studies on the slow component of uptake (i.e., the component remaining in the presence of EGTA) suggested that the apparent affinity for extravesicular calcium increased about fivefold (i.e., the $K_{0.5}$ decreased from 43.1 to 8.8 μM) as E_K was raised from −67 to 0 mV with little change in the maximal rate of influx (Hungerford et al., 1984). Since the assays for Figs. 7 and 10 were carried out in 39.2 μM extravesicular calcium, sites binding extravesicular calcium would move from 48 to 82% saturation as E_K increased from −67 to 0 mV. Thus, further increases in affinity for extravesicular calcium as E_K became positive would not explain the sharp increase in sodium-dependent calcium influx. Using a sarcolemma vesicle preparation isolated from 1-day-old chick hearts, Wakabayashi and Goshima (1981) also concluded that the stimulation of sodium-dependent calcium uptake by positive membrane potentials was not due to the slight changes in $K_{0.5}$ that they observed. Rather, they suggested that the stimulation of influx was due to an increase in the maximal rate.

If the pathways for sodium-dependent calcium influx and for calcium influx in the absence of sodium were, in fact, independent, the sharp breaks upward in Figs. 7 and 10*B* may have been kinetic manifestations that are linked, presumably through Haldane relationships (e.g. *see* Segel, 1975), to the equilibrium equation for electrogenic sodium/calcium exchange (Eq. 1; $r > 2$). Eq. (1) with $r \geqq 3$

requires a greater absolute increase in the equilibrium level of internal calcium when the membrane potential is moved from 0 to +30 mV compared to when the potential is moved from −100 to 0 mV. This is true for the assay concentrations used here or for physiological concentrations of external and internal sodium and external calcium.

The extent to which sodium-dependent calcium influx contributes to the rise in myocardial intracellular free calcium upon membrane depolarization remains unknown. The sharp increase at positive potentials reported herein, however, raises the possibility that this contribution is markedly enhanced during the portion of the cardiac action potential which resides in the positive region. It is interesting to note that it is in this region that the inward calcium current (i.e., thought to reflect calcium influx through calcium channels) may reach a plateau or begin to decrease in cardiac tissue (e.g., Lee & Tsien, 1982; Josephson et al., 1984).

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