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Voltage-Sensitive Calcium Flux Promoted by Vesicles in an Isolated Cardiac Sarcolemma Preparation

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Summary. The effect of membrane potential on the vesicular uptake of calcium in an isolated cardiac sarcolemma preparation from canine ventricle was evaluated. Membrane potentials were developed by the establishment of potassium gradients across the vesicular membranes. In the presence of valinomycin, the fluorescence changes of the voltage sensitive dye, diS-C_3-(5) were consistent with the development of potassium equilibrium potentials. Using EGTA to remove endogenous calcium from the preparation and to maintain a low intravesicular calcium concentration over time, the uptake of calcium was linear from 5 to 100 sec, in the absence of sodium, at both −98 and −1 mV. The rate of calcium uptake (calcium influx) was approximately twofold greater at −1 mV than at −98 mV, and prepolarization of the membrane potential to −98 mV did not enhance calcium influx upon subsequent depolarization to −1 mV. Hence, calcium influx was voltage-sensitive but not depolarization-induced and did not inactivate with time. Furthermore, the calcium influx was not inhibited by the organic calcium antagonists, which suggests that this flux did not occur via the transient calcium channel. Evaluation of calcium influx over a wide range of membrane potentials produced a profile consistent with the hypothesis that calcium entered the vesicles through the pathway responsible for the persistent inward current observed in voltage-clamped isolated myocytes. A model was proposed to account for these results.

Key Words calcium channel · sarcolemma · membrane potential · heart · sodium-calcium competition · calcium antagonists

Introduction

Although the importance of extracellular calcium to the excitation-contraction sequence of the mammalian heart is widely recognized, the mechanisms or pathways by which calcium enters the cell during each cardiac cycle are not well understood. It now seems likely that there are at least three pathways for calcium entry into the myocardial cell which activate upon membrane depolarization. *First,* a voltage-sensitive channel, which activates with a time to peak current of 3-10 msec and then decays or shuts off completely within 100 msec, has been observed in voltage-clamp studies of isolated myocytes from guinea pig (Lee & Tsien, 1982), bovine ventricle (Isenberg & Klöckner, 1982*b*), and bullfrog atria (Hume & Giles, 1983) and in cesium-loaded calf Purkinje fibers (Marban & Tsien, 1982). The amplitude of this transient current suggests that enough calcium may enter the myocardial cell through this pathway to account for full activation of the contractile proteins. As pointed out by Hume and Giles (1983), this inward current cannot be responsible for maintenance of the action potential plateau, since the plateau in bullfrog is generally 300–500 msec in duration. Likewise, in isolated ventricular myocytes, the fast transient inward calcium current was found to inactivate well before the termination of the plateau, which lasted on the average 400 msec (Isenberg & Klöckner, 1982*a,b*). *Second,* a persistent inward current which lacks time-dependent inactivation has been observed in voltage-clamped, internally perfused bullfrog atrial cells (Hume & Giles, 1983). A component of the slow inward current, I_{si}, which lacks time-dependent inactivation has also been observed in mammalian cardiac tissue (*see* Discussion for refs.). This inward current, which activates at approximately −50 mV, may contribute significantly to the maintenance of the plateau potential and may represent a second type of voltage-sensitive calcium channel. *Third,* electrogenic sodium/calcium exchange may operate in a reverse mode to transport one calcium into the cell in exchange for more than two sodium ions, i.e., net outward current (Mullins, 1981). With a coupling ratio of 4 (4 Na per Ca), the reversal potential for the exchange would be approximately −40 mV. Thus, as Mullins suggests, the sodium/calcium exchanger could function to deliver cal-

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cium to the cytosol during systole and to remove calcium during diastole.

As summarized by Winegrad (1979), three different types of mechanical responses have been observed in mammalian heart upon constant depolarizations for different lengths of time: (i) a process triggered by short depolarizations (<40 msec), (ii) a process triggered by depolarizations of moderate duration (40–200 msec), and (iii) a process triggered by long depolarizations (>200 msec). The role that each of the calcium entry pathways may play in the mechanical responses elicited by membrane depolarization is not known but it seems likely that each will play a specific role in the overall contractile response observed.

The physical nature of these pathways for calcium entry has not been elucidated. Although it has been suggested that calcium may cross the membrane through aqueous channels or pores and that the sodium/calcium exchanger is a carrier, confirmation of these proposals awaits more complete biochemical characterization. Towards this ultimate end, isolation schemes have been developed to yield highly enriched cardiac sarcolemma preparations (Jones et al., 1979; Caroni, Reinlib & Carafoli, 1980; Philipson, Bers & Nishimoto, 1980; Van Alstyne et al., 1980). These preparations exhibit sodium-dependent calcium movements, consistent with the presence of sodium/calcium exchange, as first demonstrated by Reeves and Sutko (1979) and by Pitts (1979). To date, however, there has been no demonstration of a calcium flux with appropriate voltage-sensitivity, ion selectivity, or pharmacological characteristics necessary to classify the flux as either the transient calcium channel or the persistent inward current. Furthermore, initial attempts to demonstrate a calcium channel flux in isolated membrane vesicles, carried out in the presence of sodium (Bartschat, Cyr & Lindemayer, 1980; Rinaldi, LePeuch & Demaille, 1981), are complicated by the presence of the electrogenic sodium/calcium exchanger.

In the present study we have used radiotracer techniques to evaluate the voltage-sensitive movements of calcium across the membrane of vesicles in a highly enriched sarcolemma preparation from canine ventricle. With EGTA as an intravesicular calcium "sink," and a low extravesicular calcium concentration of high specific radioactivity, we have been able to demonstrate a sodium-independent calcium flux, which does not inactivate with time but which has voltage-sensitive characteristics similar to those previously reported for calcium channel current in cardiac tissue.

Materials and Methods

Isolation of the Sarcolemma Preparation

A vesicular membrane preparation, highly enriched in sarcolemma markers and depurified to some extent with respect to sarcoplasmic reticulum and mitochondrial markers, was isolated from canine ventricle as previously described (Van Alstyne et al., 1980). This preparation consists of membrane vesicles (75–80% sealed right side out; 20–25% relatively permeable and/or inside-out vesicles) which are osmotically responsive and which exhibit activities consistent with the presence of the sodium/calcium exchange, calcium/calcium exchange (Bartschat & Lindenmayer, 1980), ouabain-sensitive sodium, potassium-ATPase (Wellsmith & Lindenmayer, 1980), and beta-receptor-coupled adenylate cyclase (Van Alstyne et al., 1980). In addition, transmembrane potentials can be developed by outward directed potassium gradients in the presence of the potassium-selective ionophore, valinomycin (Bartschat et al., 1980).

Protocol for Measurement of Membrane Potential

Sarcolemma preparations were "loaded" by incubation of the preparation at 4°C with a solution containing 50 mM KCl, 152 mM choline-Cl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM Tris-Cl, pH 7.4 (for 37°C), for 15–18 hr. In order to uniformly load the vesicles with EGTA, the tube containing the suspension was subsequently frozen in a dry ice/acetone bath and then immediately thawed by gentle agitation in a water bath at room temperature. The freeze-thaw procedure was repeated a total of five times. In the presence of high ionic strength, this procedure appears to allow relatively large, charged molecules (e.g., EGTA, ATP) to equilibrate with the intravesicular space without any apparent change in the permeability characteristics of the preparation (Fig. 1). The loaded suspension was placed in solutions of different potassium concentrations to establish potassium diffusion potentials in the presence of valinomycin. Membrane potential was monitored by changes in fluorescence of the dye, diS-C_3-(5), as described previously (Bartschat et al., 1980; Schilling, Schuil, Bagwell & Lindenmayer, 1984).

Protocol of Measurement of Calcium Influx

Aliquots of the sarcolemma preparation, loaded as described above, were added to reaction media containing ^{45}Ca. The free calcium concentration was calculated by assuming that the Ca · EGTA dissociation constant = 0.05 μM (calculated from equations of Harafuji and Ogawo, 1980). The uptake of calcium was terminated at the times indicated by the addition of an ice-cold stopping solution (Bartschat & Lindenmayer, 1980), which contained 225 mM KCl, 1 mM $CaCl_2$, 0.5 mM $LaCl_3$ and 10 mM Tris-Cl, pH 7.4. The samples were filtered through Millipore filters (type HA; 0.45 μm pore size) on a Hoeffer filtration apparatus and washed ten times with 5-ml aliquots of the stopping solution. The radioactivity trapped on the filter was then determined by standard liquid scintillation technique and corrected for back-

ground counts by filtration and washing of the reaction medium with protein added after the stopping solution.

Results

Evaluation of the effects of membrane voltage on calcium uptake by the sarcolemma preparation must consider a number of potential complications. *First,* previous attempts to discover the manifestation of a calcium channel in this preparation were complicated by the presence of a voltage-sensitive, sodium-dependent calcium movement which, at least in part, could reflect electrogenic sodium/calcium exchange (Bartschat et al., 1980). With one exception (*see below*), the experiments in the present study were carried out in the absence of sodium. *Second,* measurement of endogenous calcium in the sarcolemma preparation has suggested an intravesicular calcium concentration of 194 μM (Bartschat & Lindenmayer, 1980). Inhibition of the calcium channel by internal calcium in a variety of excitable tissues appears to occur at calcium concentrations less than this value (Brehm & Eckert, 1978; Brehm, Eckert & Tillotson, 1980; for review, Hagiwara and Byerly, 1981). In addition, endogenous calcium associated with the preparation appears to be exchangeable through a calcium/calcium exchange mechanism (Bartschat & Lindenmayer, 1980) which would complicate the interpretation of ^{45}Ca movements anticipated for the present study. *Third,* significant back flux of radiotracer calcium may occur as the intravesicular calcium concentration increases, producing nonlinearity in calcium uptake with time (Bartschat & Lindenmayer, 1980). In order to eliminate these problems, we used EGTA to remove endogenous calcium and to maintain a low intravesicular free calcium concentration over the time course of the experiments. In addition, we used low concentrations of external free calcium (4–40 nM) to minimize background calcium flux. Radioactive calcium (^{45}Ca) of high specific activity (approximately 2500 cpm/pmol calcium) was used to maximize detection of voltage-dependent calcium flux at the low concentrations employed.

In the presence of the potassium-selective ionophore, valinomycin, membrane potentials that approached the potassium equilibrium potential could be established across the membrane of the isolated cardiac sarcolemma vesicle (Schilling et al., 1984). In the present study, the first experiment was designed to evaluate the capacity of the isolated cardiac sarcolemma vesicles to develop membrane potentials under the conditions which were to be employed for the study of voltage-sensitive calcium uptake. In order to estimate the magnitude of the membrane potential, we have employed the fluorescent probe, diS-C_3-(5). The percent change in fluorescence varied in a linear manner with the logarithm of the external potassium concentration (Fig. 1). The slope of the line relating external potassium

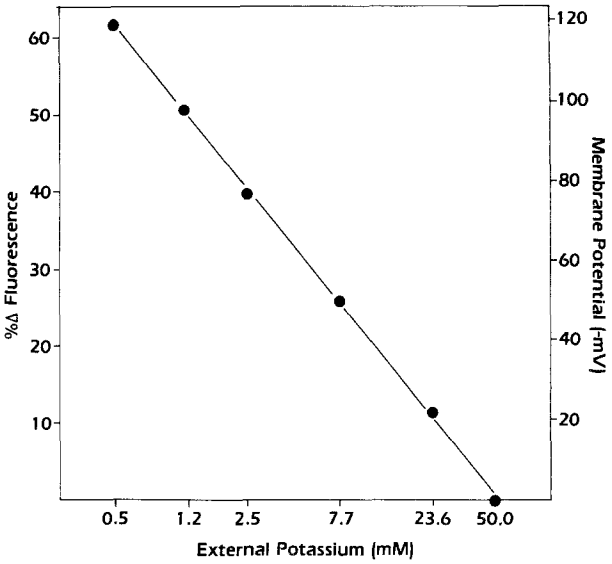

Fig. 1. Effects of variation in external potassium concentration on membrane potential measured by the fluorescence of the dye, diS-C_3-(5). An aliquot of the sarcolemma-enriched preparation was incubated at 4°C in a loading medium containing 50 mM KCl, 152 mM choline-Cl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM Tris-Cl (adjusted to pH 7.4 for 37°C). After 15–18 hr, this suspension was subjected to five freeze-thaw cycles as described in the text. Aliquots of the loaded suspension were added to reaction media in a quartz cuvette pre-equilibrated to 37°C in an Aminco-Bowman spectrophotofluorometer. The reaction media contained various concentrations of KCl (external potassium *plus* choline was kept constant at 208 mM), 100 nM valinomycin, 0.125 mM EGTA, 0.025 mM $MgCl_2$, and 10 mM Tris-Cl, pH 7.4. The dye was added immediately (1 μM final concentration) and the fluorescence recorded (excitation and emission wavelengths were 622 and 670 nm, respectively). Fluorescence at each potassium concentration was normalized (as the percent decrease in fluorescence, $\%\Delta F$) with respect to the fluorescence at zero membrane potential (equal intra- and extravesicular potassium concentrations). The $\%\Delta F$ was then equated to membrane potential for each external potassium concentration by the following equation:

$$\%\Delta F \sim E_m = E_K = 61.5 \log \frac{[K^+]_o}{[K^+]_i}$$

and the mean value for $E_K/\%\Delta F$ was used to calibrate the membrane potential scale. Data shown represents the average of experiments performed on two preparations, each in duplicate

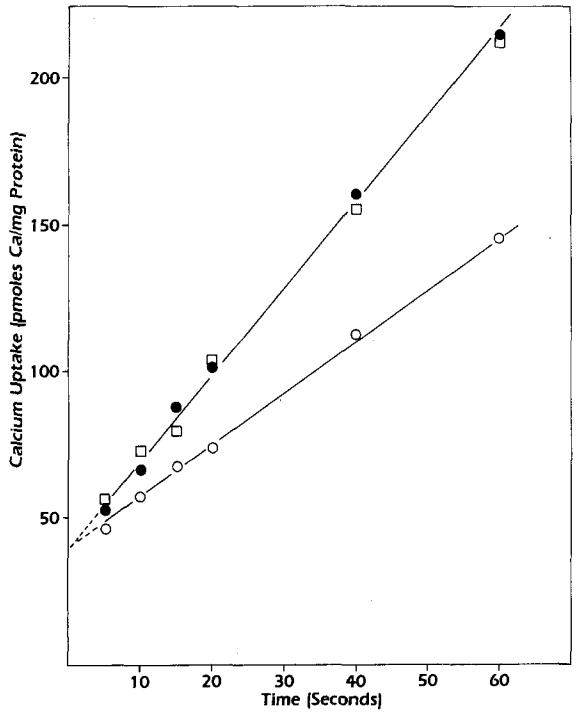

Fig. 2. Time course of calcium uptake by the sarcolemma-enriched preparation. The preparations were loaded as described in the legend of Fig. 1. Aliquots of the loaded sarcolemma suspension (10 μl; 20–50 μg protein) were added to 390 μl of a polarization media at 37°C which contained (final concentrations) 100 nM valinomycin, 0.125 mM EGTA, 0.025 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, and either 1.25 mM KCl plus 204 mM choline-Cl (○ and ●: $E_K = -98$ mV) or 48.25 mM KCl plus 157 mM choline-Cl (□: $E_K = -1$ mV). After 15 sec (time zero), 1.6 ml of a solution was added to the reaction medium to yield final concentrations of 0.025 mM EGTA, 0.005 mM $MgCl_2$, 100 nM valinomycin, 10 mM Tris-Cl, pH 7.4, 3.65 μM $CaCl_2$ (total) with ^{45}Ca (4 μCi/ml) and either 48.25 mM KCl plus 157 mM choline-Cl (● and □: $E_K = -1$ mV) or 1.25 mM KCl plus 204 mM choline-Cl (○: $E_K = -98$ mV). Uptake of calcium was terminated at the times indicated by addition of 5 ml of ice-cold stopping solution containing 225 mM, KCl, 1 mM $CaCl_2$, 0.5 mM $LaCl_3$, and 10 mM Tris-Cl, pH 7.4. The samples were then filtered through a Millipore filter (type HA; 0.45 μm pore size) and washed ten times with 5-ml aliquots of the stopping solution. Radioactivity trapped on the filter was then determined by liquid scintillation technique and corrected for background counts by filtration and washing of the reaction medium with protein added after the stopping solution. The results shown are from one of three experiments performed in duplicate.

to the calculated (legend to Fig. 1) potential was 59.1 mV per 10-fold change in potassium concentration with an x-intercept of 52.6 mM potassium. Hence, an excellent correlation between membrane potential and the potassium equilibrium potential was obtained in the presence of valinomycin.

The effect of voltage on the time-dependent association of calcium with the sarcolemma preparation was first determined at two membrane potentials (values similar to resting and plateau levels). The uptake of calcium with time was linear from 5 to 60 sec at a membrane potential of either −98 or −1 mV (Fig. 2) but the rate of calcium uptake at −1 mV was twice that observed at −98 mV. Thus, calcium uptake with time appeared to be voltage sensitive. This result is similar to the findings of Nachshen and Blaustein (1980) for the effect of membrane potential on calcium uptake by isolated synaptosomal preparations. Those authors, however, also observed a depolarization-induced component of calcium uptake that was apparently complete by 1 sec. In order to determine whether a depolarization-induced component of calcium uptake was promoted by the sarcolemma preparation, the membrane potential was hyperpolarized to −98 mV for 15 sec and then depolarized to −1 mV. The rate of calcium uptake after depolarization was the same as for the −1 mV condition without prior polarization (Fig. 2). This suggested that while calcium uptake was voltage-sensitive, it was not induced by depolarization (i.e., the sarcolemma preparation did not manifest a component of calcium flux that recovered from inactivation with time when held at negative potentials). Further, a component of calcium influx, which inactivated with time after a step-depolarization from negative potentials, was not observed.

One reason for the lack of a depolarization-induced component may have been that the time of hyperpolarization prior to depolarization was inadequate. To test this possibility, the vesicles were held at −98 mV for 45 sec before depolarization during which time the vesicles were allowed to accumulate calcium. We anticipated one of two possible results: (a) The rate of calcium uptake would simply increase (as in Fig. 2); or (b) there would be a very rapid increase in vesicular calcium (e.g., from the −98 to the −1 mV line at 45 sec in Fig. 2) which would be followed by a new rate of calcium uptake. The results of this experiment showed that depolarization after 45 sec produced an increased rate of calcium uptake without an "instantaneous" movement of calcium (Fig. 3). These data suggested, therefore, that this pathway for calcium uptake is voltage-sensitive, does not inactivate with time, and is not enhanced by prepolarization to −98 mV. Furthermore, the uptake was linear over this time frame, which suggested that significant backflux of radiolabel did not occur and that internal EGTA was an effective sink for intravesicular calcium, maintaining the free calcium concentration at a low level.

The purpose of the next experiment was twofold. *First,* if EGTA was effectively maintaining the intravesicular calcium concentration low, then ad-

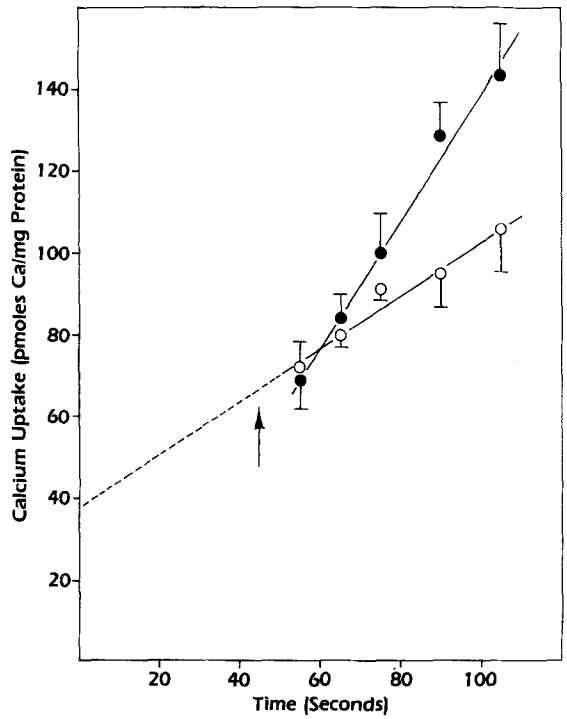

Fig. 3. Time course of calcium uptake by the sarcolemma preparation: 45 sec polarization. The reaction was initiated by the addition of 10 μl of the loaded suspension to 390 μl of a polarization medium at 37°C containing (final concentrations) 100 nM valinomycin, 1.25 mM KCl, 204 mM choline-Cl, 0.125 mM EGTA, 0.025 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, and 13.2 μM $CaCl_2$ (total) plus ^{45}Ca (E_K = −98 mV). After 45 sec (at the arrow), 1.6 ml of a solution was added to the reaction medium to yield final concentrations of 0.025 mM EGTA, 0.005 mM $MgCl_2$, 100 nM valinomycin, 10 mM Tris-Cl, pH 7.4, 3.65 μM $CaCl_2$ (total) with ^{45}Ca and either 48.25 mM KCl plus 157 mM choline-Cl (●: E_K = −1 mV) or 1.25 mM KCl plus 204 mM choline-Cl (○: E_K = −98 mV). The reaction was stopped at the indicated times, filtered, washed, and the radioactivity associated with the filters was measured. Each point is the mean ± SEM of experiments performed on three preparations, each in duplicate

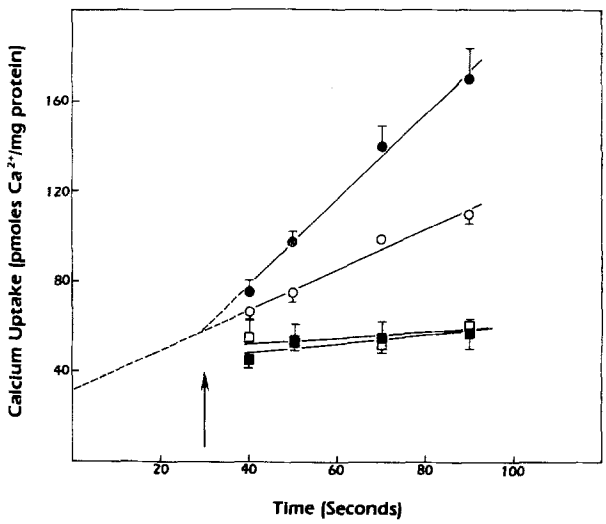

Fig. 4. Effect of sodium on the voltage-dependent uptake of calcium by the sarcolemma-enriched preparation. The reaction was initiated by the addition of 10 μl of the loaded suspension to 390 μl of a polarization medium at 37°C containing (final concentrations) 100 nM valinomycin, 1.25 mM KCl, 204 mM choline-Cl, 0.125 mM EGTA, 0.025 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, and 13.2 μM $CaCl_2$ (total) plus ^{45}Ca (E_K = −98 mV). After 30 sec (at the arrow), 1.6 ml of a solution was added to the reaction medium to yield final concentrations of 0.025 mM EGTA, 0.005 mM $MgCl_2$, 100 nM valinomycin, 10 mM Tris-Cl, pH 7.4, 3.65 μM $CaCl_2$ (total) with ^{45}Ca and either 48.25 mM KCl plus 157 mM choline-Cl (●: E_K = −1 mV) or 1.25 mM KCl plus 204 mM choline-Cl (○: E_K = −98 mV), 48.25 mM KCl plus 40.8 mM choline-Cl and 116.2 NaCl (■: E_K = −1 mV), or 1.25 mM KCl plus 40.8 mM choline-Cl and 163.2 NaCl (□: E_K = −98 mV). The reaction was stopped at the indicated times, filtered, washed, and the radioactivity associated with the filters was measured. Each point is the mean ± SEM of experiments performed on three preparations, each in duplicate

dition of external sodium sometime after calcium uptake has taken place should have little effect on calcium already associated with the vesicles. (This is because calcium in the vesicles should be well below the $K_{0.5}$ for electrogenic sodium/calcium exchange.) *Second,* it is thought that sodium can pass through the calcium channel (Reuter & Scholz, 1977*a*). Therefore, addition of external sodium should, in effect, compete with calcium for entry; i.e., calcium influx should be inhibited in the presence of sodium. Both results are demonstrated in Fig. 4. Vesicles were allowed to accumulate calcium for 30 sec, at which time they were diluted with either a choline-containing solution or a sodium-containing solution. Sodium blocked the uptake of calcium at both −98 and 0 mV. Note also that sodium did not induce an efflux of calcium from the vesicles as would be expected if sodium/calcium exchange were operative. This result suggested that either the calcium taken up in these experiments was into vesicles that do not contain the sodium/calcium exchanger or, the more likely possibility, that EGTA was acting as an effective calcium sink and the intravesicular calcium concentration was well below the $K_{0.5}$ for activation of the exchange reaction. The latter explanation was further supported by the fact that the profile in the absence of sodium (Fig. 4) continued to be linear from 40 to 90 sec, again suggesting that this flux was unidirectional (i.e., no radiotracer backflux).

Note that extrapolation of the calcium uptake between 10 and 60 sec (Fig. 2) and 45–100 sec (Figs. 3 and 4) to time zero did not pass through the origin. This result was variable from preparation to preparation, ranging from 0 to 75 pmol/mg. However,

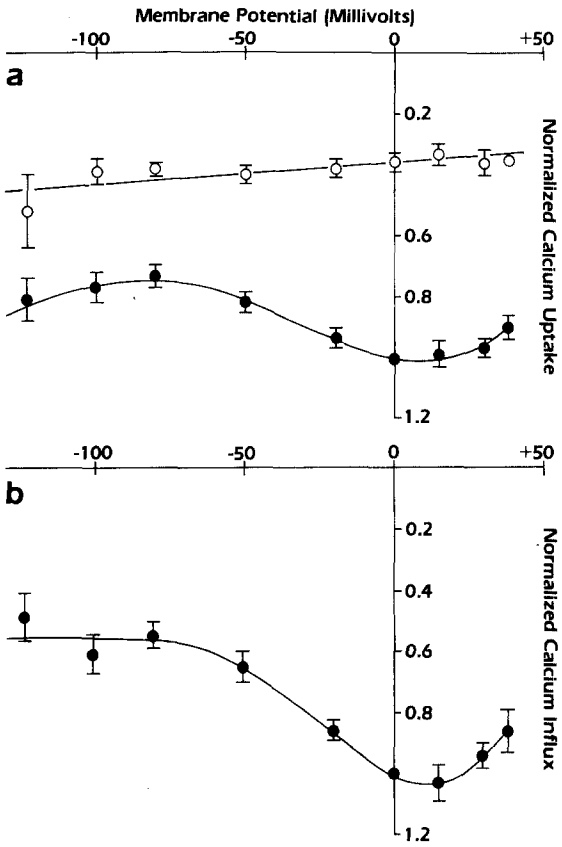

Fig. 5. Effect of membrane potential on calcium uptake and influx in sarcolemma vesicles. An aliquot of sarcolemma preparation was loaded as described in the legend of Fig. 1. The reactions were initiated by adding aliquots of the loaded suspension to media containing various concentrations of KCl (i.e., to yield different membrane potentials; external potassium *plus* choline was kept constant at 208 mM), 100 nM valinomycin, 0.050 mM EGTA, 0.010 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, plus 4 μM $CaCl_2$ (total) with ^{45}Ca. The reactions were stopped at 10 sec (○, panel *a*) or 60 sec (●, panel *a*) by addition of 5 ml of the stopping solution. After filtration and washing, the radioactivity associated with the filters was measured as described in the legend of Fig. 2. Calcium uptake at 10 sec was subtracted from the amount accumulated at 60 sec and divided by 50 sec for conversion to influx units (pmol/mg protein/sec; panel *b*). Each point represents the mean ± SEM of experiments performed on six preparations, each in duplicate. All data have been normalized with respect to values obtained at zero membrane potential: 96.8 ± 10.6 pmol Ca/mg protein (uptake at 60 sec, panel *a*) and 1.25 ± 0.16 pmol Ca/mg/sec (influx, panel *b*)

overnight incubation at 5°C with 5 mM ATP resulted in ~ fivefold stimulation of the extrapolated time zero point (data not shown). Little effect of ATP was observed with acute or short incubations (<2 hr), and ATP was without apparent effect on the subsequent rate of calcium uptake between 10 and 60 sec.

The effect of membrane potential on calcium influx was determined over a wide range of voltages (Fig. 5). Calcium uptake at 10 sec (Fig. 5*a*, open circles) decreased slightly as membrane potential was varied from −123 to +38 mV. Calcium uptake at 60 sec (Fig. 5*a*, filled circles) was not linear with membrane potential. The nonlinearity became even more pronounced when calcium uptake was converted to influx (i.e., value at 60 sec minus value at 10 sec; Fig. 5*b*). Recently, Lee and Tsien (1982), using suction pipette techniques, reported voltage-dependent profiles for calcium currents recorded in isolated guinea pig heart cells. The general shape of the calcium influx curve in sarcolemma vesicles (Fig. 5*b*) was similar to that observed for current in the heart cells. Appreciable calcium influx occurred over the range of −123 to −60 mV although it appeared to be voltage-insensitive. Influx began to increase at about −50 mV, peaked between +10 and +15 mV, and then declined at more positive voltages; between −80 and +15 mV calcium influx increased 1.9-fold.

The voltage sensitivity of the calcium influx shown in Fig. 5*b* suggests that the calcium permeability coefficient (P_{Ca}) changes as a function of membrane potential. The relationship between the logarithm of P_{Ca} and membrane potential is shown in Fig. 6. As membrane potential increased from −40 to +40 mV, a linear 10-fold increase in P_{Ca} occurred per 50 mV change in membrane potential. At membrane potentials more negative than −40 mV, the change in P_{Ca} with membrane potential decreased and P_{Ca} may approach some constant, low value at more negative potentials. The 100-fold change in calcium permeability between −123 and +38 mV underlines the dramatic influence of membrane potential on calcium flux into the membrane vesicles.

It should be noted that the permeability constant for calcium did not reach a maximal, limiting value as the membrane potential was increased up to +38 mV. The voltage range over which these experiments could be performed was governed primarily by the internal potassium concentration. Extention of the voltage range to more positive potentials would require dropping inside potassium below 50 mM. Using valinomycin to establish the potential, we found that concentrations below 50 mM did not appear to allow adequate control of the membrane potential. An internal potassium concentration of 50 mM was the lowest at which we have obtained data consistent with the establishment of Nernst potentials with minimal dissipation of the potential with time. This limited the upper limit of potentials to +38 mV (at physiological ionic strength). However, over the voltage range studied, the permeability change as a function of membrane

potential was as expected from previous work. Measurement of tail currents for the transient calcium channel shows a maximum only above +60 mV (Fenwick, Marty & Neher, 1982). Over the potential range of −20 to +60 mV, tail current amplitude was found to increase dramatically in a fashion similar to that shown in Fig. 6.

An alternate interpretation of the results presented above was that calcium flux was a function of extravesicular potassium instead of membrane potential since the former was used to set the latter equal to the potassium Nernst potential. Reuter and Scholz (1977*a*) and Lee and Tsien (1982) suggested that potassium can pass through the calcium channel. Several considerations, however, seemed to eliminate the possibility that the flux of calcium was linked to the flux of potassium. First, membrane potential was always set equal to E_K. Hence no net flux of potassium should have occurred through a channel. Second, over the voltage range where calcium influx increased (−50 to 0 mV), there existed and outwardly directed potassium concentration gradient. Over the positive voltage range, where calcium influx decreased, there was an inwardly directed potassium concentration gradient. Together these results rule out the possibility that calcium was being "swept" in the direction of potassium flux. Furthermore, the decrease in calcium influx in the positive and negative voltage ranges was not consistent with an electrogenic, coupled transport of calcium and potassium in opposite directions. An electrogenic process, carrying net outward charge, would be characterized by an increase in calcium influx at more positive potentials. A process, carrying net inward charge, would be characterized by an increase in calcium influx at more negative potentials. Neither profile was observed.

The calcium influx shown in Figs. 2–5 was not inhibited by tetrodotoxin, verapamil, nitrendipine or nifedipine, so it seems unlikely that this calcium flux was through open sodium channels or via the transient calcium channel. It is possible that the flux occurred through a channel which was modified by the freeze-thaw treatments (required to load the vesicles with EGTA) or by EGTA *per se*. Other possibilities are discussed below. Interestingly, depolarization-induced calcium flux in synaptosomal preparations was only partially inhibited by verapamil and unaffected by nifedipine (Nachshen & Blaustein, 1979). The effects of inorganic calcium antagonists (e.g., La^{3+}, Mn^{3+}) are difficult to access in the presence of calcium · EGTA buffers. Magnesium, however, which has minimal effects on free calcium concentration in the buffer, was found to inhibit this calcium influx. Finally we found that this calcium flux is temperature labile; preincubation of the loaded preparation between 30 and 37°C for 15 min inhibits this voltage-sensitive flux.

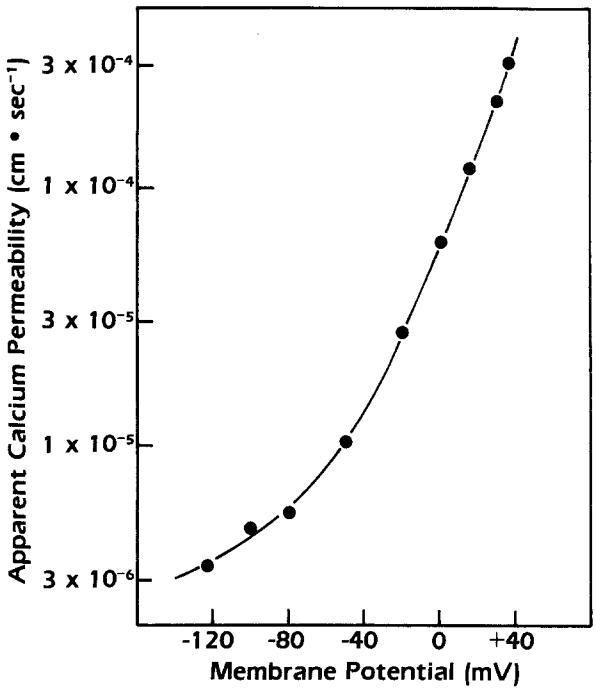

Fig. 6. Effect of membrane potential on the apparent calcium permeability coefficient. The mean values of calcium influx in pmol Ca/mg/sec from Fig. 5, panel *b* were converted to pmol Ca/cm²/sec by assuming a vesicular volume of 7.5 μl/mg (Schilling et al., 1984) and an average radius of 50 nm (Van Alstyne et al., 1980). The calcium permeability coefficient (P_{Ca}) at each membrane potential (E_m) was calculated by the following equation for unidirectional flux:

$$P_{Ca} = J_{Ca} \frac{RT}{zE_mF} \frac{1 - \exp(zE_mF/RT)}{[Ca]_o}$$

where J_{Ca} is the measured calcium influx, z is the ionic valance for calcium, $[Ca]_o$ is the extravesicular free calcium concentration (4.5 nM), and R, T, and F have their usual meaning. Curve was fit by eye

Discussion

In the present study, we measured the manifestation of a voltage-sensitive calcium pathway in an isolated cardiac sarcolemma preparation from canine ventricle. In the presence of intravesicular EGTA and a low extravesicular calcium concentration, the uptake of calcium by the vesicles was linear with time for at least 2 min. The influx of calcium upon membrane depolarization was not affected by prior polarization of the membrane to negative potentials. Hence, while the influx was

voltage-sensitive it lacked any apparent voltage- or time-dependent inactivation.

Lack of Inactivation of Calcium Influx

The calcium influx demonstrated in the present study appears to have voltage characteristics similar to the persistent inward current observed by Hume and Giles (1983) in bullfrog atrial cells. A component of inward current which either lacks inactivation or has a very long time constant for inactivation has been reported previously in Purkinje fibers (Gibbons & Fozzard, 1975; Kass, Siegelbaum & Tsien, 1976; Kass & Scheuer, 1982) and in nerve (Eckert & Lux, 1975; Schwindt & Crill, 1980; Byerly & Hagiwara, 1982; Brown & Griffith, 1983). A slow, apparently noninactivating calcium influx has also been observed in isolated synaptosomal preparations (Nachshen & Blaustein, 1980) and in the clonal, PC12 nerve cell (Stallcup, 1979; Toll, 1982). In both of these isolated preparations, depolarization-induced calcium influx continued for extended times (>60 sec) after depolarization. The lack of inactivation of calcium influx in the present study is consistent with the hypothesis that this calcium flux occurs through the pathway responsible for the persistent inward current observed in intact cardiac tissue.

To our knowledge, values for calcium permeability of the persistent inward current have not been reported. Values for calcium influx/beat have been tabulated by Chapman (1979), and they range from 2.5×10^{-14} to 200×10^{-14} moles/cm^2/beat. Peak calcium influx reported herein (from Fig. 5) was 2.9×10^{-16} moles/cm^2/sec, which is at least 100-fold lower. However, calcium influx in Fig. 5 was measured with a very low external calcium concentration (i.e., 0.00025% of physiological extracellular free calcium). The comparison is further complicated by the lack of sodium and the presence of very low magnesium in the external solution. For these reasons the P_{Ca} in Fig. 6 is termed "apparent."

The lack of inactivation of calcium influx in the present experiments could reflect the low intravesicular calcium concentration maintained in the preparation by the presence of intravesicular EGTA. A calcium-sensitive component of inactivation of the calcium channel has been reported in *Paramecium* (Brehm & Eckert, 1978; Brehm et al., 1980), molluscon neuron (Brown, Morimoto, Tsuda & Wilson, 1981; Eckert & Tillotson, 1981) and in cardiac Purkinje fibers (Isenberg, 1977). Likewise, Linden and Brooker (1980; 1982) have obtained evidence for internal calcium effects on the inward plateau current in Purkinje fibers. It seems possible that the calcium influx observed in the present experiments may occur through a voltage-sensitive calcium channel that is dependent on internal calcium for inactivation. The influence of internal calcium on the persistent inward current is not known at this time.

Lack of Organic Calcium Antagonist Effect

If the observed calcium influx occurred via the voltage-sensitive transient calcium channel, then it should have been inhibited by the organic calcium antagonists nitrendipine, nifedipine, or verapamil. In the present experiments we failed to demonstrate sensitivity to these agents. High affinity nitrendipine binding has been reported in isolated membrane preparations from heart (Bellemann, Ferry, Lubbecke & Glossmann, 1981; Bolger et al., 1982; DePover et al., 1982; Ehlert, Roeske, Itoga & Yamamura, 1982; Murphy & Snyder, 1982; Williams & Tremble, 1982; Sarmiento et al., 1983); to date, however, there has been no demonstration of a nitrendipine-sensitive, voltage-dependent calcium flux in an isolated membrane preparation from heart. Nachshen and Blaustein (1979) report no effect of nifedipine and only a partial effect of verapamil on the depolarization-induced calcium uptake in synaptosomes. The only experiments to our knowledge that demonstrate a correlation between antagonist binding and inhibition of calcium influx were performed in PC12 cells (Toll, 1982). In heart there appears to be a 100 to 1000-fold discrepancy between the affinity of antagonist binding to isolated membrane preparations and the I_{50} for inhibition of I_{Ca} (Lee & Tsien, 1983), and it has been shown that nitrendipine binding sites do not co-purify with markers traditionally ascribed to the cell surface membrane (Depover et al., 1983; Williams & Jones, 1983).

Lack of a Depolarization-Induced Calcium Influx

In synaptosomes a component of calcium influx ("fast") which inactivates within 1 sec after depolarization has characteristics of the voltage-sensitive transient calcium channel current observed in nerve with voltage-clamp techniques (Nachshen & Blaustein, 1980). The fact that we have been unable to demonstrate a similar result in the isolated car-

diac sarcolemma vesicle suggests that either the experimental conditions are inappropriate for the expression of the channel flux [e.g., Saimi & Kung (1982) have shown that divalent cations are involved in the channel gating mechanism] or that the channel is either absent or in a nonactivatable state in the preparation. Nachshen and Blaustein reported that the "fast" component of calcium influx was not observed if the synaptosomes were depolarized with veratridine, batrachotoxin, or gramicidin in low potassium solutions and suggest that this effect may result from the rather slow rate of depolarization induced by these agents in comparison with the rate of inactivation of the channel. Likewise in the present experiments, we have relied on the potassium-valinomycin system for polarization and depolarization of the membrane. It seems likely, in light of the synaptosome results, that valinomycin may not depolarize the vesicles with sufficient rapidity to observe activation of the transient calcium channel. Alternatively, the transient calcium channel may be inactivated by the lack of some endogenous cytoplasmic factor. Calcium channel "run down" has been observed in internally perfused nerve cell (Byerly & Hagiwara, 1982) and in bovine chromaffin cells (Fenwick et al., 1982). This endogenous factor may be cAMP, ATP or a specific protein kinase since adenylate cyclase activity appears to influence calcium channel function (Reuter & Scholz, 1977*b*; for review *see* Reuter, 1983) via channel phosphorylation (Osterrieder et al., 1982; Iwasa & Hosey, 1983). In *Paramecium,* a cytoplasmic protein of molecular weight less than 50,000 daltons appears to be responsible for the expression of the calcium channel in mutants that do not normally express this activity (Haga & Hiwatashi, 1982). An interesting observation by Schramm et al. (1983) suggests that slight structural modifications of the dihydropyridine calcium antagonist, nifedipine, can result in stimulation of the calcium channel. These authors suggest the presence of an endogenous regulator of the calcium channel. Such a regulator may have been removed from the sarcolemma during isolation. This could account for the apparent discrepancy between the affinity of antagonist binding to membrane preparations and the inhibition of calcium current in heart cells. Nonetheless, the lack of nifedipine or nitrendipine effect on the calcium flux in the present study further supports the conclusion that this flux is not via the transient calcium channel. If indeed this flux occurred through the pathway responsible for the persistent inward current, our data suggests that this current may not be sensitive to the organic calcium antagonists.

A Modified Form of the Calcium Channel?

The following hypothesis, as an alternative to the above discussion, seems to account for a number of observations made about calcium channel current, calcium flux, and nitrendipine binding and inhibition of current flow in cardiac tissue. The calcium channel may exist in two forms: (1) a high conductance state with rapid turn on and turn off kinetics and low affinity for the dihydropyridine antagonists; and (2) a low conductance state that does not inactivate with time but which exhibits voltage-sensitivity. This form of the channel would have high affinity for the dihydropyridine antagonists but would not be blocked by these compounds. The low conductance state would represent a relatively inactivated form of the high conductance state. An endogenous regulator may convert the calcium channel from the low conductance, high affinity form to the high conductance, low affinity form. In order to evaluate this hypothesis the identification of the endogenous factor seems obligatory. This is similar to proposals for the acetylcholine receptor-operated channel, in which the endogenous activator (acetylcholine) has a low affinity for the receptor when the receptor is in the "sensitized" form (high conductance state of channel) and a high affinity for the receptor when the receptor is in the "desensitized" form (low conductance state of channel) (for review, *see* Conti-Tronconi & Raftery, 1982).

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