Inner Sarcolemmal Leaflet Ca^{2+} Binding: Its Role in Cardiac Na/Ca Exchange

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ABSTRACT A recently completed model of Ca concentration and movements in the cardiac cell diadic cleft space predicts that removal or neutralization of inner sarcolemmal (SL) leaflet anionic Ca-binding sites at the sarcolemmal border of this space will greatly diminish Na/Ca exchange-mediated Ca efflux. The present study tests this prediction using the local anesthetic dibucaine as a probe. It is shown, in isolated SL, that dibucaine competitively displaces Ca specifically from anionic phospholipid headgroups. Dibucaine also displaces Ca from the SL when applied to intact cells. It does not affect the content or release of Ca from sarcoplasmic reticulum (SR) in these cells. This eliminates a primary effect on SR Ca as a contributing factor to dibucaine's effect on Na/Ca exchange-mediated Ca efflux. Measurement of this efflux from whole cells shows a highly significant reduction of 58% ($p < 0.001$) by 0.5 mM dibucaine. The inhibiting effect of dibucaine on Na/Ca exchange-mediated Ca efflux can be significantly reversed by augmentation of Ca release from SR by caffeine at the time of activation of Na/Ca exchange. This supports the contention that the dibucaine-SL interaction is a competitive one vis-à-vis Ca. The results are supportive of the model in which inner SL leaflet Ca-binding sites account for the delay of Ca diffusion from the diadic cleft, thereby prolonging the time for which [Ca] remains elevated in the cleft. The prolonged increased [Ca] significantly enhances the ability of Na/Ca exchange to remove Ca from the cell during the excitation-contraction cycle.

INTRODUCTION

A model for calcium movement in the cardiac cell's cleft space between the junctional sarcoplasmic reticulum (SRC) cistern and the inner leaflet of the sarcolemma (SL) has recently been completed (Langer and Peskoff, 1996). The model predicts that a large fraction of the cell's sodium-calcium (Na/Ca) exchange takes place in this diadic cleft space. Moreover, the model indicates that experimentally demonstrated (Post et al., 1988; Post and Langer, 1992) SL inner-leaflet anionic Ca-binding sites are of critical importance to the efficient operation of Na/Ca exchange in the space. If these sites, predominantly phospholipid in nature, are neutralized or removed, the model predicts that cellular Na/Ca exchange will be markedly slowed. By contrast, there is also experimental evidence in the literature for marked increase of Na/Ca exchange produced by incorporation of anionic phospholipids (e.g., phosphatidyl-serine) into the SL membrane (Vemuri and Philipson, 1987; Hilgemann and Collins, 1992).

The inner SL leaflet Ca-binding sites serve to delay the diffusion of Ca from the diadic cleft after its release from the SR. This maintains [Ca] in the cleft space at levels that permit optimal operation of the Na/Ca exchangers located in the cleft SL. In the absence or neutralization of the binding sites the model predicts [Ca] in the space would fall to diastolic levels (100 nM) within 500 μs after cessation of SR release. This would result in insufficient [Ca] in the cleft space to more than minimally activate the exchanger (K_d(Ca) $\sim$ 5 μM; Hilgemann and Collins, 1992). With operative anionic Ca-binding sites present, the model predicts that [Ca] in the space is >5 μM 100 ms after cessation of release and still >1 μM after 150 ms (Langer and Peskoff, 1996). This would provide the exchangers with [Ca] levels sufficient to significantly stimulate Ca efflux via Na/Ca exchange for most of diastole.

The goal of the present study is to test, in the whole functional cell, the prediction of the cleft model as to the role of the anionic inner SL leaflet sites. A recently developed istopic ^{45}Ca washout technique permits measurement in the whole cell of a component of Ca efflux specifically dependent upon Na/Ca exchange (Langer and Rich, 1992, 1993; Post et al., 1993). The technique enables the use of a single cell sample for accurate measurement of Na/Ca-mediated Ca efflux under control conditions and after various interventions. In the present study we measure the effect on Na/Ca exchange-mediated Ca efflux of the potent local anesthetic dibucaine. Dibucaine is used as a probe for this purpose on the basis of its reported ability to penetrate membranes, interact with anionic phospholipids, and displace Ca (Low et al., 1979; Ohki, 1984; Paphadjopoulos, 1970; Browning and Akutsu, 1982).

We first document the ability of dibucaine to specifically displace Ca from phospholipid-binding sites in sarcolemma isolated from the cultured cells. Next we demonstrate that the anesthetic does the same when applied to whole cells. It is also shown that the drug does not deplete caffeine-releasable stores of Ca in the sarcoplasmic reticulum. Fi-

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nally, the action of dibucaine on Na/Ca exchange-mediated Ca efflux is measured. Results are consistent with the prediction that inner SL leaflet Ca-binding sites are of critical importance in the operation of the cells' Na/Ca exchangers.

METHODS

Cell culture

The culture procedure has been described previously (Post and Langer, 1992). Briefly, cardiac myocytes were isolated from 1–3-day-old Sprague-Dawley rats and cultured with a modified method of Harary and Farley (1963). During the isolation process fibroblasts were removed with a 2-h pre-plating step in growth medium (Gibco, Ham F10) supplemented with 10 μM arabinose-C, 10% fetal calf serum, 10% horse serum, penicillin (100,000 units/liter), and streptomycin (100 mg/liter) according to the method of Blondel et al. (1971). This procedure produces a virtually 100% myoblastic culture. Cells grown on Primaria disks (Falcon, Sumter, SC) were used for preparation of "gas-dissected" sarcolemmal membranes, and cells on Primaria dishes at low density were used for measurement of contractile function. Cells on glass coverslips were used for measurement of intracellular Ca transients. Cells grown on Primaria slides were used to determine ^{45}Ca efflux with a newly developed perfusion technique (see below). All cultures were primary and were used after 3–4 days.

Measurement of Na-Ca exchange-mediated Ca efflux

The principle for measurement of ^{45}Ca washout is similar to that previously described (Langer and Rich, 1992, 1993), but with a newly developed flow cell attached to the washout system it is possible to measure ^{45}Ca washout from neonatal cultured cells. A slide (7 × 3 cm) cut from a large Primaria dish served as the base for culture of a cell monolayer. After 3–4 days' culture the slide with cells was fitted into a cutout of a Lexan plate. Another plate was placed over the cell-containing plate and tightened down to prevent leakage. The inlet and outlet paths are shaped so as to form a square-wave flow front across the cells. The inlet and outlet paths are flush with the cell surface. The flow cell washes out with a $t_{1/2}$ = 400 ms at the perfusion rate used, close to nonperfusion limitation for these cells. With the slide in place the flow cell is closed and the inlet is connected to the output of step-motor-driven syringes, which were set to deliver one pulse of 3 ml each second. A microcomputer controls the timing of the step motors, pulse volume, pulse duration, and interval between pulses. Before washout the cells were labeled with the desired solution containing ^{45}Ca (usually 30 μCi/μmol Ca). At the end of the labeling the solution was drained. The slide with cells was mounted in the flow cell, and washout was started by activation of the desired step motor. During the course of washout the perfusate can be switched to one of a different composition within 1 s by activation of a second step motor syringe system. During the washout each pulse is collected in a separate vial for later scintillation counting.

Fig. 1 shows two successive washout curves obtained from the same cells. Cells were labeled with ^{45}Ca. The first washout (*unfilled circles*) commenced with zero Na, zero Ca isotope-free perfusate for 35 s. Na and Ca were returned to perfusate at the 36th second of the washout. The same cells were relabeled with ^{45}Ca, and a second washout (*filled circles*) was performed under the same conditions. The second washout curve is superimposable.

It has been thoroughly documented in the cultured cells used in this study that when ^{45}Ca washout is commenced without Na nor Ca in the washout solution, a component of ^{45}Ca-labeled Ca is "trapped" within the cells at unchanged specific activity for at least 2 min until Na and Ca are returned to the perfusate (Post et al., 1993; Langer et al., 1995). When Na/Ca exchange is "turned on," a clearly defined increase in ^{45}Ca activity is recorded in the effluent. Integration of this 15-s transient of labeled Ca gives an accurate measurement of Ca efflux specifically dependent upon Na/Ca exchange.

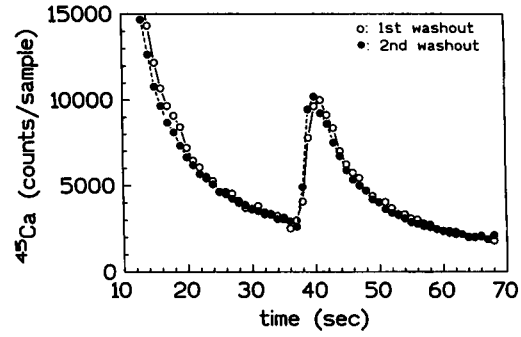

FIGURE 1 ^{45}Ca washout under control conditions. Two successive ^{45}Ca washouts are performed in the same cells. Cells were labeled with 30 μCi ^{45}Ca/μmol Ca for 20 min. The first washout (○) commenced with zero Na, zero Ca isotope-free perfusate for 35 s, at which time the solution was switched to one containing Na and Ca for the remainder of the washout. The same cells were then relabeled with ^{45}Ca and the washout was repeated. The curves are superimposable.

Isolation of sarcolemma

The "gas-dissection" technique has been described previously (Post and Langer, 1992; Langer et al., 1995). Briefly, the custom-made Primaria disk with cultured cells is placed at the center of a platform within a stainless steel chamber. After the chamber is closed, the platform is elevated so that a valve, which extends into the chamber, makes firm contact with the center of the disk. The distal end of the valve is flat and sits flush on the center of the monolayer on the disk. The aperture of the valve is a circumferential slit immediately above its flat end. The valve is connected in series with another inlet valve outside of the chamber, which controls the entry of N_2 gas at a pressure of 2100–2200 psi. Upon rapid opening of the inlet valve, N_2 exits from the slit of the distal valve in a high-velocity stream parallel to the surface of the monolayer. This gas stream shears off the upper surface of the cells and blows out the cellular material. The sarcolemma is left attached to the disk. The purity and recovery of this SL preparation have been described previously (Post et al., 1988). It gives the best combination of yield and purity of SL currently available. Specifically, it contains no SR contaminant (Langer et al., 1995).

Measurement of sarcolemmal ^{45}Ca binding

In membranes after ^{45}Ca labeling of whole cells

Cells grown on the Primaria culture disk are incubated with ^{45}Ca-containing solution (30 μCi/μmol Ca) at room temperature for 10 min. After ^{45}Ca labeling the solution is drained from the disk and the disk is washed in a series of five beakers for 10 s each (total 50 s). Sampling of the solution from the final beaker as well as sampling of the activity remaining on blank disks after washout shows that the wash sequence removes all extracellular ^{45}Ca. The cell-containing disks are divided into two groups for each treatment. The two groups are labeled in identical solution with ^{45}Ca, but one group is washed in standard solution (with Na and Ca), while the other is washed in 0Na-0Ca solution. Therefore, one group is washed out with the Na-Ca exchanger "on," the other group with the exchanger "off." The difference (binding with exchanger "off" minus binding with exchanger "on") represents sarcolemmal Ca binding when the cells' Na-Ca exchange is turned off and the ^{45}Ca destined to leave the cell via Na-Ca exchange is trapped.

After the wash in the fifth beaker, fluid is drained from the disk and the moist disk is placed in the gas-dissection chamber. The chamber is closed

and SL membranes are isolated. The chamber is carefully decontaminated after each experimental sequence. The disk with dissected membrane attached is removed from the chamber. The area of undissected, compressed cells under the valve as well as a 1-mm region at the extreme periphery of the disk where the membrane was found incompletely dissected are scraped clean. The membranes are sampled for lipid content (Boettcher, 1961), and this is used for normalization of ^{45}Ca activity among the disks and for converting membrane-bound ^{45}Ca activity to μmol Ca/kg dry weight cell. This conversion makes it possible to compare the whole cell Ca data with the gas-dissected SL data. Residual membrane is carefully scraped from the disk, which is then carefully washed with buffer to ensure complete removal. Washing and scraping are repeated four times for a complete removal of all membrane. The ^{45}Ca activity in the scrapings is determined by scintillation spectrometry. The result is expressed as cpm/Pi. The data are then converted to μmol Ca/kg dry weight cell. The correction factor is obtained according to previous results (Post et al., 1988; Post and Langer, 1992; Langer et al., 1995).

In membranes labeled after isolation

Gas-dissected membranes were washed in standard perfusate buffer to remove any adherent material. They were then incubated in 150 mM KCl buffer (pH 7.2) containing 30 μC_i ^{45}Ca for 15 min with or without 1 mM dibucaine hydrochloride or before ^{45}Ca labeling with a mixture of phospholipases C from *B. cereus* (0.5 IU/ml) and *C. welchii* (1.0 IU/ml) (Sigma, St. Louis, MO) to remove the phospholipid headgroups (Post and Langer, 1992). The membranes were exposed to the phospholipases for 30 min at 37°C, which has been shown to be sufficient to remove all phospholipid Ca-binding sites. After ^{45}Ca labeling the disks to which the membranes were bound were washed three times (10 s each wash) in isotopic-free and Ca-free buffer. This was sufficient to remove all extramembranous ^{45}Ca. The membranes were then incubated in an accurately measured buffer volume containing 1 mM $LaCl_3$ (pH = 6.8) for 15 min to displace all membrane-bound ^{45}Ca. Multiple samples of the supernatant were taken for measurement of the displaced ^{45}Ca activity. The membranes were scraped from the disks, and protein was measured according to the method of Lowry et al. (1951) for normalization of ^{45}Ca binding. Binding is expressed in cpm/μg membrane protein.

Measurement of contraction

The video technique is used for measurement of cell contraction. The system has been described previously (Wang et al., 1993). Briefly, the dish with the cultured cells is mounted on the stage of an inverted phase interference microscope. The change of perfusate is controlled by a microcomputer with exchange time <500 ms. The image of a selected area is captured via an Electrical Industries Assoc. video camera and amplified on a video screen. The cell contraction is detected by a position detector (raster line). The movement of the cell border relative to the raster is measured as the time required for the scanning dot to intersect the cell edge. This is converted to a voltage, amplified, filtered, and recorded on a chart recorder. The cells are not stimulated. Spontaneous contraction and caffeine-induced contracture are measured under various conditions.

Intracellular calcium measurement

Cells grown on a glass coverslip are incubated for 15 min in standard perfusate including 4 μM fura 2-AM (Calbiochem, La Jolla, CA) at room temperature. The cells are then washed with fura-free buffer and kept in the dark for 15 min before the experiment. The coverslip is transferred to the stage of the microscope. Light from a 100-W mercury arc lamp is focused onto a scanning mirror oscillating at 60 Hz, which deflects the beam alternately through either a 335-nm or a 405-nm interference filter. The filtered beam passes through a fused silica fiberoptic light guide (Edmund Scientific) attached to the epifluorescence port of a Zeiss Axovert 10 microscope. The beam is reflected by a 425-nm bypass dichroic mirror (Omega Optical) and then passes through the objective lens (100× oil, NA 1.3), which focuses the beam on the cell. Light returning from the preparation passes through the objective and is reflected by a 580-nm pass dichroic mirror into an image intensifier (Xybion, San Diego, CA). The video image is digitized using a DT 3851-8 image processor (Data Translation, Marlboro, MA) and stored in a volatile memory by a Northgate DX-250 microcomputer. Each ratio image is built from a short-wavelength image (335 nm) and the following long-wavelength image (405 nm). A window of 5 × 5 pixels from ratio images of the tested cell is chosen for the construction of a Ca transient curve. The Ca transient is represented by the fluorescence ratio. Each point of the Ca transient is the mean ratio in the selected window. The time resolution is about 30 ms.

Statistics

The results are reported as mean ± SE if not otherwise indicated. For the paired experiments the paired *t*-test was used to do the statistical analysis. For the unpaired experiments the results were statistically analyzed with Student's *t*-test.

RESULTS

Ca binding on isolated sarcolemma

Fig. 2 shows the effects of 1 mM dibucaine, applied directly to isolated SL, on Ca bound. Binding in the absence of dibucaine in the presence of 40 μM free [Ca] is set at 100%. Application of dibucaine significantly ($p < 0.001$) reduced binding to 52 ± 5.3% and is illustrated in the second bar. A combination of phospholipase C from *B. cereus* and *C. welchii* was previously shown to remove all phospholipid Ca-binding headgroups (Post and Langer, 1992). Treatment of the membranes with PLC reduced binding to 58% of control, as shown in the third bar. The effect of dibucaine on Ca binding to membranes pretreated with PLC is shown in the fourth bar. It is clear that dibucaine had no additional effect on binding. This can only be interpreted to mean that Ca displacement is specific for that Ca bound to phospho-

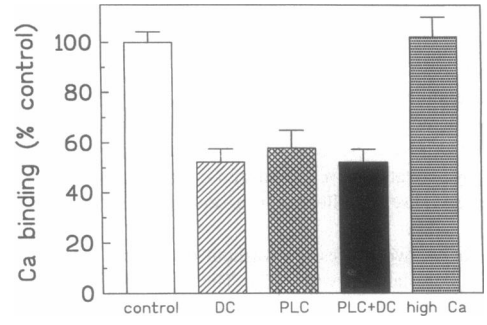

FIGURE 2 Ca bound to isolated sarcolemma (in vitro binding). Binding in the presence of 150 mM KCl and 40 μM Ca is set at 100% (control n = 7). Addition of 1 mM dibucaine (DC) to the KCl solution reduces binding to 52 ± 5.3% of control ($p < 0.001$; n = 7). In another set of membranes pretreatment with phospholipases C (PLC) reduces binding to 58 ± 7.1% (n = 6) of control, similar to the effect of dibucaine (DC). The addition of dibucaine to membranes previously treated with phospholipases (PLC + DC) does not displace additional Ca (n = 6). Increased Ca concentration by 10 × (400 μM) in the presence of 1 mM dibucaine (high Ca) returns Ca binding to control level (n = 5). Bar = 1 SE of mean.

lipid headgroups by dibucaine and has no effect on other (approximately 50% of total) Ca-binding sites.

The competitive nature of dibucaine's effect on Ca binding is shown in the fifth bar. Here the action of 1 mM dibucaine is measured in the presence of 400 μM free [Ca], a 10-fold increase over that used in the experiments presented in bar 2. Note that the binding is returned to the control level when free [Ca] is increased.

Ca binding on sarcolemma labeled in whole cells

The results presented above demonstrated the ability of dibucaine to competitively displace Ca from phospholipid headgroups in SL isolated from cultured myocardial cells. Because it is possible to instantaneously isolate SL from intact cells, the effect of dibucaine on SL binding in these intact cells can be measured. Therefore, the effect on the sarcolemmal bound fraction destined for Na-Ca exchange (see Introduction) can be specifically measured.

Whole cells are labeled with ^{45}Ca in standard perfusate. As outlined in Methods, extracellular ^{45}Ca is then washed away either with or without Na and Ca in the washout solution. Membranes are then isolated by "gas-dissection" and ^{45}Ca binding is measured. The difference in ^{45}Ca activity between the two groups is the amount of Ca dependent upon Na/Ca exchange attributable to sarcolemmal binding. This value is taken as the control and is represented by the first bar in Fig. 3. The same sequence is then repeated with another set of membranes but with 0.5 mM dibucaine in the ^{45}Ca-labeling solution but not in the washout solution. The effect on SL binding is shown in the second bar and is highly significant ($p < 0.001$, $n = 9$). Therefore the effect of dibucaine on previously isolated membranes can be duplicated on membranes isolated after dibucaine has been applied to the intact cell. These results suggest that dibucaine is capable of displacing Ca from the SL when applied to the intact cell as well as when it is applied to the isolated membranes.

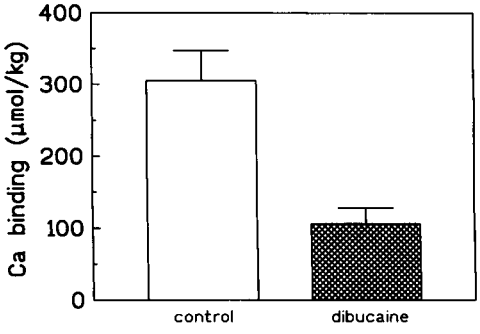

FIGURE 3 Ca bound to sarcolemma of whole cells (in vivo binding). Sarcolemmal Ca binding component of that Ca trapped by turning off Na-Ca exchange in whole cells. Binding in control perfusate (normalized per kilogram dry weight cells) is shown in the first bar. When ^{45}Ca labeling is done in the presence of 0.5 mM dibucaine there is a marked reduction ($p < 0.001$) in Ca binding to the subsequently isolated sarcolemmal membranes (*second bar*).

Response to caffeine as affected by dibucaine

Charged local anesthetics have been reported to have diverse effects on Ca release from SR in skinned skeletal muscle fibers and in isolated SR vesicles (Pike et al., 1989; Shoshan-Barmatz and Schut, 1993; Volpe et al., 1983; Yagi and Endo, 1980). In most cases, under in vitro conditions, the anesthetics have been found to inhibit SR Ca release. Because the SR is proposed to provide a component of Ca to Na-Ca exchange-mediated flux, it is necessary to determine whether dibucaine affects SR Ca content and release under the experimental conditions of the present study. We used caffeine as a probe of SR content. Fig. 4 shows the effect of 0.5 mM dibucaine on the caffeine-induced contracture in a cultured myocyte. The introduction of 10 mM caffeine elicited a significant contracture in a spontaneously beating cell, which relaxed in a few seconds, despite the continuance of caffeine in the perfusate. Caffeine is then removed, the cell is allowed to recover, and 0.5 mM dibucaine is applied for 20 min. This is the maximum time for which cells were exposed to the anesthetic in the isotopic experiments. Dibucaine, as expected, produces cessation of the spontaneous contraction. It does not, however, cause significant change in the amplitude of the caffeine-induced contracture. The relaxation phase is prolonged and partial contracture persists, consistent with partial inhibition of Na/Ca exchange by dibucaine. Similar results were documented in a total of seven cells.

Although the contraction was inhibited because of the effect of dibucaine on sarcolemmal ion channels, Ca content in the SR as manifested by caffeine contracture was not significantly altered during prolonged treatment with dibucaine. This is further confirmed by study of intracellular Ca transients with the Ca-sensitive fluorescent dye fura-2. The Ca transients were measured with a ratio imaging system constructed as described (see Methods). The effect of caffeine on the Ca transient in neonatal cultured cells was observed under control conditions and under treatment with 0.5 mM dibucaine.

A representative result is shown in Fig. 5. The change in intracellular Ca concentration is represented by the change in fluorescent ratio (F_{335}/F_{405}) at two excitation wavelengths (335 mm and 405 mm). The cells loaded with fura-2 AM showed no spontaneous beating in standard perfusate at room temperature, in contrast to the non-fura-2-loaded cells. This inhibitory effect on spontaneous cell contraction is probably due to the buffering effect of fura-2 on intracellular free Ca, which cannot reach the threshold necessary for triggering Ca release from SR under nonstimulated conditions. Upon application of 10 mM caffeine the cell manifested a transient intracellular Ca increase due to Ca release from the SR, as shown by an increase in the fluorescence ratio (Fig. 5, the first Ca transient). The intracellular Ca level decreased after the initial peak and reached the level before caffeine exposure. Because SR cannot retain Ca in the presence of caffeine, the decline in the intracellular Ca level should be due largely to the outward transport of Ca

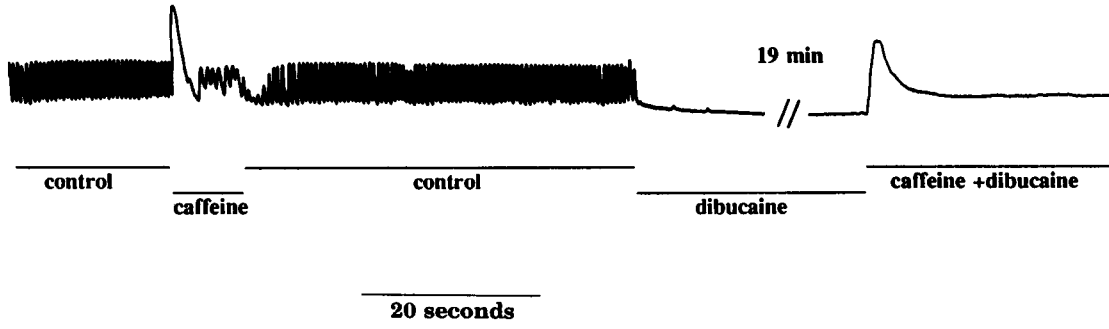

FIGURE 4 Caffeine-induced contracture. Contractile amplitude of a single cultured myocyte beating spontaneously. Caffeine (10 mM) is added to control perfusate, producing a contracture as recorded. Caffeine is removed and the cell resumes spontaneous contraction. Dibucaine (0.5 mM) is then added and perfusion is continued for 20 min to match the period of dibucaine exposure used for the isotopic experiments. Caffeine is again added, and a contracture of amplitude nearly equal to that of the previous one is produced.

via SL Na-Ca exchange. The cell was then perfused with the standard perfusate for 1 min. This step eliminates the effect of caffeine (see Fig. 4). During this period the cell was allowed to regain its SR Ca store. The cell was then continuously perfused with dibucaine-containing solution (0.5 mM dibucaine in the standard perfusate) for 20 min. Caffeine was reintroduced (10 mM caffeine plus 0.5 mM dibucaine in the standard perfusate). The intracellular Ca transient induced by caffeine under these conditions has essentially the same amplitude as that under control conditions (Fig. 5, the second Ca transient), but the rate of decrease in intracellular Ca level is slowed. Because the ability of SR to retain Ca is inhibited with caffeine under both conditions, the slower decline of intracellular Ca after treatment with dibucaine is ascribed to slower outward transport of Ca through the SL (see later discussion). A similar response was obtained from six cells under identical experimental conditions. These results confirm that the SR Ca content is little changed after dibucaine treatment and that Ca can still be released by caffeine.

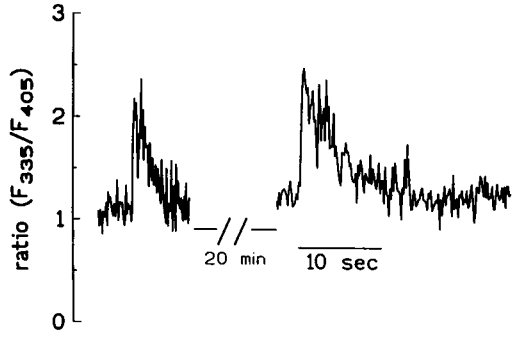

FIGURE 5 Caffeine-induced intracellular Ca transient. Cells cultured on a glass coverslip were loaded with fura-2 AM at 4 μM for 15 min at 23°C. Cells were perfused with standard perfusate after loading. The first Ca transient was induced by the addition of 10 mM caffeine in standard perfusate. Caffeine was removed, and the cells were then exposed to standard perfusate plus 0.5 mM dibucaine for 20 min. Caffeine (10 mM) was again added in the presence of dibucaine, and a transient of amplitude similar to that of the first was recorded.

^{45}Ca washout from cells as affected by dibucaine and dibucaine + caffeine

Fig. 6 illustrates three ^{45}Ca washouts from the same cells. The cells on a slide (see Methods) were labeled for 20 min with ^{45}Ca in standard perfusate. The first washout (*open circles*) was started with ^{45}Ca-free perfusate that contained zero Na and Ca. Thus Na-Ca exchange was inoperative. At the 36th second both Na and Ca were returned to the perfusate and the accumulated ^{45}Ca was cleared from the cells over the next 15 s. Integration of the ^{45}Ca activity during the transient indicates that 647 μmol Ca/kg dry weight cells was exchanged. We have shown in Fig. 1 that

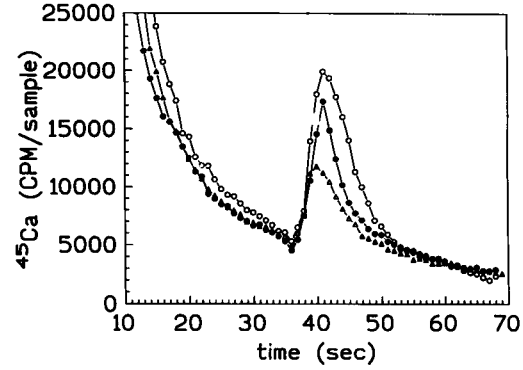

FIGURE 6 ^{45}Ca washout. Three successive ^{45}Ca washouts in the same cells. Cells were labeled with 30 μCi ^{45}Ca/μmol Ca for 20 min. Rapid washout (3 ml pulses/s) with zero Na, zero Ca isotope-free perfusate was continued for 36 s when Na and Ca were returned (within 1 s) to the perfusate, and washout continued until the 70th second (○). The transient increase in ^{45}Ca activity represents an efflux of Ca from the cells of 647 μmol/kg dry weight via Na/Ca exchange. The cells were then relabeled with ^{45}Ca in an identical manner but in the presence of 0.5 mM dibucaine, and the washout sequence was repeated. Activation of Na/Ca exchange now produced an efflux of only 233 μmols (△), just 30% of control. A third ^{45}Ca labeling, still in the presence of dibucaine, was performed, followed by 36 s of Na, Ca free isotopic washout (●). At this time 10 mM caffeine was added when Na, Ca were returned to activate the exchange. The addition of caffeine increased the flux to 413 μmol, an increase of 77% over the previously recorded transient.

another identical ^{45}Ca labeling and washout procedure on the same cells produces a superimposable washout and Na-Ca exchange-dependent response. Therefore, the cells were relabeled with ^{45}Ca, but in the presence of 0.5 mM dibucaine, and then washed out as previously (*open triangles*). Note the clear decrease in the size of the transient, which now represents 233 μmol Ca/kg dry weight cells. The reduction produced by dibucaine in a total of eight experiments was 404 μmol/kg dry weight or a decrease of 58%, which is highly significant ($p < 0.001$). The effect of dibucaine is, then, to decrease the amount of Ca cleared from the cell when Na-Ca exchange is activated. The cells were then ^{45}Ca-labeled for a third time, still in the presence of dibucaine. Washout was again initiated without Na or Ca until the 36th second, when Na and Ca plus 10 mM caffeine were returned. The result is clear. The addition of caffeine causes additional Ca to be released for a total of 413 μmol Ca/kg cells or an increase of 180 μmol over that with dibucaine alone. The release of additional Ca from the SR can partially overcome the dibucaine effect in intact cells. The addition of caffeine in the presence of dibucaine increased the release to 68% control in six experiments ($p < 0.002$), as seen in Fig. 7. The unlikely possibility that caffeine itself competes directly with dibucaine and thereby counteracts its effect was tested on isolated SL. Caffeine at 10 mM had no effect on dibucaine's ability to displace Ca from these membranes.

^{45}Ca washout from cells as affected by procaine

The amount of Ca displacement by charged local anesthetics from biomembranes has been found to be dependent on the partition coefficient of the anesthetic (Low et al., 1979). If the inhibitory effect of dibucaine on Ca efflux is secondary to Ca displacement at the inner SL leaflet, then a local anesthetic with low partition coefficient should be less effective than dibucaine. Procaine, with a partition coefficient 50-fold less than that of dibucaine but with the same single cationic charge, is such an anesthetic. Procaine applied to the cells in the same manner as dibucaine (0.5 mM) had no effect on Na-Ca exchange-mediated Ca efflux in six experiments.

Effect of beat rate on Ca content

Local anesthetics have inhibitory effects on membrane ion channel currents. They exert their anesthetic effect in neurons and suppress the excitation-contraction coupling in skeletal and cardiac muscles by blockade of sodium channels. In this study we found that dibucaine completely eliminates cell beating (Fig. 4). This raises the possibility that dibucaine, through its effect on beating rate, might influence the Na/Ca-dependent flux. To investigate this possibility we examined the effect of low-dose dibucaine on the spontaneous beat rate of the cultured cells. We found that 50 μM dibucaine, 10% the dose used in all other protocols, completely stopped the cells. We then measured the effect of 50 μM dibucaine on the Na/Ca exchange-mediated flux. Cells were ^{45}Ca-labeled in 50 μM dibucaine and washed out with dibucaine in both the 0Na-0Ca and Na-Ca containing washout solutions. The results are summarized in Fig. 8. The induction of quiescence produced a 10% reduction of ^{45}Ca release when Na/Ca exchange was activated. The reduction produced by 0.5 mM dibucaine was 6 times greater ($p < 0.0001$). Therefore cessation of beating can account for only a small fraction of the effect of 0.5 mM dibucaine on Na/Ca exchange-mediated flux.

DISCUSSION

The elements of the current "cleft space" model (Langer and Peskoff, 1996) pertinent to the operation of Na/Ca exchange are shown in Fig. 9. It is generally agreed that Ca release is triggered from JSR "feet" into the cleft space (Ikemoto et al., 1991; Jorgensen et al., 1993); this is shown in the figure. A release sufficient to produce maximum myofilament activation (>90 μmol/kg wet weight cells; Fabiato, 1983) will result in ~90% of the

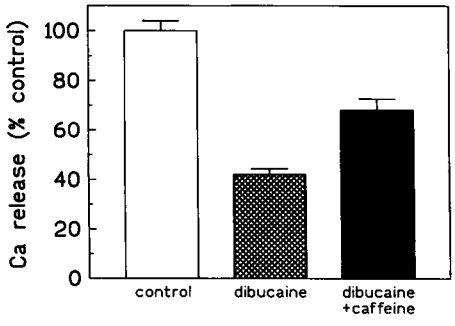

FIGURE 7 Na-Ca exchange-mediated Ca flux summary. Flux after ^{45}Ca labeling and washout in control solution (set at 100%), in the presence of dibucaine, and in the presence of dibucaine but with 10 mM caffeine added to the solution activating Na/Ca exchange. The differences between control and dibucaine and between dibucaine and dibucaine + caffeine are highly significant ($p < 0.001$ and < 0.0017, respectively).

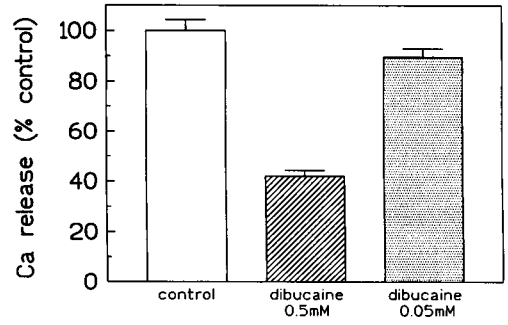

FIGURE 8 Effect of quiescence on Na-Ca exchange-mediated flux. Na-Ca mediated ^{45}Ca exchange in the presence of 0.5 mM and 50 μM dibucaine, both of which caused complete quiescence. The highly significant difference ($p < 0.0001$) of the higher dose is clearly evident.

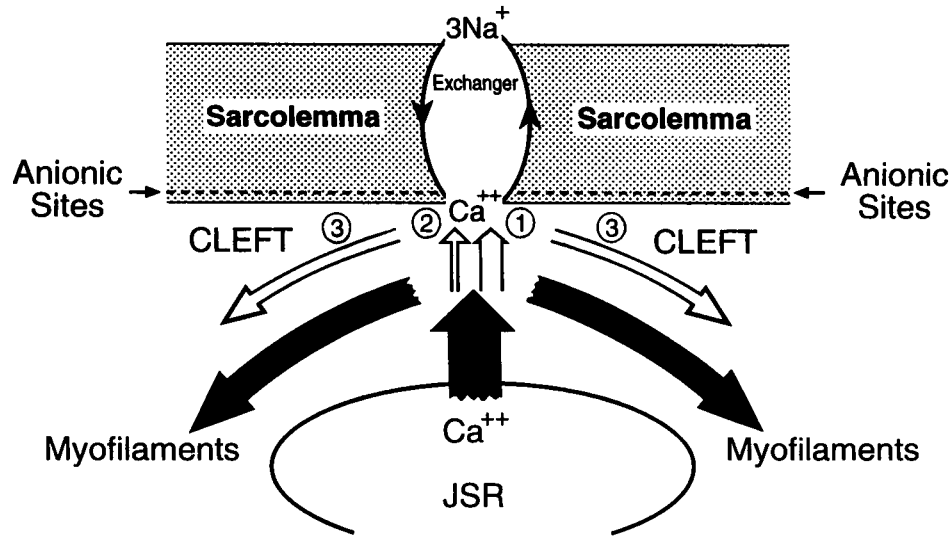

FIGURE 9 Model of Ca movement in the diadic cleft region. Ca exits the SR into the cleft space, as indicated by the vertical solid arrow. A fraction of this Ca exits the cleft via Na/Ca exchange (*open arrow 1*), and a larger fraction exits to the cytoplasm and myofilaments (*curved solid arrows*). If the anionic sites on the inner SL are removed or neutralized (as with dibucaine), less Ca will exit via Na/Ca exchange (*open arrow 2*) and more Ca will exit to the cytoplasm (*open arrows 3*). See text for further discussion.

released Ca diffusing from the cleft to the myofilaments and ~10% being transported out of the cell via the exchangers according to the model. The operation of the exchangers under this condition is indicated by the numeral "1" in the figure; it is the condition of operation when a full complement of anionic sites is present on the inner SL leaflet. The qualitative response of Na/Ca exchange to removal or neutralization of the anionic sites is shown by the arrow labeled "2" in the diagram. The model predicts that if all anionic sites were to be removed or neutralized, efflux via the exchangers would decrease to 30% of control levels during the course of an excitation-contraction cycle. This reduction occurs because, in the absence of the anionic-binding sites, the diffusion rate of Ca from the cleft space is greatly increased. [Ca] in the cleft would fall to diastolic levels (100 nM) within 500 μs after cessation of the approximately 20-ms SR release. This results in an elevated [Ca] in the cleft space for too brief a time for more than minimum activation of the exchangers (K_d(Ca) $\approx$ 5 μM; Hilgemann and Collins, 1992) to occur. Thus the Ca normally transported out of the cell via the exchangers (arrow 1) diffuses out of the space to the cytoplasm (arrow 3) and would, eventually, be resequestered by the longitudinal SR. The model predicts that total neutralization of the anionic sites would diminish Ca efflux via Na/Ca exchange by 70% during a single cardiac cycle.

We selected dibucaine as a candidate to interact with inner-leaflet phospholipid sites on the basis of its reported characteristics. The molecule consists of a hydrophobic portion connected to a tertiary amine. In its free-base form it is able to penetrate biomembranes by a hydrophobic interaction with the membrane phospholipids. Once inside the cells, at reduced pH, it assumes its univalent cationic form. It is known to be a potent displacer of Ca from model phospholipid membranes and has been shown to interact competitively with divalent cations, especially Ca, at negatively charged phospholipid polar sites with displacement of membrane-bound Ca (Blaustein and Goldman, 1966; Feinstein and Paimre, 1966; Papahadjopoulos, 1972). In the erythrocyte membrane Low et al. (1979) found dibucaine to be a potent displacer of Ca. They proposed that the hydrophilic portion of the molecule is positioned in the membrane by interaction of membrane phospholipid with the positively charged amine oriented at the lipid:water interface. This interaction results in neutralization of the negative charge of the membrane and reduces Ca binding. Competitive interaction between local anesthetics and Ca has also been demonstrated in nerve cells (Blaustein and Goldman, 1966). A very recent study (Shibata et al., 1995), using infrared spectroscopy, proved that tetracaine cation interacts with phospholipid polar groups in competition with water. The implication is that tetracaine binds strongly to the phosphate moiety of the phospholipids and that the binding is competitive. Tetracaine is a tertiary amine local anesthetic with actions very similar to those of dibucaine.

The present study indicates that dibucaine acts on cultured myocytes in the manner outlined above. In vitro studies of the SL membrane show (Fig. 2) that dibucaine specifically displaces Ca from binding sites on anionic phospholipid, 85% of which are found on the inner SL leaflet (Post et al., 1988). The competitive nature of the interaction is supported by the ability of high Ca concentration to overcome the dibucaine effect (Fig. 2). The effect of dibucaine on previously isolated membranes was duplicated on membranes isolated after dibucaine had been applied to intact, functional cells (Fig. 3), suggesting that its in vitro and in vivo actions are similar. The lack of effect of procaine on Na-Ca-mediated Ca efflux adds support to the proposal that the anesthetic must penetrate the membrane and act at its inner surface to be effective. Procaine, with a cationic charge equal to

that of dibucaine but with 50-fold less ability to penetrate lipid bilayers (Low et al., 1979), is without effect on Na-Ca exchange-mediated Ca efflux.

Local anesthetics have been reported to affect SR activity. This effect can be inhibitory or stimulatory. Most of the observed effects of local anesthetics on SR are obtained from either skinned skeletal muscle or isolated SR vesicles (Pike et al., 1989; Shoshan-Barmatz and Schut, 1993; Volpe et al., 1983; Yagi and Endo, 1980). The effect of dibucaine on SR is not evident in our cultured cell model. Because of the complex effect of the local anesthetics we therefore carefully monitor the SR function in this study. We found that neither the Ca content nor the Ca release ability of SR is significantly altered by dibucaine. The exact mechanism for this difference is not entirely clear, but it seems possible that dibucaine distribution to the SR is more limited in the intact cell. Our results support the contention that, under conditions of this study, dibucaine does not significantly diminish Ca in the SR or inhibit its release (Figs. 4 and 5). This means it is unlikely that the effects of dibucaine on Ca efflux mediated by Na/Ca exchange (Fig. 6) are due to effects on SR content or release. Neither can the effects be attributed, to any significant extent, to the ability of dibucaine to stop the spontaneous beating of the cells (Fig. 8).

The competitive nature of dibucaine/Ca interaction (Fig. 2) and the characteristics of the cleft space model predict that elevation of Ca in the cleft space and at the inner SL leaflet should restore a significant fraction of the exchanger-mediated Ca efflux inhibited by dibucaine. Augmentation of Ca release by caffeine simultaneously with activation of Na/Ca exchange (Figs. 6 and 7) clearly produces a significant restoration of dibucaine-inhibited Ca efflux. These results also indicate that dibucaine does not produce an irreversible direct modification of the exchanger molecule. If such were the case, elevation of Ca would not be expected to restore exchanger function to the extent demonstrated.

The present results support the importance of phospholipid-based inner SL leaflet Ca binding in the operation of the cardiac cell's Na/Ca exchangers, as proposed by the "cleft space" model (Langer and Peskoff, 1996). The presence of these low-affinity (K_d = 1.1 mM; Post and Langer, 1992) binding sites is critical to the operation of the Na/Ca exchangers present in the cleft's SL membrane. The binding is responsible for delay of Ca diffusion from the cleft and maintenance of [Ca] at levels orders of magnitude greater than in the general cytoplasm for prolonged (100–150 ms) periods after JSR Ca release ceases. These high [Ca] levels greatly facilitate Ca efflux via the exchangers and thereby enable the cell to maintain steady-state levels of Ca.

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