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Sarcolemmal Calcium Binding Sites in Heart: I. Molecular Origin in "Gas-Dissected" Sarcolemma

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Summary. Calcium in the myocardial cell is highly compartmentalized and a fast, an intermediate, a slow and a nonexchangeable calcium pool have been described. The fast pool contains 66% of the total cell exchangeable calcium in cultured neonatal rat heart cells with a $t_{1/2}$ of < 1.5 sec. Though the cellular origin of this fast pool is unknown, its rapidity and its displacement by La^{3+} most likely places it at the sarcolemma or at least in rapid equilibrium with the sarcolemma.

We isolated the sarcolemma of cultured neonatal rat heart cells using the gas-dissection technique, which yields a pure sarcolemmal preparation in less than a second, thereby precluding membrane changes which might occur during conventional plasma membrane isolation. We determined the calcium binding characteristics of these membranes, using an on-line technique to monitor ^{45}Ca, which allows measurement of ^{45}Ca binding characteristics in the presence of unbound ^{45}Ca. Two classes of calcium binding sites were determined: (i) K_d of 13 μM, capacity 7 nmol/mg and (ii) K_d of 1.1 mM, capacity of 84 nmol/mg. To assess the molecular origin of the sarcolemmal calcium binding we treated the membranes with a variety of enzymes. Protease or neuraminidase treatment did not cause large changes in these parameters. Simultaneous treatment with two different phospholipases C or the extraction of the lipids with isopropanol resulted in a dramatic loss of the low-affinity binding sites.

These results, in association with previously defined sarcolemmal phospholipid distribution, places the low-affinity binding sites at the cytoplasmic leaflet. The physiological implication of this localization as it pertains to cellular calcium exchange is discussed.

Key Words gas dissection · sarcolemma · neonatal rat heart · Ca^{2+} binding sites · phospholipids

Introduction

Calcium ions (Ca^{2+}) play a key role in the excitation-contraction (EC) coupling sequence of the myocardium. Therefore, further definition and understanding of the subcellular Ca^{2+} compartmentation is essential in order to completely understand the sequence and its attendant Ca^{2+} movements. A large part of the cellular Ca^{2+} in myocardial cells is very rapidly exchangeable. In cultured neonatal rat heart cells this Ca^{2+} compartment exchanges with a $t_{1/2} <$ 1.5 sec and contains at least 66% of the cells' total exchangeable Ca^{2+} (Kuwata & Langer, 1989). The content of this pool, 3.2 mmol Ca^{2+}/kg dry wt (or 550 μmol Ca^{2+}/kg wet wt) (Kuwata & Langer, 1989) is more than sufficient to cause contraction of the contractile apparatus, were it released into the cytosol (Fabiato, 1983). The rapidity of this exchange, as well as the accessibility of this pool to La^{3+}, places this pool at the sarcolemma and/or in an intracellular compartment, which is in rapid equilibrium with the sarcolemma.

Several studies do, indeed, point to the sarcolemma as a possible, functional Ca^{2+} store. Polymyxin B, a highly positively charged amphiphatic peptidolipid, competes for Ca^{2+} binding sites on synthetic lipids, as well as on isolated sarcolemma (Burt & Langer, 1983). Treatment of the sarcolemma with phospholipase D produces the anionic phospholipid phosphatidic acid in the sarcolemma and increases the sarcolemmal Ca^{2+} binding (Burt, Rich & Langer, 1984). Finally, positively charged dodecyltrimethylammonium and negatively charged dodecylsulfate decreases and increases, respectively, the sarcolemmal Ca^{2+} binding (Philipson, Langer & Rich, 1985; Post et al., 1991). An indication that this sarcolemmal-bound Ca^{2+} plays a role in excitation-contraction coupling is the fact that in all the studies mentioned above an increase in the sarcolemmal-bound Ca^{2+} coincides with an increase in cell contraction, whereas a decrease in sarcolemmal-bound Ca^{2+} coincides with a diminution of cell function (Philipson et al., 1985; Langer & Rich, 1986; Post et al., 1991).

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Since the sarcolemma is a possible storage site for this pool of rapidly exchangeable Ca^{2+} we studied the Ca^{2+} binding characteristics of the sarcolemma from the cell type in which we kinetically analyzed this rapid pool, the cultured neonatal rat heart cell (Kuwata & Langer, 1989). Several studies have been done on calcium binding to isolated sarcolemma (Scarpa & Williamson, 1974; Limas, 1977; Bers & Langer, 1979; Philipson et al., 1980*b*). However, all these studies were performed using sarcolemmal vesicles prepared by tissue homogenization and gradient centrifugation. In the present study we use the unique "gas-dissection" technique (Langer, Frank & Philipson, 1978; Post et al., 1988*a*), which gives rise to a membrane preparation of high purity in less than a second. This technique prevents any major disturbances in the sarcolemmal organization, which might occur during more elaborate and time-consuming techniques. Secondly, these Ca^{2+} binding studies involved wash steps to remove the excess of $^{45}Ca^{2+}$, which might lead to either a loss of bound Ca^{2+} (especially in view of the fast exchange characteristics of part of myocardial Ca^{2+}) or to a contamination by incomplete removal of $^{45}Ca^{2+}$. We used a different technique which monitors the sarcolemmal Ca^{2+} binding on-line (Frank et al., 1977), which then allows the assessment of sarcolemmal $^{45}Ca^{2+}$ binding in the presence of unbound $^{45}Ca^{2+}$.

Our results show that the sarcolemma isolated from cultured neonatal rat heart cells has two classes of Ca^{2+} binding sites. We were not able to establish the origin of the high-affinity, low-capacity class. The low-affinity, high-capacity class has, most likely, a phospholipid origin. Part of this study has been presented at the 1991 FASEB meetings (Post & Langer, 1991).

Materials and Methods

Cell Culture

Culturing of the cells was done according to a modification of the method of Harary and Farley (1963). Neonatal rats (1–2 days old) were decapitated, and the hearts were excised and minced. The mince was incubated in a spinner flask at 37°C with 0.1–0.05% trypsin (in 137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose and penicillin (100,000 U/liter)/streptomycin (100 mg/liter). The incubation fluid was decanted, and new medium was added. The supernatant from the first three incubations (15 min each) was discarded; during the following 6–8 incubations (10 min each), the mince was almost completely digested. The cell pellets were spun (8 min, 430 × *g*) and resuspended in growth medium (Gibco, Ham F10, supplemented with 10% fetal calf serum, 10% horse serum, penicillin (100,000 U/liter)/streptomycin (100 mg/liter), arabinose C (10 μM, to inhibit fibroblast growth) and $CaCl_2$ (final concentration 1 mM)). The cells were plated on Falcon 3000 dishes for 2–3 hr, during which time fibroblasts adhere and myocytes remain freely suspended (Blondel, Roijeir & Cheneval, 1971). Finally, the myocytes were plated on Primaria-treated discs (Falcon Plastics) and within three days a confluent monolayer of spontaneously beating, virtually pure myocytes was formed.

Isolation of the Sarcolemma

The gas-dissection technique has been previously described (Langer et al., 1978; Post et al., 1988*a*). Briefly, the disc with cell monolayer attached is placed at the center of a platform in a stainless steel chamber and the chamber is closed. The platform is then elevated so that a valve, which extends into the chamber, makes firm contact with the center of the disc. This valve is in series with an inlet valve outside the chamber, controlling the entry of N_2 gas at 2000–2100 psi. The distal valve is conical such that its flat lower surface sits flush on the center of the monolayer on the disc. Upon rapid (<1 sec) opening of the inlet valve N_2 exits in a high velocity stream parallel to the surface of the monolayer, giving a gas velocity that is optimal for membrane dissection. As the N_2 stream travels radially over the surface of the monolayer the upper surface of the cells is sheared open, the cellular material blown out and the sarcolemma left attached to the discs. Purity and recovery of this sarcolemma preparation has been described before (Post et al., 1988*a*) and is summarized as follows: a 50-fold purification of the Na^+/K^+ ATPase, an increase of the cholesterol/phospholipid ratio from 0.35 to 0.49 (mol/mol), an increase of the phospholipid-to-protein ratio of 0.24 to 1.4 (μmol P_i/mg protein), absence of sarcoplasmic reticulum, a small mitochondrial contamination and a recovery of 43%. This unique sarcolemmal preparation represents the highest combination of yield and purity yet reported and provides a virtually instantaneous isolation.

Ca^{2+} Binding to the Gas-Dissected Membranes

The $^{45}Ca^{2+}$ binding to the gas-dissected membranes is monitored by a scintillation flow cell technique, described in detail by Frank et al. (1977). The plastic discs, on which the cells are grown, contain a scintillant material. After the gas-dissection procedure the discs are mounted in a flow cell, which then is placed in the well of a specially designed scintillation counter. The following perfusate was used (in mM):133 NaCl, 3.6 KCl, 0.3 $MgCl_2$, 16 glucose and 5 tris(hydroxymethyl)aminomethane maleate (pH 7.20/NaOH) and various $CaCl_2$ concentrations. To this solution $^{45}Ca^{2+}$ (ICN) was added at 1 μCi/ml, except at $CaCl_2$ concentrations higher than 2 mM where more $^{45}Ca^{2+}$ was added. The $^{45}Ca^{2+}$ bound to the membranes, in close proximity of the scintillation disc, counts with a much higher efficiency (39%) than the $^{45}Ca^{2+}$ in the bulk solution (<5%). By the addition of La^{3+} to the solution all the calcium bound to the membranes is displaced, whereas the La^{3+} addition does not affect the signal of the bulk solution. By comparing the signal at the different Ca^{2+} concentrations and under the conditions of no binding to the membranes (obtained by the addition of La^{3+}) we can calculate the amount of Ca^{2+} bound to the membrane and construct appropriate Scatchard plots.

Protein Analysis

At the end of the experiment the discs were removed from the flow cell and dried. Subsequently, the membranes were dissolved in a small volume of 1 N NaOH and the protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin in 1 N NaOH as a standard. Results were then expressed as nmol Ca^{2+}/mg sarcolemmal protein.

Membrane Modifications

Neuraminidase Treatment

To remove a large part of the sialic acid residues of the membranes, we incubated the membranes, attached to the discs, with neuraminidase from *Clostridium* perfringens (Sigma, product no. N2133, or Worthington, product no. 4761) for 30 min, at 37°C and 0.25 IU/ml. These enzymes, at this concentration are known to remove over 50% of the sarcolemmal sialic acid residues (Frank et al., 1977).

Protease Treatment

To remove part of the sarcolemmal proteins (Leonards, 1989) we incubated the membranes with protease from *S. aureus* (Worthington, product no. 3605) for 30 min, 37°C, 70 IU/ml. In a separate set of incubations the membranes were analyzed for their protein/phospholipid ratio, to assess the activity of the enzyme.

Phospholipase Treatment

To remove the sarcolemmal phospholipid headgroups the membranes were incubated with a mixture of phospholipases C from *B. cereus* (0.5 IU/ml) and *C. welchii* (1.0 IU/ml) (both Sigma, product no. P7147 and P4039, respectively) for 30 min, 37°C. In a separate set of incubations the lipids were extracted by isopropanol immersion and separated by one-dimensional thin-layer chromatography as described before (Post et al., 1988*b*).

Isopropanol Treatment

To remove all lipidic components, without large change in the protein content, we incubated the membranes for 30 min in isopropanol (Post et al., 1988*a*).

Results

Figure 1 shows the effect of 1 μM A_{23187} (Calbiochem) on the $^{45}Ca^{2+}$ binding to the gas-dissected membranes. Addition of the Ca^{2+} ionophore after reaching plateau labeling, indicating complete turnover of the flow cell solution and complete equilibration between free and bound $^{45}Ca^{2+}$, does not increase the $^{45}Ca^{2+}$ signal. The absence of any effect shows that in the membranes isolated by "gas dissection," both the intracellular and the extracellular surface of the membrane are participating in the $^{45}Ca^{2+}$ binding and that no sealed structures are present. The figure also shows that 1 mM La^{3+} displaces all the $^{45}Ca^{2+}$ bound (at a Ca^{2+} concentration of 1 mM), since the addition of 5 mM La^{3+} does not further decrease the $^{45}Ca^{2+}$ signal. At higher Ca^{2+} concentrations (up to 5 mM) 5 mM La^{3+} was shown to be sufficient to displace all the $^{45}Ca^{2+}$ (*not shown*). Note that the ^{45}Ca bound to the membranes represents only about 2% of the total ^{45}Ca signal. Though the ^{45}Ca in the bulk solution is counted with <5% efficiency, its relatively large volume contributes 98% of the counts. Its level is, however, absolutely stable and unaffected by interventions which affect Ca binding. This makes the ^{45}Ca signal from the membranes clearly apparent.

Figure 2 shows the data obtained from a typical experiment. At the indicated time points solution changes are made to solutions containing different amounts of $^{40}Ca^{2+}$ and a constant concentration of $^{45}Ca^{2+}$. The changes observed in the $^{45}Ca^{2+}$ are the first indication that this method does, indeed, show $^{45}Ca^{2+}$ binding to the membranes. Most of the experiments are ended by switching to a solution containing La^{3+}, which displaces all Ca^{2+} bound to the membranes. The difference between the signal obtained in the La^{3+} solution (shown by the dashed line in the graph) and the signal obtained in the different Ca^{2+} solutions represents the amount of Ca^{2+} bound to the sarcolemma after correction for the appropriate specific activity. Figure 2 also shows that there is no hysteresis in the $^{45}Ca^{2+}$ binding during the experiment. When at the end of the experiment the switch is made to the initial solution, instead of to the La^{3+}-containing solution, the original $^{45}Ca^{2+}$ signal is obtained.

The Ca^{2+} binding to the gas-dissected sarcolemma is shown in Fig. 3. The inset shows near saturation of this binding at a value of 65 nmol Ca^{2+}/mg sarcolemmal protein, which occurs at a Ca^{2+} concentration of 5 mM. Scatchard analysis of these binding data is also shown in Fig. 3. It is clear that there are two classes of Ca^{2+} binding sites: one with a K_d of 13 μM and a capacity of 7 nmol/mg and one with a K_d of 1.1 mM and a capacity of 84 nmol/mg.

Because the K_d of the low affinity site is approximately 1 mM, we studied the effect of the sarcolemmal modifications on $^{45}Ca^{2+}$ binding for Ca^{2+} concentrations only up to 2 mM, in order to reduce the amount of isotope needed.

Figure 4*A* shows the effect of the neuraminidase treatment on the sarcolemmal Ca^{2+} binding. Although the binding characteristics seemed to have changed slightly, no gross changes occurred. There are still two classes of binding sites present: K_d of 22 μM and 1 mM with capacities of 8 and 47 nmol/

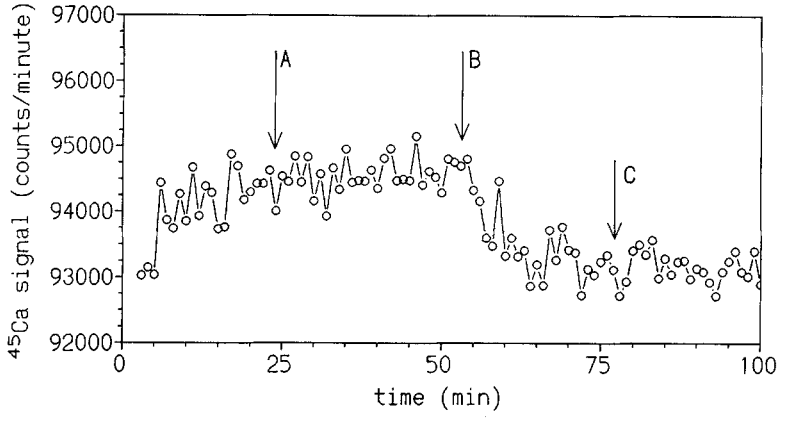

Fig. 1. The effect of the Ca^{2+} ionophore A_{23187} on the $^{45}Ca^{2+}$ binding to the gas-dissected membranes. At point *A* 1 μM of the ionophore was added and no significant effect was observed. At point *B* 1 mM La^{3+} was added to displace the sarcolemmal-bound Ca^{2+}, which led to a decrease in the signal. Finally at point *C* 5 mM La^{3+} was added and no further decrease in the signal was observed, indicating complete displacement at 1 mM La^{3+}.

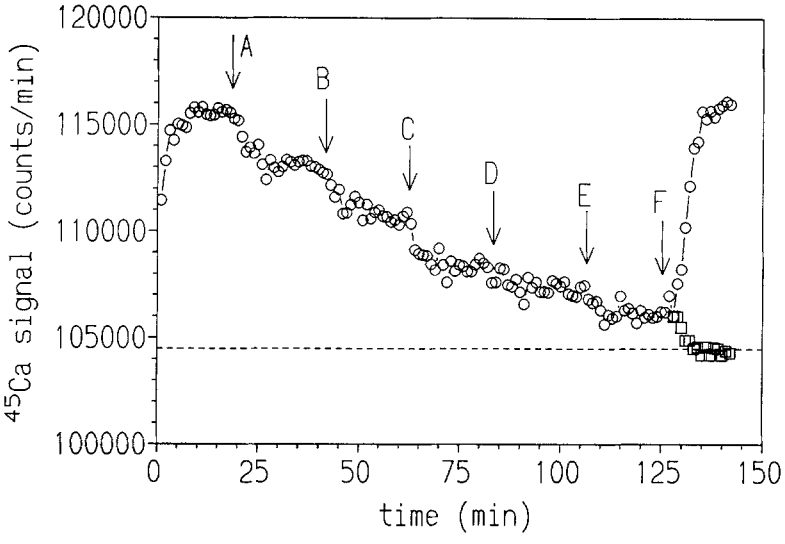

Fig. 2. The experimental protocol used. At the start of the experiment the gas-dissected membranes were superfused with the buffer, containing $^{45}Ca^{2+}$ ($\approx$1 μCi/ml) and a Ca^{2+} concentration of 8 μM. At points *A–E* $^{40}Ca^{2+}$ was added to obtain a Ca^{2+} concentration of 13, 33, 108, 258 and 1008 μM, respectively. At point *F* 1 mM La^{3+} was added (open squares) to displace all the bound Ca^{2+}. The difference between the $^{45}Ca^{2+}$ with and without La^{3+} then represented the amount bound to the sarcolemma. In a separate experiment we switched (at *F*) to the initial solution (open circles) and the signal returned to its original value.

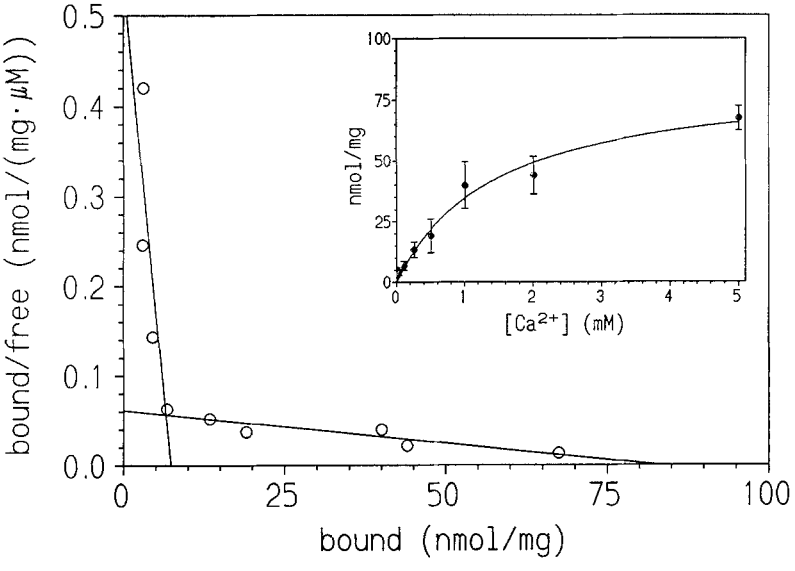

Fig. 3. Scatchard analysis of the data obtained from control gas-dissected membranes. Two distinct classes of binding sites can be observed. The inset shows the saturation curve for these membranes.

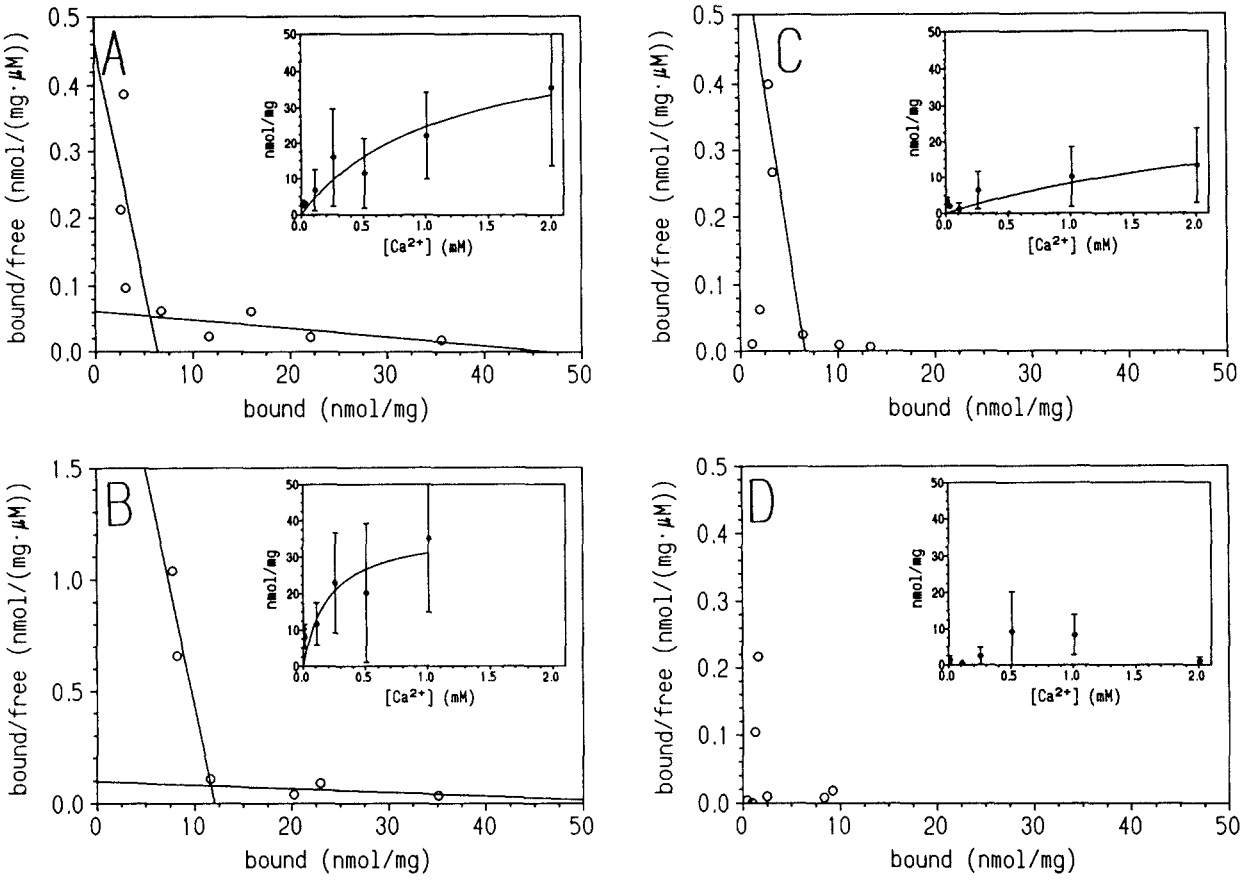

Fig. 4. Scatchard analysis of the data and the saturation curves obtained after the various treatments: (*A*) neuraminidase treatment, (*B*) protease treatment, (*C*) phospholipase C treatment and (*D*) isopropanol extraction.

mg, respectively. However, it is also clear from the binding curve that, despite several advantages of the technique used (as mentioned in Introduction), there is a rather large standard deviation in the data, using this method. For this reason we did not statistically analyze the relatively small differences between K_d values and capacity and decided to focus on the qualitative changes in the binding characteristics upon the different treatments, in order to probe the molecular origin of the two binding sites.

The protease treatment of the gas-dissected membranes increased the phospholipid/protein ratio from 1.0 to 2.6, indicating a 60% loss of sarcolemmal protein and thus the activity of the enzyme preparation. Gel electrophoresis (*not shown*) showed the degradation of many high molecular weight proteins, as found before in a vesicular preparation (Leonards, 1989). Figure 4*B* shows the Ca^{2+} binding to the protease-treated membranes. Since it is not justified to use the protein content of these treated membranes to express the Ca^{2+} binding data and to compare them with the other experiments, we extracted the lipids of these treated membranes, quantified the phospholipids and with the use of the known protein/phospholipid ratio of control membranes (Post et al., 1988*a*) we calculated the original amount of protein. This value then was used to express the Ca^{2+} binding and is shown in Fig. 4*B*). It is clear that there are still two classes of binding sites present (K_d = 5 μM and 1 mM, capacity = 12 and 60 nmol/mg). By exposing the protease-treated membrane to the different Ca^{2+} concentrations we made an interesting observation. Ca^{2+} binding was normal up to 1 mM Ca^{2+}; however, when we raised the concentration to 2 mM, in all series we performed this way, a sudden, large irregularity in the $^{45}Ca^{2+}$ signal occurred, which made the series unusable for the $^{45}Ca^{2+}$ binding assessment. Therefore, concentration series were stopped at 1 mM Ca^{2+}. The change in the $^{45}Ca^{2+}$ signal upon exposure to 2 mM Ca^{2+} will be addressed in Discussion.

The combination of the two phospholipases C clearly removed most of the headgroups of the sarcolemmal phospholipids, as can been seen in Fig. 5. This figure shows the thin-layer chromatogram of the phospholipids of the same amount of control and

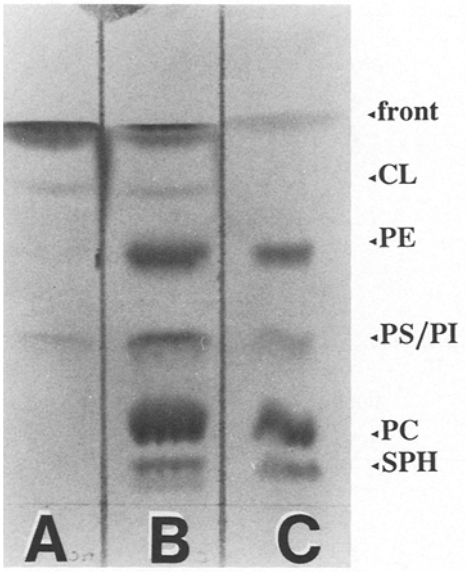

Fig. 5. Iodine staining of a thin-layer chromatogram of synthetic lipids (lane *C*), lipid extract of control membranes (lane *B*) and of phospholipase C-treated membranes (lane *A*). (*SPH* = sphingomyelin, *PC* = phosphatidylcholine, *PE* = phosphatidylethanolamine, *PS/PI* = phosphatidylserine/phosphatidylinositol, *CL* = cardiolipin and *front* contains neutral lipids such as cholesterol and diacylglycerol.

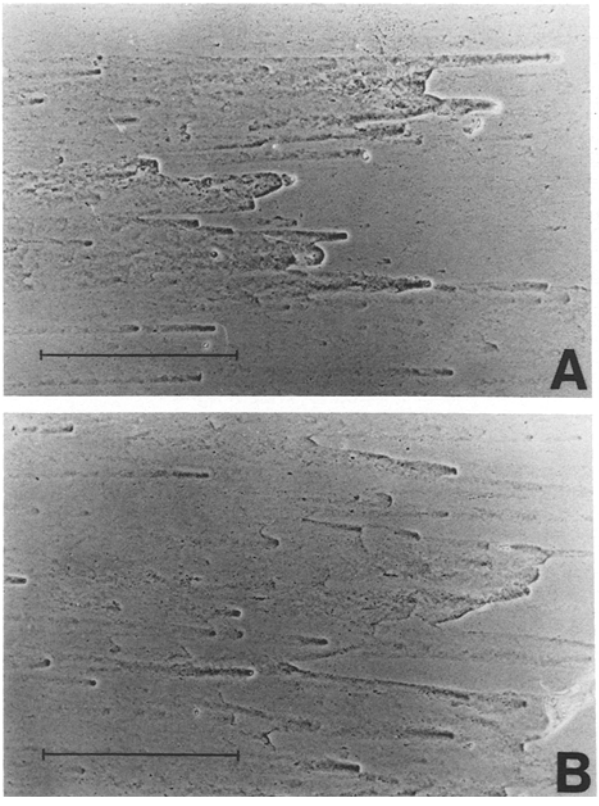

Fig. 6. Light interference micrograph of different fields from one disc of gas-dissected membranes before (*A*) and after (*B*) extraction of the lipids by isopropanol. The bar represents 120 μm.

lipase-treated membranes and shows that almost all the phospholipids were degraded. The two spots visible on the chromatogram of the treated membranes are cardiolipin and phosphatidylinositol/phosphatidylserine (which comigrate in this system). Because of the absence of a reaction with ninhydrin, which reacts with free amine groups, we conclude that this second spot contains mainly phosphatidylinositol (*PI*). Figure 4*C* shows the effect of the lipase treatment on the $^{45}Ca^{2+}$ binding. The total binding to the membranes is greatly diminished to about 10 nmol/mg protein, and the low-affinity sites have disappeared. The high-affinity sites are still present, with an approximate K_d of 10 μM and a capacity of 7 nmol/mg.

Extraction of all the lipids of the sarcolemma by isopropanol has a dramatic effect on the $^{45}Ca^{2+}$ binding, which is now almost nonexistent. As can be seen in Fig. 4*D*, it is difficult to discern any low-affinity Ca^{2+} binding site classes, although the high-affinity class seems to be present. Interestingly, the general membrane structure, as seen with phase-contrast microscopy, is unchanged (Fig. 6) by extraction of the lipids.

Discussion

This study shows the existence of at least two classes of calcium binding sites in the myocardial sarcolemma, using an on-line $^{45}Ca^{2+}$ binding technique, which is independent of the presence of unbound $^{45}Ca^{2+}$. The high-affinity sites have a K_d of 13 μM and a capacity of 7 nmol Ca^{2+}/mg sarcolemmal protein. The K_d of the low-affinity binding sites is 1.1 mM and the capacity is 85 nmol/mg sarcolemmal protein.

The membranes used in this study were isolated from cultured neonatal rat heart cells. These cells show many characteristics identical to the intact tissue, indicating that no gross changes occur in these cells during culturing. We isolated the sarcolemma of these cells using the unique "gas-dissection" technique developed in our laboratory (Langer et al., 1978). Ca^{2+} binding to membranes depends on the method of isolation; for instance, the severity of homogenization, the presence of Ca^{2+} chelators, differences in ionic strength and the length of the procedure. The "gas-dissection" technique is a pure physical technique, which yields purified sarcolemma (Post et al., 1988*a*) in less than a second, in a configuration which can be immediately used for the $^{45}Ca^{2+}$ binding assay. The simplicity of this method and the absence of high ionic strength and

Table 1 Calcium binding characteristics in cardiac sarcolemma

	High affinity K_d (μM)	nmol/mg	Low affinity K_d (mM)	nmol/mg
Bers and Langer (1979)	22	54	1.2	216
Scarpa and Wiliamson (1974)	16	11	0.8	123
Limas (1977)	5	17	1.8	680
Post and Langer (present study)	13	7	1.1	85

Ca^{2+} chelators during the isolation procedure, very likely result in a membrane preparation having characteristics close to the sarcolemma *in situ*.

Ca^{2+} binding characteristics also depend on the method used to monitor the Ca^{2+} binding. The method used in this study is independent of the free $^{45}Ca^{2+}$, since the $^{45}Ca^{2+}$ bound to the sarcolemma is counted with a much higher efficiency than the $^{45}Ca^{2+}$ in the bulk solution and because of the fact that removal of the $^{45}Ca^{2+}$ from the sarcolemma (by the addition of La^{3+}) will not significantly affect the background signal of the bulk solution. Subtraction of the two values will then yield the amount of $^{45}Ca^{2+}$ bound to the sarcolemma, independent of the presence of unbound $^{45}Ca^{2+}$. Other methods involve washing of the membranes after the incubation, resulting either in a removal of part of the bound Ca^{2+} or in incomplete removal of unbound Ca^{2+}. Furthermore the Ca^{2+} binding is dependent on the incubation medium (ionic strength (Bers & Langer, 1979; Philipson et al., 1980*b*), as well as pH (Langer, 1985)).

Because of these many variables in the methods applied, comparison of different data has to be done cautiously. Nevertheless several studies showed the presence of two distinct classes of sarcolemmal Ca^{2+} binding sites, with characteristics comparable to the ones found in this study (*see* the Table). The K_d's reported in this study are in the range of the ones reported before; the capacities are among the lowest.

In the past, several studies have been performed to analyze the molecular origin of the sarcolemmal Ca^{2+} binding sites. However, these either studied the effect of membrane modifications at one Ca^{2+} concentration (Langer & Nudd, 1983), worked with vesicular preparations (Bers & Langer, 1979; Philipson, Bers & Nishimoto, 1980*a*; Philipson et al., 1980*b*) and/or isolated membrane components (Philipson et al., 1980*b*). This study used the sarcolemma within minutes after the cells were taken out of culture and at a series of Ca^{2+} concentrations, to study the effect of selective removal of specific sarcolemmal components.

With the use of the calcium ionophore A_{23187} it is shown that there are no sealed structures present in this preparation (Fig. 1). Furthermore, the $^{45}Ca^{2+}$-containing solutions have access to both sides of the membrane, since we have shown before that large enzymes have access to both sides (Post et al., 1988*a*). Treatment of the "gas-dissected" membranes with neuraminidase did not qualitatively change the Ca^{2+} binding characteristics of the membranes. Using the same membrane preparations it was indeed found that neuraminidase treatment did not have a significant effect on the Ca^{2+} binding at 1 mM free Ca^{2+} (Langer & Nudd, 1983). Using isolated rabbit sarcolemmal vesicles, neuraminidase treatment did not result in any significant effect on Ca^{2+} binding (Philipson et al., 1980*b*). Therefore, it is unlikely that neuraminic acid residues play a significant direct role in sarcolemmal Ca^{2+} binding, in the concentration range tested.

Protease-treated "gas-dissected" membranes showed qualitatively unchanged Ca^{2+} binding characteristics. This agrees with the observation that identical protease treatment did not affect the cation-induced aggregation behavior of sarcolemmal vesicles (Leonards, 1989). This means that it is unlikely that proteins play an important role in the sarcolemmal Ca^{2+} binding. Protease treatment, however, had a secondary effect on the sarcolemma. Performing the experiment, as shown in Fig. 1, resulted in a sudden change in the $^{45}Ca^{2+}$ signal when the Ca^{2+} concentration was raised to 2 mM. We observed this in four out of four experiments and therefore ended the subsequent experiments at 1 mM Ca^{2+}. This sudden change in signal might be due to a direct effect

of high Ca^{2+} concentrations on the sarcolemmal architecture, because of removal of stabilizing factors during the protease treatment. Under normal conditions the sarcolemma is likely in a metastable configuration (Post et al., 1988*b*) and removal of certain protein components and an increase in Ca^{2+} might disturb the membrane configuration (Verkleij, Post & Schneijdenberg, 1990), resulting in completely different characteristics.

Treatment of the sarcolemma with a mixture of two phospholipases C resulted in the removal of most of the phospholipids (Fig. 5) and resulted in a major loss of the low-affinity binding sites. The maximal binding was 10 nmol Ca^{2+}/mg protein, being only 12% of the control value. The lipid analysis showed that after the lipase treatment some phosphatidylserine and cardiolipin was present, which might be responsible for the remaining Ca^{2+} binding.

In a subsequent series of experiments we removed all of the lipid components of the sarcolemma (Post et al., 1988*a*), which had no effect on the overall morphology of the sarcolemma (Fig. 6). This resulted in an almost complete removal of the low-affinity binding sites, although the high-affinity sites still seemed to be present. These data very strongly point in the direction of the sarcolemmal phospholipids as being the origin of the low-affinity Ca^{2+} binding sites. In a previous study, applying only one phospholipase C, no significant change in the Ca^{2+} binding was observed at 1 mM Ca^{2+}; however, it was shown that the enzyme mainly degraded phosphatidylcholine, a neutral phospholipid molecule (Langer & Nudd, 1983). Ca^{2+} binding to vesicles made from lipid extract from isolated sarcolemma was shown to be 80% of the amount bound to the original vesicles (Philipson et al., 1980*b*), indicating Ca^{2+} binding to lipids. This agrees with our finding; however, the configuration of the lipid vesicles is most likely completely different from sarcolemmal vesicles.

Therefore, the results of this study indicate that the low-affinity Ca^{2+} binding sites are of phospholipid origin. The origin of the high-affinity sites could not be established but are unlikely of lipidic origin. Using isolated phospholipids it has been shown that not only negatively charged phospholipids but also neutral phospholipids can bind Ca^{2+} (Seelig, 1990). However, in a previous study (Langer & Nudd, 1983) it was shown that removal of the neutral phosphatidylcholine did not affect the Ca^{2+} at 1 mM Ca^{2+}. Furthermore, we found that treatment of the gas-dissected membranes with sphingomyelinase C, which degrades the other choline-containing phospholipid sphingomyelin, does not affect the Ca^{2+} binding either (J. A. Post, *unpublished results*). Therefore, it is unlikely that the choline-containing phospholipids play a role in the sarcolemmal Ca^{2+} binding.

We have shown that the phospholipids in the sarcolemma of cultured neonatal rat heart cells are highly asymmetrically distributed (Post et al., 1988*a,b*). Sphingomyelin and phosphatidylcholine make up 85% of the phospholipids of the outer monolayer of the sarcolemma, whereas all the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, and most of the phosphatidylethanolamine are present in the cytoplasmic leaflet. Since removal of the choline-containing phospholipids did not affect the Ca^{2+} binding to the gas-dissected membranes, whereas partial or complete removal of all the lipids does, we must conclude that the noncholine-containing phospholipids are responsible for the low-affinity Ca^{2+} binding sites and that these Ca^{2+} binding sites reside in the cytoplasmic leaflet of the sarcolemma. In view of these results we performed a series of calcium binding experiments in which a buffer was used, whose composition approximated cytoplasmic ion concentrations (120 mM KCL, 10 mM NaCl, 1mM $MgCl_2$, 2 mM K_2HPO_4, and 5 mM trizma-maleate, pH 6.95/KOH). This was to determine whether the binding characteristics are dramatically changed in the presence of a more "cytosolic" environment. This change in buffer system did not produce significant changes in the calcium binding characteristics.

The presence of these low-affinity sites in the inner monolayer of the sarcolemma clearly poses a problem for the in vivo condition. Intracellular Ca^{2+} concentrations in the myocytes vary from 5×10^{-6} to 10^{-7} M during the contraction cycle. This means that Ca^{2+} bound to the low-affinity sites would be very low, about 0.5 nmol/mg protein and these binding sites would then be unlikely to represent a significant fraction of the kinetically defined fast compartment. However if there is a diffusion-restricted space under the sarcolemma (Lederer, Niggli & Hadley, 1990; Langer & Rich, 1992), a microenvironment close to the inner monolayer could be created, in which the Ca^{2+} concentration is increased. This could lead to a much higher Ca^{2+} concentration in the vicinity of the inner monolayer and would result in significant binding to low-affinity sites. Under these conditions the low-affinity binding sites and maybe even the high-affinity binding sites could bind sufficient Ca^{2+} to play an important role in the rapid Ca^{2+} movements between the cell and its external environment.

The latter hypothesis is put forward and discussed in the accompanying paper, in which we, based on experimental data, model this diffusion-restricted compartment.

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References

Bers, D.M., Langer, G.A. 1979. Uncoupling cation effects on cardiac contractility and sarcolemmal Ca^{2+} binding. *Am. J. Physiol.* **237:**H332–H341

Blondel, B., Roijeir, T., Cheneval, J.P. 1971. Heart cells in culture: A simple method for increasing the proportion of myoblasts. *Experientia* **27:**356–358

Burt, J.M., Langer, G.A. 1983. Ca^{2+} displacement by polymyxin B from sarcolemma isolated by 'gas dissection' from cultured neonatal rat myocardial cells. *Biochim. Biophys. Acta* **729:**44–52

Burt, J.M., Rich, T.L., Langer, G.A. 1984. Phospholipase D increases cell surface Ca^{2+} binding and positive inotropy in rat heart. *Am. J. Physiol.* **247:**H880–H885

Fabiato, A. 1983. Calcium-induced release of calcium from cardiac sarcoplasmic reticulum. *Am. J. Physiol.* **245:**C1–C14

Frank, J.S., Langer, G.A., Nudd, L.M., Seraydarian, K. 1977. The myocardial cell surface, its histochemistry, and the effect of sialic acid and calcium removal on its structure and cellular ionic exchange. *Circ. Res.* **41:**702–714

Harary, L., Farley, B. 1963. In vitro studies of single rat heart cells. I. Growth and organization. *Exp. Cell. Res.* **29:**451–465

Kuwata, J.H., Langer, G.A. 1989. Rapid, non-perfusion-limited calcium exchange in cultured neonatal myocardial cells. *J. Mol. Cell. Cardiol.* **21:**1195–1208

Langer, G.A. 1985. The effect of pH on cellular and membrane calcium binding and contraction of myocardium. A possible role for sarcolemmal phospholipid in EC coupling. *Circ. Res.* **57:**374–382

Langer, G.A., Frank, J.S., Philipson, K.D. 1978. Preparation of sarcolemmal membrane from myocardial tissue culture monolayer by high-velocity gas dissection. *Science* **200:**1388–1391

Langer, G.A., Nudd, L.M. 1983. Effects of cations, phospholipases, and neuraminidase on calcium binding to "gas-dissected" membranes from cultured cardiac cells. *Circ. Res.* **53:**482–490

Langer, G.A., Rich, T.L. 1986. Augmentation of sarcolemmal Ca by anionic amphiphile: Contractile response of three ventricular tissues. *Am. J. Physiol.* **250:**H247–H254

Langer, G.A., Rich, T.L. 1992. Identification and characterization of a discrete Na-Ca exchange-dependent calcium compartment in rat ventricular cells. *Am. J. Physiol. (in press).*

Lederer, W.J., Niggli, E., Hadley, R.W. 1990. Sodium-calcium exchange in excitable cells: Fuzzy space. *Science* **248:**283

Leonards, K.S. 1989. Role of proteins in cation/membrane interactions of isolated rat cardiac sarcolemmal vesicles. *Mol. Cell. Biochem.* **95:**31–42

Limas, C.J. 1977. Calcium-binding sites in rat myocardial sarcolemma. *Arch. Biochem. Biophys.* **179:**302–309

Lowry, O.H., Rosebrough, M.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–273

Philipson, K.D., Bers, D.M., Nishimoto, A.Y. 1980*a*. The role of phospholipids in the Ca^{2+} binding of isolated cardiac sarcolemma. *J. Mol. Cell. Cardiol.* **12:**1159–1173

Philipson, K.D., Bers, D.M., Nishimoto, A.Y., Langer, G.A. 1980*b*. Binding of Ca^{2+} and Na^{+} to sarcolemmal membranes: Relation to control of myocardial contractility. *Am. J. Physiol.* **238:**H373–H378

Philipson, K.D., Langer, G.A., Rich, T.L. 1985. Charged amphiphiles regulate heart contractility and sarcolemmal-Ca^{2+} interactions. *Am. J. Physiol.* **248:**H147–H150

Post, J.A., Ji, S., Leonards, K.S., Langer, G.A. 1991. Effects of charged amphiphiles on cardiac cell contractility are mediated via effects on Ca^{2+} current. *Am. J. Physiol.* **260:**H759–H769

Post, J.A., Langer, G.A. 1991. Molecular origin of sarcolemmal calcium binding sites. *FASEB J.* **5:**A1051

Post, J.A., Langer, G.A., Op den Kamp, J.A.F., Verkleij, A.J. 1988*a*. Phospholipid asymmetry in cardiac sarcolemma. Analysis of intact cells and 'gas-dissected' membranes. *Biochim. Biophys. Acta.* **943:**256–266

Post, J.A., Verkleij, A.J., Roelofsen, B., Op den Kamp, J.A.F. 1988*b*. Plasmalogen content and distribution in the sarcolemma of cultured neonatal rat myocytes. *FEBS Lett.* **240:**78–82

Scarpa, A., Williamson, J.R. 1974. Calcium binding and calcium transport by subcellular fractions of heart. *In:* Calcium Binding Proteins. W. Drabikowski, H. Strzelecka-Gobaszewska, and E. Carafoli, editors, pp. 547–585. Elsevier, Amsterdam

Seelig, J. 1990. Interaction of phospholipids with Ca^{2+} ions. On the role of the phospholipid head groups. *Cell Biol. Int. Rep.* **14:**353–360

Verkleij, A.J., Post, J.A., Schneijdenberg, T.W.M. 1990. Is acidosis the clue to the loss of structure and functioning of the sarcolemma? *Cell Biol. Int. Rep.* **14:**335–341

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