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Calcium at the Surface of Cardiac Plasma Membrane Vesicles: Cation Binding, Surface Charge Screening, and Na-Ca Exchange

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Summary. Calcium binding and Na-Ca exchange activity were measured in isolated cardiac plasma membrane vesicles under various ionic conditions. A model was developed to describe the Ca binding characteristics of cardiac sarcolemmal vesicles using the Gouy-Chapman theory of the diffuse double layer with specific cation binding to phospholipid carboxyl and phosphate groups. The surface association constants used for Ca, Na, K and H binding to both of these groups were 7, 0.63, 0.3 and 3800 M^{-1}, respectively. This model allows the estimation of surface [Ca] under any specific ionic conditions. The effects of the divalent screening cation, dimethonium, on Ca binding and Na-Ca exchange were compared. Dimethonium had no significant effect on Ca binding at high ionic strength (150 mM KCl), but strongly depressed Ca binding at low ionic strength. Dimethonium had no significant effect on Na-Ca exchange (Na-inside dependent Ca influx) at either high or low ionic strength. These results suggest that the Ca sites of the Na-Ca exchanger are in a physical environment where they are either not exposed to or not sensitive to surface [Ca].

Key Words sarcolemma · Ca binding · Na-Ca exchange · double layer · dimethonium · surface Ca

Introduction

The disposition of Ca ions at and around the surface of the plasma membrane is likely to be important in the regulation of Ca transport, membrane fusion and other Ca-dependent membrane processes. We have previously characterized Ca binding to isolated cardiac sarcolemmal vesicles (Bers & Langer, 1979; Philipson, Bers & Nishimoto, 1980*a*; Philipson, Bers, Nishimoto & Langer, 1980*b*; Bers, Philipson & Langer, 1981). In these studies, correlations were demonstrated between the amount of sarcolemmal bound Ca and contractility of cardiac muscle preparations under various ionic conditions. It was suggested that the amount of Ca bound might be directly related to the amount of Ca which entered the cell and thus activated contraction (either directly or via stimulation of Ca release from the sarcoplasmic reticulum, e.g. Fabiato (1983)). It has been suggested that the sarcolemmal-bound Ca could provide a local source of Ca to support Ca channel current which might otherwise be diffusion limited (Bers & Peskoff, 1984). Wilson, Morimoto, Tsuda and Brown (1983) have suggested that peak current through Ca channels in helix nerve cell bodies is determined by the surface concentration of the divalent cations Ca, Sr and Ba (which carry current) at the mouth of the channel. Since the amount of Ca bound is directly related to the surface [Ca], this might also explain in part the correlations between Ca binding and contractility (Bers & Langer, 1979; Philipson et al., 1980*b*; Bers et al., 1981).

Sodium-calcium exchange probably plays an important role in the regulation of intracellular Na and Ca in cardiac muscle (*see* e.g., Langer, 1982), but its role in the beat-to-beat regulation of contractile force is unknown. Sodium-calcium exchange has been extensively characterized in isolated plasma membranes (*see* Philipson, 1985, for review). It is of interest to determine whether the Na-Ca exchange is sensitive to the surface [Ca] rather than to the bulk [Ca]. This may have important implications for understanding the physical environment of the ionic binding sites on the exchanger.

In the present study we have developed a model which can describe Ca and Na binding characteristics of isolated sarcolemmal vesicles. This model is based on the Gouy-Chapman-Stern theory of the diffuse double layer with specific cation binding and is similar to that used by McLaughlin, Mulrine, Gresalfi, Vaio and McLaughlin (1981) to describe the adsorption of divalent cations to phospholipid vesicles. This model takes into account membrane surface charge and screening effects and allows prediction of surface concentrations of the ionic species present (e.g. Ca, Na, H). We estimate Ca binding parameters, surface [Ca] and double layer Ca

under various ionic conditions. In addition, we evaluate the effects of surface [Ca] on Na-Ca exchange activity.

Materials and Methods

Sarcolemmal Isolation

Sarcolemmal vesicles were isolated from rabbit and dog ventricles by modified versions of the method described by Bers (1979). Rabbit hearts were perfused with 25 mM KCl, 39 mM Na-tetraborate, 5 mM EDTA, pH 7.0 at 4°C. Ventricles were trimmed, minced and homogenized (glass-Teflon) in 250 mM sucrose, 20 mM Tris, 1 mM dithiothreitol, pH 7.6 (HM). Filtered homogenate plus 150 mM KCl, 25 mM pyrophosphate was centrifuged 30 min at 177,000 × g_{av}, the pellet resuspended in HM, incubated with deoxyribonuclease I (~6 mg/g wet weight) for 45 min at 30°C, cooled and homogenized with a Polytron (PT20ST, two 4-sec bursts). This material was then layered over 34% sucrose, centrifuged for 40 min at 83,000 × g_{av} and the interface and above was diluted with HM, pelleted (177,000 × g_{av}, 30 min), resuspended in 45% sucrose and layered under a sucrose gradient (11%, 26%, 29%, 32%, 34% sucrose). Gradients were centrifuged 16–18 hr at 77,000 × g_{av}. The sarcolemmal fraction was recovered in the upper part of the 26% phase, spun down and resuspended in HM or other solution appropriate for Ca binding experiments. Compared to the filtered homogenate, the rabbit sarcolemmal vesicles were 30- to 50-fold higher in both ouabain-sensitive (Na + K)ATPase and K-*p*-nitrophenylphosphatase specific activity. Succinate dehydrogenase specific activity was 0 to 0.4-fold that in homogenate.

A similar procedure was used to isolate canine sarcolemmal vesicles and has been recently detailed by Frank, Philipson and Beydler (1984). Canine sarcolemmal vesicles were used for all Na-Ca exchange experiments and the Ca binding experiments in Figs. 5 and 8. All other Ca binding experiments were performed with rabbit sarcolemmal vesicles.

Na-Ca Exchange

Sodium-calcium exchange was measured as intravesicular Na (Na_i)-dependent Ca uptake using techniques we have described in detail elsewhere (Philipson, 1984*b*). Briefly, 0.005 ml of Na (140 mM)-loaded sarcolemmal vesicles (or 140 mM K-loaded vesicles for blanks) were diluted to 0.25 ml in a solution containing 140 mM KCl, choline chloride, or LiCl, or 280 mM sucrose. All solutions had 10 μM $CaCl_2$ and 0.3 μCi $^{45}CaCl_2$ and 10 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer (pH 7.4 at 37°C). After 1.5 sec of Na_i-dependent Ca uptake, the reaction was quenched by the addition of 0.03 ml of 140 mM KCl, 1.0 mM $LaCl_3$, and the vesicles were recovered by Millipore filtration (0.45 μm). The filters were washed with two 3-ml aliquots of 140 mM KCl, 0.1 mM $LaCl_3$. Blanks, which accounted for less than 10% of the Ca uptake, allow correction for remaining superficially bound Ca and for any Na_i-independent Ca uptake. All solutions were at 37°C.

Ca Binding

Our techniques for the measurement of Ca bound superficially to the sarcolemmal membrane are described in detail elsewhere (Bers & Langer, 1979; Philipson et al., 1980*b*). Briefly, vesicles were incubated in the solution of interest, variable [Ca], and ^{45}Ca for 4–10 min at 26–37°C. Bound Ca was near maximal by 1.0 min and remained stable to 10 min. Aliquots were filtered (Millipore, 0.45 μm) and then washed with a small volume of H_2O (either 1 ml or 2 × 0.7 ml). These wash conditions were essential for maintaining weakly bound Ca on the sarcolemma. This small water rinse produces the same results as no rinse at all, but with substantially lower variability (Philipson et al., 1980*b*). Other conditions are given in the figure legends.

Theory

A simple model which might be hoped to explain the Ca binding characteristics and provide insight into [Ca] at the surface of sarcolemmal membranes would include Ca binding to and neutralization of negatively charged sites on the membrane surface. A net surface charge (σ) would create a surface potential (ψ_o). The relationship is most readily described by the general form of the Grahame (1947) equation from the Gouy-Chapman theory of the diffuse double layer.

$$\sigma = \left\{2\varepsilon_r\varepsilon_o RT \sum C_i[\exp(-z_i F\psi_o/RT) - 1]\right\}^{1/2} \quad (1)$$

where ε_r is the dielectric constant of the solution, ε_o the permittivity of free space, C_i the bulk concentration of ion i; T, z, F and R have their usual meanings and σ is in Coul/unit area. The surface concentration (CS) of each ion can be obtained from the Boltzmann relation:

$$CS_i = C_i\exp(-z_i F\psi_o/RT). \quad (2)$$

The surface concentration of an ion (e.g. Ca) will in turn determine the amount of binding to the membrane:

$$\text{Bound}_i = (\sigma_t/z_i)/(1 + (1/K_i CS_i)) \quad (3)$$

where K_i is a surface concentration association constant (M^{-1}) and σ_t is the total surface charge. The value of σ and σ_t can also be expressed in units of charges/Å^2 or nmol/mg protein (e.g. in Eq. (3), the Table and below). Calcium binding partially neutralizes the surface charges, σ [in Eq. (1)] causing σ to become less than σ_t and also decreasing the magnitude of ψ_o. This, of course, then limits the cation concentrating effect of the surface potential [Eq. (2)].

The simplest case considered will be where Ca, Na and Cl are the only ionic species and only Ca binds to the negative fixed charges. There are two adjustable parameters in this case, σ_t and K_{Ca}. The value of σ_t was assumed to be twice the maximum

Ca binding capacity (~300 nmol/mg protein, e.g. *see* Bers et al., 1981) and thus K_{Ca} could be estimated. This model was inadequate to explain Ca binding characteristics, but illustrates a fundamentally important point about the analysis of binding data involving charged species. Figure 1 shows a Scatchard plot of Ca binding predicted by Eqs. (1)–(3) with σ_t = 600 nmol/mg and K_{Ca} = 7 M^{-1}. The Ca binding is by definition to one class of independent sites, but the Scatchard plot is curved and might be erroneously resolved into two classes of binding sites. This curved Scatchard plot results from the nonproportional concentrating effect of the surface charge and the diminution of surface charge as Ca binds at higher [Ca] (e.g., increasing bulk [Ca] from 10 μM to 10 mM increases surface [Ca] by only 43-fold). The appropriate ordinate would be Ca bound/surface [Ca] (shown in Fig. 1 inset) which produces a straight line. Scatchard plots of this type are normally not practical since surface concentrations are usually not known.

The model as so far described is inadequate for several reasons. (i) It requires an unrealistically high σ_t for a biological membrane (600 nmol/mg would correspond to approximately 1 charge/166 Å^2). (ii) It cannot adequately explain Ca binding as a function of [Ca] (*not shown*). (iii) It does not account for the effects of pH or monovalent cations on Ca binding. We will therefore describe another model which (although still undoubtedly an oversimplification of the real system) may be more realistic, account for a broad spectrum of experimental results with sarcolemmal vesicles and make some predictions about surface concentrations. Equations (1)–(3) can be readily extended to include other cations (Na, K, H).

The surface charge of sarcolemmal membranes may be largely due to acidic phospholipids (e.g. phosphatidylserine). However, anionic phospholipids constitute at most 20% of the membrane phospholipids (~200 nmol/mg protein) and thus would be insufficient to account for the quantity of Ca binding observed. Calcium may also bind at the phosphate group of membrane zwitterionic phospholipids. For simplicity, the membrane will be considered to be composed of only three representative phospholipids: (i) phosphatidylserine (PS, 20%), an acidic phospholipid, (ii) phosphatidylethanolamine (PE, 40%), a zwitterionic phospholipid with a titratable amino group, and (iii) phosphatidylcholine (PC, 40%), a zwitterionic phospholipid with a nontitratable tertiary amine group. These values approximate the phospholipid composition of sarcolemmal vesicles previously measured by Philipson et al. (1980*a*).

Calcium, Na, K and protons are allowed to bind to either the carboxyl or phosphate groups of the

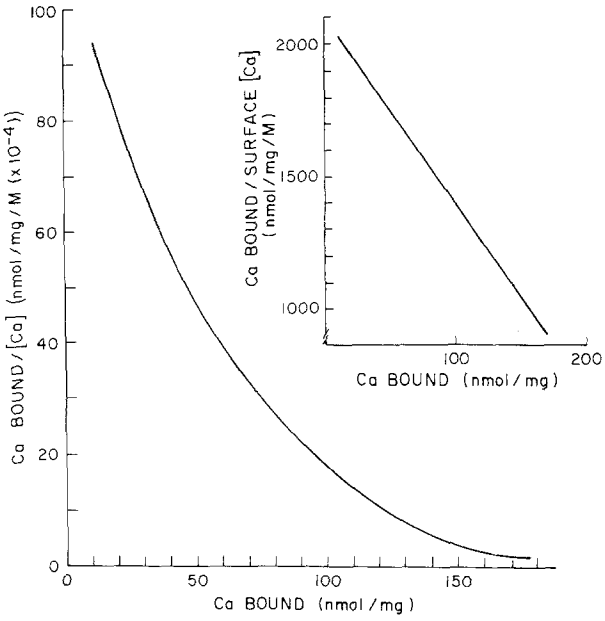

Fig. 1. Scatchard plot of Ca binding to sarcolemmal vesicles predicted by a simple initial model. In this model there is only one class of noninteracting Ca binding sites. The Scatchard plot is curved because the surface [Ca] at the binding sites is not directly proportional to bulk [Ca] (due to surface charge effects, *see* text). The inset shows that the Scatchard plot is linear when surface [Ca] is considered to be the free concentration. The conditions for both curves are 0.01–10 mM $CaCl_2$, 140 mM NaCl, pH = 7.4, 28°C

phospholipids with a single surface association constant for both sites (K_{Ca}, K_{Na}, K_K and K_H). Thus Eq. (3) for Ca binding would be:

$$CaB = (PL/2)\Big/\left[1 + \frac{(1 + K_{Na}CS_{Na})(1 + K_KCS_K)(1 + K_HCS_H)}{K_{Ca}CS_{Ca}}\right] \quad (4)$$

where CaB is Ca bound and PL is the total number of anionic sites at which Ca or other cations can bind (in nmol/mg protein or charges/Å^2) (PL = PE + PC + 2 × PS). Note that this differs from Eq. (3) in that the maximum Ca binding is now $PL/2$ rather than σ_t/z_i. Similar equations are used to describe binding of the other cations (e.g. NaB, KB and HB). The binding of any cation will reduce the surface charge:

$$\sigma = \sigma_t - (2CaB + NaB + KB + HB) \quad (5)$$

where σ_t is the total available surface charge in the absence of cation binding.

In addition to affecting σ by binding to anionic groups, protons will also determine σ_t by titrating the amino groups of the phospholipids. As the surface pH is increased, these amino groups would be-

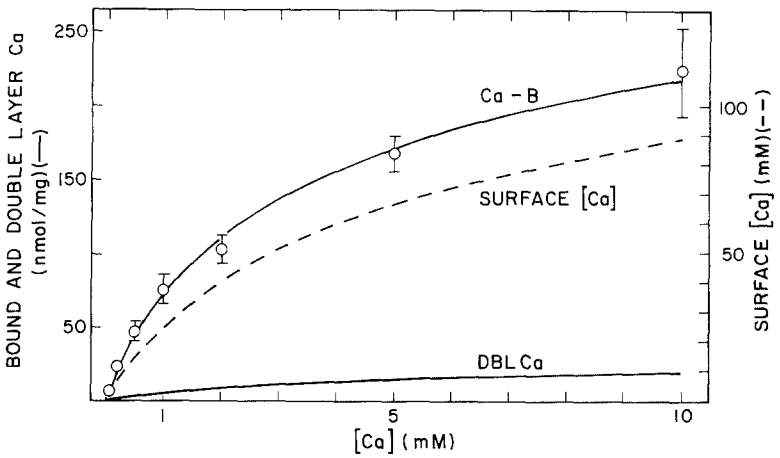

Fig. 2. Ca binding to rabbit cardiac sarcolemmal vesicles as a function of [Ca] is indicated by the open circles (±SEM). The three curves are predicted from the model described in the text and using the parameters indicated in the Table. The upper solid curve (*Ca-B*) is Ca binding, the broken curve is surface [Ca] and the lower solid curve (*DBL Ca*) is the amount of Ca accumulated in the diffuse double layer due to surface charge. NaCl = 140 mM, pH = 7.4 and 28°C

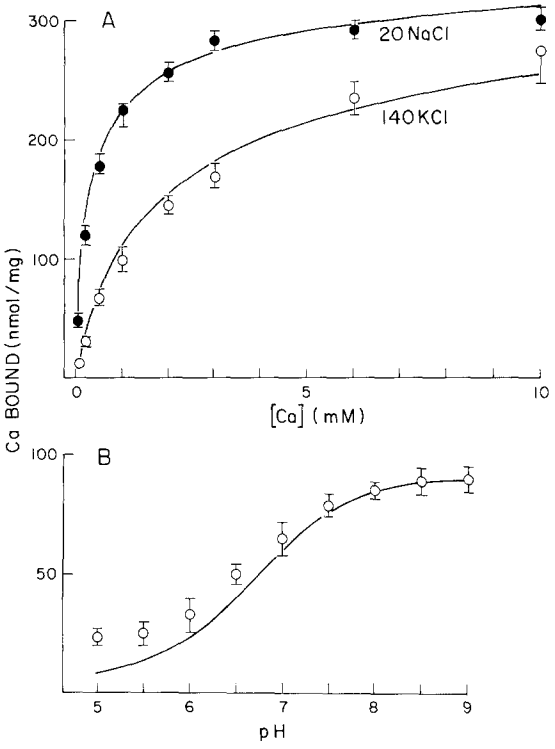

Fig. 3. Ca binding measured in rabbit ventricular sarcolemmal vesicles is indicated by the open and filled circles. The curves are predicted by the model described in the text using the parameters in the Table. (*A*) The upper curve is Ca binding in 20 mM NaCl, 240 mM sucrose. The lower curve is Ca binding in 140 mM KCl. Both are with 5 mM Tris at pH = 7.4 and 28°C. Some data for 20 mM NaCl were taken from Bers et al. (1981). (*B*) The effect of pH on sarcolemmal Ca binding at 140 mM NaCl, 1 mM $CaCl_2$, 28°C. The pH was buffered with 5 mM Tris-MES (2(N-morpholino)-ethanesulfonate)

come deprotonated with a surface association constant (K_{NH}) and surface charge would increase

$$\sigma_t = PS + [(PE + PS)/(1 + K_{NH} \cdot CS_H)]. \quad (6)$$

Thus, in this model σ_t is a function of pH and does not directly affect the number of cation binding sites. At higher pH, more amino groups are deprotonated, but the number of anionic binding sites (*PL*) is unchanged. Amino group deprotonation makes surface charge more negative, and thus the surface concentration of cations will be higher. This increases binding which in turn decreases σ.

All equations can be solved simultaneously for each ion for a given set of ionic conditions using an iterative procedure (TK! Solver; Software Arts). The parameters which must be selected are the phospholipid surface density and surface association constants. The model can then predict the surface charge (σ and σ_t), ψ_o, surface concentrations and amounts of bound cations. It can also predict the quantity of ions accumulated in the diffuse double layer (*see* Appendix).

Results

Figures 2–4 illustrate that by using the single set of parameters in the Table this simple model can describe reasonably well the Ca binding characteristics of isolated cardiac sarcolemmal vesicles. Figure 2 shows Ca binding data (points) and the Ca binding predicted by the model (upper curve) as a function of bulk [Ca] in the presence of 140 mM NaCl at pH 7.4. Also shown in Fig. 2 is the predicted surface [Ca] and the amount of excess Ca in the diffuse double layer (DBL Ca) which in part balances the residual negative surface charge (σ). Despite the fact that Ca is highly concentrated at the negatively charged membrane surface most of the surface charge compensation is due to Na under these conditions. For example, at 1 mM Ca, surface [Ca] is 25 mM (25-fold increase) while surface [Na] is 706 mM

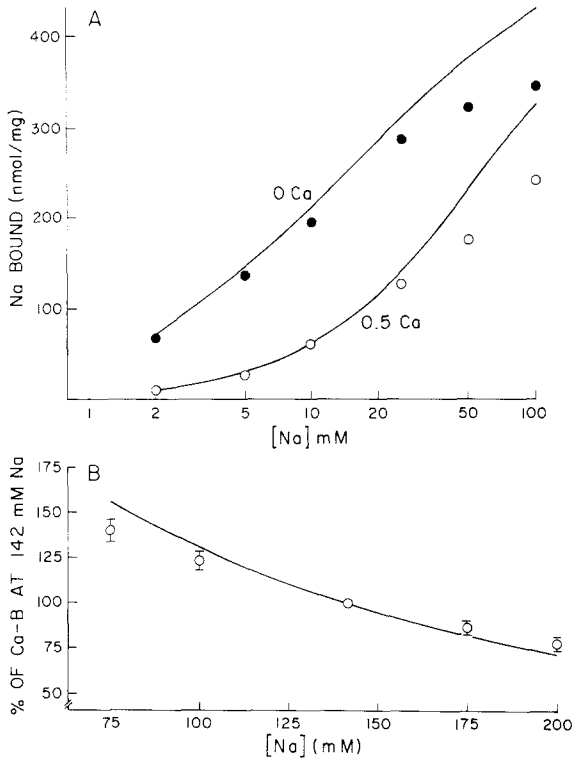

Fig. 4. Na and Ca binding data (circles) measured in rabbit ventricular sarcolemmal vesicles (from Philipson et al., 1980*b*). The curves are predicted by the model described in the text using the parameters in the Table. (*A*) The upper curve is Na binding in nominally 0 mM Ca (1 μM is used in the model) and the lower curve in 0.5 mM Ca. At high [Na] there was large variation in Na binding measurements, presumably due to the low specific activity of ^{22}Na. (*B*) The effect of [Na] on Ca binding as percent of that bound at 142 mM. Sucrose was used as isosmolar replacements for NaCl at [Na] below 200 mM. $CaCl_2$ = 1.5 mM. For both panels pH = 7.4 buffered with 5 mM Tris, 26°C

Table. Surface association constants and representative phospholipid density used in model calculations

K_{Ca}		7 M^{-1}
K_{Na}		0.63 M^{-1}
K_K		0.30 M^{-1}
K_H		3800 M^{-1}
K_{NH}		8×10^5 M^{-1}
PS	(20%)	208 nmol/mg prot. (1/500 Å^2)
PE	(40%)	416 nmol/mg prot. (2/500 Å^2)
PC	(40%)	416 nmol/mg prot. (2/500 Å^2)

The values chosen for K_{Ca}, K_{Na} and K_K are similar to those estimated by McLaughlin et al. (1981). The K_H value is similar to that estimated by Seimiya and Ohki (1973). The value of K_{NH} was selected to best fit the pH dependence of Ca binding. In the natural sarcolemma (containing cholesterol and protein) the density of phospholipid molecules was assumed to be 1/100 Å^2. To convert these phospholipid densities to nmol/mg protein it was also assumed that there are 1.6 μmol phospholipid/mg protein (Philipson, Frank & Nishimoto, 1983) and that 65% of the phospholipids are on the outer surface. The phospholipid densities are thus nmol of externally located phospholipid per mg of sarcolemmal protein.

(a five-fold increase). Under these conditions only 3.5% of the surface charge compensation is due to Ca accumulation in the double layer, 66% is due to Na accumulation and 30% is due to Cl depletion. As [Ca] increases from 10 μM to 10 mM, σ compensation by Ca increases from 0.04 to 20%, σ compensation by Na falls from 71 to 48% and σ compensation by Cl remains at about 30%. The excess of cations in the double layer maintains macroscopic electroneutrality over the membrane surface-diffuse double layer region. The method used to calculate these diffuse double layer ionic integrals is discussed in the Appendix. The value of ψ_o in Fig. 2 goes from −45 to −27 mV as [Ca] is increased from 10 μM to 10 mM

Figure 3*A* shows data and predicted curves of Ca binding as a function of [Ca] when all but 20 mM Na is replaced with isotonic sucrose and when 140 mM KCl replaces the NaCl. Also shown are the data and calculated values for the effects of pH on Ca binding (Fig. 3*B*), Na binding as a function of [Na] at two Ca concentrations (Fig. 4*A*), and the effects of [Na] on Ca binding (Fig. 4*B*).

This model and the parameters chosen are not necessarily a unique solution and the fit to the data is not perfect. Nevertheless, the parameters chosen are close to values which have been reported by others. The data are fit reasonably well if one considers the simplification, constraints and variety of binding results fit with the same limited number of adjustable parameters. This model thus may be useful in the interpretation of surface Ca effects with respect to Ca transport mechanisms.

Dimethonium is a divalent cation which has been reported to exert only a screening effect and not bind at sites to which Ca binds (McLaughlin, Grathwohl & McLaughlin, 1983). Figure 5 demonstrates the effects of dimethonium on Ca binding to cardiac sarcolemmal vesicles at both low and high ionic strength. At high ionic strength, dimethonium has no significant effect on Ca binding but decreases Ca binding markedly at low ionic strength. This is consistent with a purely screening effect of dimethonium and is readily explained by the model. This is illustrated in Fig. 6 where the predicted values for Ca binding are shown in the absence and presence of dimethonium (as a nonbinding divalent cation). The depression of Ca binding produced by dimethonium in a low ionic strength medium is indirect. That is, dimethonium screens negative surface charges decreasing the magnitude of ψ_o which re-

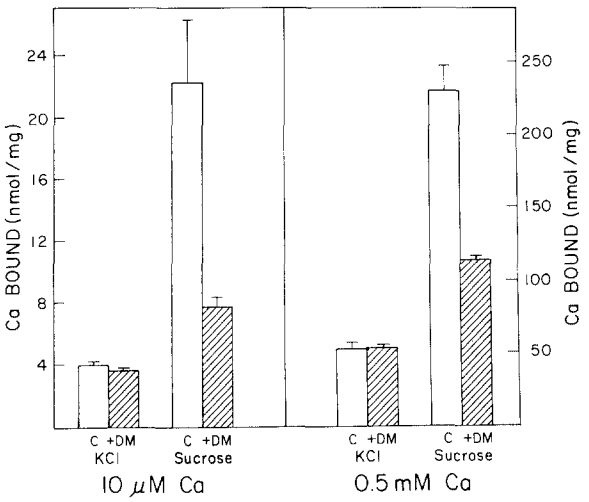

Fig. 5. The effect of 10 mM dimethonium on Ca binding to canine sarcolemmal vesicles at high and low ionic strength at two Ca concentrations. Results are shown in the absence (control, C) and presence of 10 mM dimethonium bromide (+*DM*). The high ionic strength medium contained 140 mM KCl and the low ionic strength medium contained 280 mM sucrose. The pH was buffered at 7.4 with 10 mM Tris-MOPS (sucrose solutions) or 10 mM K-MOPS (KCl solutions), 37°C

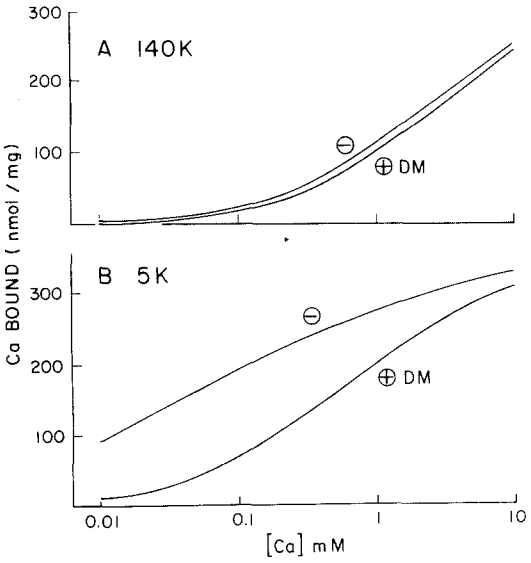

Fig. 6. The Ca dependence of Ca binding at high and low ionic strength in the absence (−) and presence of dimethonium (+*DM*) predicted by the model described in the text using the parameters in the Table. (*A*) High ionic strength medium (140 mM KCl). (*B*) Low ionic strength (5 mM KCl). For all curves pH = 7.4, 28°C

duces surface [Ca] and thus Ca binding. Figure 7 shows that similar curves are predicted for surface [Ca].

Calcium transport mechanisms in the sarcolemma might be expected to be sensitive to the surface [Ca] rather than that in bulk phase. Such a possibility has been proposed for Ca channels in helix nerve cell bodies (Wilson et al., 1983). We have investigated the effect of surface [Ca] on the Na-Ca exchange system in cardiac sarcolemma. Sodium-calcium exchange activity was measured at high and low ionic strength in the presence and absence of dimethonium (Fig. 8*A*). Neither dimethonium nor high ionic strength depressed Na-Ca exchange significantly despite large changes of surface [Ca]. Thus, it seems that Na-Ca exchange is directly sensitive to bulk [Ca] rather than surface [Ca]. Figure 8*B* shows the effects of different monovalent salt substitutions for sucrose on Na-Ca exchange and Ca binding. It can be seen that KCl and choline chloride do not significantly depress Na-Ca exchange while LiCl does. The relatively large standard error bars on the Na-Ca exchange data are due to variations in the absolute magnitude of exchange activity in different sarcolemmal preparations. The insignificant decrease in Na-Ca exchange produced by dimethonium (Fig. 8*A*) was not a consistent trend in individual experiments. All monovalent salts depressed Ca binding significantly, where none except Li depressed Na-Ca exchange. Calcium binding may be more sensitive to monovalent cations due to both greater direct competition for binding sites and the surface charge screening effect on surface [Ca]. That Li inhibited Na-Ca exchange may be due to a modest inhibitory action of Li binding to Na sites on the Na-Ca exchange. The results from Figs. 7 and 8 suggest that surface [Ca] is a critical determinant of Ca binding, but not Na-Ca exchange activity.

Discussion

The model described here is a formulation of Gouy-Chapman theory of the diffuse double layer with ionic absorption to fixed negative charges. We have not accounted for the finite size of the absorbing ions as was done by Stern (*see* Verwey & Overbeek, 1948, or Bockris & Reddy, 1970, for discussion). This theory has been implemented successfully to describe divalent cation induced shifts in the gating of ionic channels (Frankenhauser & Hodgkin, 1957; Begenisich, 1975; Hille, Woodhull & Shapiro, 1975; Gilbert & Ehrenstein, 1969) and changes in surface potential and cation binding at phospholipid membrane surfaces (McLaughlin et al., 1971, 1981, 1983; McLaughlin, 1977; Ohki & Suave, 1978; Lau, McLaughlin & McLaughlin, 1981; Ohki & Kurland, 1981). Thus, it seems reasonable to use this theory to describe cation binding

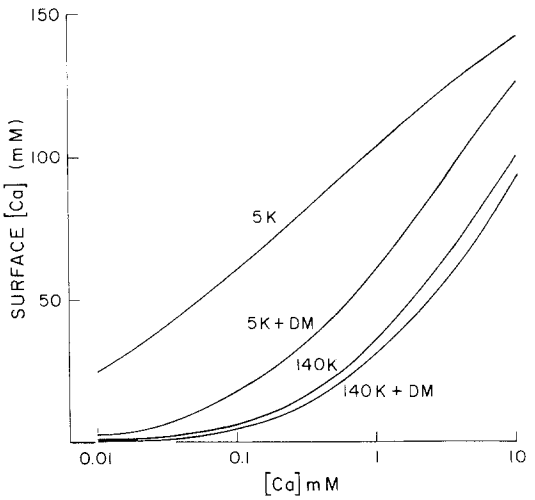

Fig. 7. Surface [Ca] predicted by the model at high (*140K*) and low ionic strength (*5K*) in the absence and presence of 10 mM dimethonium (+*DM*). The ionic conditions are high ionic strength, 140 mM KCl (*140K*) or low ionic strength (*5K*). For all curves pH = 7.4. 28°C

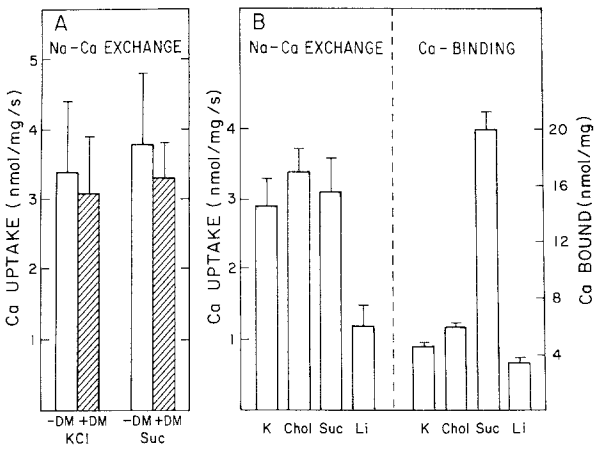

Fig. 8. (*A*) The effect of 10 mM dimethonium bromide (*DM*) on Na_i-dependent Ca influx in canine sarcolemmal vesicles at high (140 mM KCl) and low ionic strength (280 mM sucrose, *suc*). (*B*) The effects of different monovalent cations and sucrose on Na-Ca exchange and Ca binding in canine sarcolemmal vesicles. Concentrations of K, Li and choline were 140 mM as chloride salts and sucrose was 280 mM. For all panels [Ca] = 10 μM, 37°C with pH adjusted to 7.4 with 10 mM K-MOPS (KCl solutions) or Tris-MOPS (all others)

and surface concentrations at cardiac sarcolemmal membranes. The model described in the present study is certainly an oversimplification of this complex biological membrane.

Adding complexities to the model which may be realistic (e.g., multiple classes of Ca binding affinity or more complex ion interactions at the surface) can produce a more precise fit to the experimental data, but increases the number of adjustable parameters without changing fundamental conclusions. The parameters used in the model (Table) to describe the data in Figs. 2–4 are not a unique solution for any one set of conditions, but are restricted by the breadth of data described in these figures. Our attempt was to find a simple model, which could adequately describe sarcolemmal Ca binding properties with a limited number of adjustable parameters that have been independently estimated. We have previously demonstrated that most of the Ca binding of isolated cardiac sarcolemmal vesicles can be attributed to the membrane phospholipids (Philipson et al., 1980*a*). In the model, we have used 20% acidic phospholipids, which is typical of many biological plasma membranes (McLaughlin, 1977). This value is higher than that which we have previously measured (14%, Philipson et al., 1980*a*), but is in the range of other values reported for cardiac sarcolemma (7–34%; Anand, Chauhan & Dhalla, 1977; Feldman & Weinhold, 1977; Nagatomo, Hattori, Ikeda & Shimada, 1980; Weglicki et al., 1980; Tibbits et al., 1981). The other two classes of phospholipids used in the model are also typical of values reported in these studies. The model can still describe the data if 12% acidic phospholipids are assumed to be present, but the other constants have to be changed slightly. The conclusions are not altered. The surface association constants for Ca, K and Na used are very similar to those estimated for phosphatidylserine by McLaughlin et al. (1981, 1983), Kurland et al. (1979) and Ohki and Kurland (1981) and for Ca binding to the phosphate group of phosphatidylcholine and phosphatidylethanolamine (Grasdalen et al., 1977; McLaughlin et al., 1978, 1981). The lumped carboxyl/phosphate K_{Ca} used in the present study (7 M^{-1}) falls between the K_{Ca} values assumed by McLaughlin et al. (1981) for these two functional groups. This single class of Ca binding sites allows us to include monovalent cation competition at all Ca binding sites without doubling the number of association constants required. McLaughlin et al. (1981) did not allow monovalent cations to bind to zwitterionic phospholipids, nor did they include consideration of the effects of pH.

The surface proton association constant used here for both the phosphate and carboxyl groups (3800 M^{-1}) is similar to those estimated by Seimiya and Ohki (1973), and thus protons do not compete for these sites around neutral pH (and variations in this K_H do not make much difference). However, the pH dependence of Ca binding can be explained by the titration of the amino groups of the phospholipid heads. The surface proton association constant used here for these amino groups (K_{NH}, 8 × 10^5 M^{-1}) was chosen to fit the pH dependence of Ca

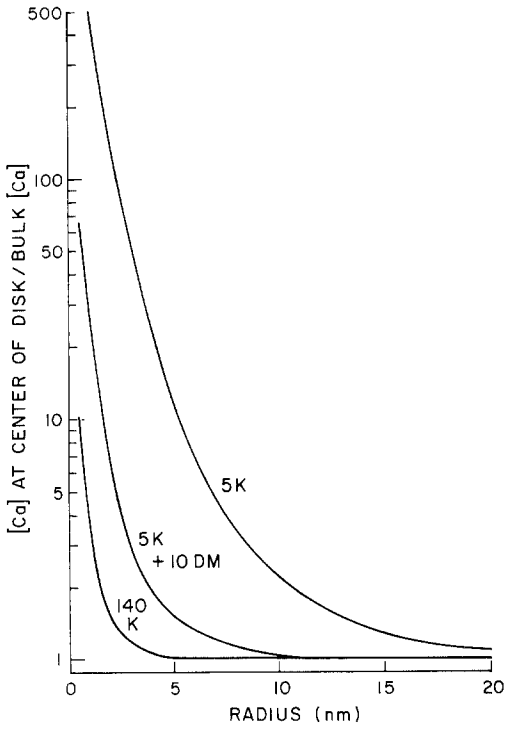

Fig. 9. The ratio of the surface [Ca] calculated at the center of a charge-free disk to the bulk [Ca] is shown as a function of the radius of the disk. The curves converge to a value of 1. That is, if the disk is large enough, the surface [Ca] will be unaffected by surface charges at the perimeter and thus surface [Ca] = bulk [Ca]. The three ionic conditions illustrated are 5 mM KCl (*5K*), 5 mM KCl + 10 mM dimethonium chloride (*5K + 10 DM*) and 140 mM KCl (*140K*). For all curves [Ca] = 10 μM, pH = 7.4

binding. The bulk pH dependence of Ca binding to sarcolemmal vesicles is quite similar to that of Ca binding to monolayers of pure phosphatidylethanolamine and phosphatidylserine reported by Seimiya and Ohki (1973) over the pH range 5–9. Seimiya and Ohki (1973) attributed this pH dependence to the titration of phospholipid amino groups with an apparent pK_a at pH 7.5 (for their ionic conditions, we would estimate surface pH to be 5.8). This apparent pK_a and the surface pK_a used in the present study are, however, substantially more acid than pK_a values estimated more directly by others for phosphatidylserine and phosphatidylethanolamine (e.g. Papahadjopoulos, 1968; Szabo, Eisenman, McLaughlin & Krasne, 1972). Thus, it is possible that the pH dependence of Ca binding in the present study may be attributable to the titration of some other sarcolemmal constituent.

When measuring Ca binding by utilizing radiotracers, it is difficult to know how much of the apparent Ca binding is true binding and how much is Ca concentrated in the diffuse double layer. Experimental evidence suggests that at least most of what is measured as Ca binding is specific absorption rather than double layer concentration. If Ca binding as measured here was due purely to Ca accumulation in the screening layer, one would expect all divalent cations to be equally effective at decreasing this Ca in the double layer. In fact, divalent cations depress Ca binding with a selectivity resembling many Ca specific binding sites (Bers & Langer, 1979). Ca binding is strongly depressed at high ionic strength by Cd, moderately by Co and Mn, weakly by Mg (Bers & Langer, 1979) and, as demonstrated in the present study, insignificantly by dimethonium (double layer Ca would also be more sensitive to dimethonium).

As seen in Fig. 2, the model predicts that the Ca concentrated in the double layer is small compared to the Ca bound (the double layer Ca is only 6–9% of that bound at 0.01–10 mM Ca). Even at low ionic strength (5 mM NaCl) the double layer Ca is only 8–17% of that bound as bulk Ca increases from 10 μM to 10 mM. It is still possible that double layer Ca is measured as part of Ca binding by the technique employed. This can readily be incorporated into the model describing the experimental data in Figs. 2–4 by small changes in the association constants and has no effect upon the conclusions.

The implication from Fig. 8 that Na_i-dependent Ca influx is not affected by the surface [Ca] may provide some insight into the physical environment of the Ca sites on the Na-Ca exchanger. One possible explanation is that a disk free of surface charge surrounds the Na-Ca exchanger site. This would decrease the magnitude of the potential at the center of the disk (ψ_c) such that there is little or no concentrating effect on cations. A simple way to estimate the effect of such a charge-free disk on [Ca] at the center is to use the linearized Poisson-Boltzmann relation as has been done by Apell, Bamberg and Läuger (1979) and Wilson et al. (1983).

$$\psi_c = \psi_o \exp[-r/(\varepsilon_r \varepsilon_o RT/2IF^2)^{1/2}] \quad (7)$$

where the quantity in parentheses is the Debye length squared, I is the ionic strength and r is the radius of the disk. The [Ca] at the center can then be obtained by substitution of ψ_c for ψ_o in Eq. (2) for Ca.

Figure 9 shows the ratio of the [Ca] at the center of a charge-free disk to the bulk [Ca] as a function of disk radius at a bulk [Ca] of 10 μM. This is the concentration at which Na-Ca exchange was measured. Curves are shown for media containing 5 mM KCl (5K), 5 mM KCl + 10 mM dimethonium bromide (5K + 10 DM) and 140 mM KCl (140K). It can be seen that either addition of dimethonium or in-

creasing ionic strength lower the [Ca] at the center of the disk substantially unless the disk is 15 nm or greater in radius (where the ratio is about 1.25). This would be a relatively large charge-free disk. At higher [Ca] these curves converge upon 1 at smaller radii (e.g. at 1 mM bulk [Ca], 5 mM KCl, the [Ca] at the center would be less than 2 mM by $r = 7$ nm).

A similar sort of effect would be achieved if the Ca-sensitive site on the Na-Ca exchanger were elevated above the plane of the phospholipid head groups and thus at a potential of lower magnitude than ψ_o. This sort of effect has been experimentally evaluated by Bell and Miller (1984) for K channels of sarcoplasmic reticulum which were reconstituted into planar phospholipid bilayers. Some combination of a raised Ca binding site on the exchanger, a charge free disk or other inhomogeneities in the membrane surface charge could explain the insensitivity of Na-Ca exchange to surface [Ca] seen in the present study.

The pH dependence of Na-Ca exchange may also contribute to the apparent insensitivity of Na-Ca exchange to surface [Ca]. At 10 μM bulk [Ca], 5 mM KCl and pH = 7.4, the model predicts that surface [Ca] would be 25 mM and surface pH 5.7. Addition of 10 mM dimethonium would decrease surface [Ca] to 2.3 mM, but would also increase surface pH to 6.2. The pH increase would thus stimulate Na_i-dependent Ca influx (Philipson, Bersohn & Nishimoto, 1982). Similarly, increasing KCl to 140 mM would decrease surface [Ca] to 0.7 mM, but increase surface pH to 6.5. It may also be noted that the activity coefficients for all the ionic species might be expected to decrease in the vicinity of the membrane surface due to the predicted high ionic strength. It is not known what net effect this might produce on Na-Ca exchange.

It has recently been reported that treatment of sarcolemmal vesicles with phospholipase D (which increases membrane phosphatidic acid) and addition of exogenous anionic amphiphiles (e.g. laurylsulfate) can dramatically increase Na-Ca exchange activity (Philipson, 1984*a*; Philipson & Nishimoto, 1984). Both of these procedures probably increase the magnitude of the negative surface charge of the membrane (by modification of endogenous phospholipids or insertion of exogenous charged amphiphiles). One explanation offered was that this increase of negative surface charge increased local Ca around the Na-Ca exchanger and thus increased Na-Ca exchange activity. However, the increased Na-Ca exchange activity produced by these techniques is still insensitive to dimethonium (Philipson, 1984*a*). Thus, it would seem that the mechanism by which Na-Ca exchange is stimulated by phospholipase D or anionic amphiphiles is not due to the increase of [Ca] which occurs at the phospholipid surface of the general membrane. Thus, the data suggest that this stimulation of Na-Ca exchange is due to some localized effect in the environment of the exchanger, although other explanations are possible.

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Appendix

Applying the Gouy-Chapman theory to a Ca, Na, Cl electrolyte, the quantities of each of these ions in the screening layer responsible for balancing the surface charge can be determined. We define a nondimensional electric potential, $\phi(x) = F\psi(x)/RT$, where $\psi(\infty) = 0$, a concentration ratio in the bulk electrolyte, $\alpha/2 = [Ca]/[Cl]$ and the debye length, $1/\kappa = (\varepsilon_r\varepsilon_o RT/2F^2C)^{0.5}$, where $C = [Cl]$ in the bulk electrolyte. Poisson's equation then becomes

$$-(2/\kappa^2)(d^2\phi/dx^2) = \alpha e^{-2\phi} + (1 - \alpha)e^{-\phi} - e^{\phi}. \quad \text{(A1)}$$

The three terms on the right-hand side of Eq. (A1) are the contributions to the nondimensional charge densities of Ca, Na and Cl, respectively (obtained by multiplying their bulk values by the appropriate Boltzmann factors).

In the usual way, using the identity $2d^2\phi/dx^2 = d(d\phi/dx)^2/d\phi$, Eq. (A1) may be integrated once, yielding, after algebraic simplification,

$$d\phi/dx = \kappa(e^{-\phi} - 1)((\alpha/2) + e^{\phi})^{0.5} \quad \text{(A2)}$$

The charge per unit area of membrane in the screening layer is due to the deviations from the bulk concentration of the i^{th} species.

$$\sigma_i = z_i F C_i(\infty) \int_0^\infty (e^{-z_i\phi(x)} - 1)dx. \quad \text{(A3)}$$

Using Eq. (A2) to express the differential, dx in Eq. (A3) in terms of $d\phi$ yields integrals over ϕ which can be evaluated for the charge in the screening layer due to Na, Ca and Cl:

$$\sigma_{Na} = \frac{CF}{\kappa}\sqrt{2/\alpha}\,(1-\alpha) \ln\left[\frac{(\sqrt{1+(2/\alpha)e^{\phi(o)}}+1)(\sqrt{1+(2/\alpha)}-1)}{(\sqrt{1+(2/\alpha)e^{\phi(o)}}-1)(\sqrt{1+(2/\alpha)}+1)}\right] \quad \text{(A4)}$$

$$\sigma_{Ca} = \frac{2CF}{\kappa}\left[e^{-\phi(o)}\sqrt{(\alpha/2)+e^{\phi(o)}} - \sqrt{(\alpha/2)+1}\right] - \sigma_{Na} \quad \text{(A5)}$$

$$\sigma_{Cl} = \frac{-2CF}{\kappa}\left[\sqrt{(\alpha/2)+e^{\phi(o)}} - \sqrt{(\alpha/2)+1}\right]. \quad \text{(A6)}$$

The total charge per unit area in the screening layer is the sum of Eqs. (A4)–(A6)

$$\sigma = \frac{-2CF}{\kappa}(1-e^{-\phi(o)})\sqrt{(\alpha/2)+e^{\phi(o)}}. \quad \text{(A7)}$$

Now the relative and absolute amount of surface charge compensation by Na, Ca and Cl can be calculated using Eqs. (A4)–(A7). This solution can also be used if other monovalent ions or divalent cations are present. For example, if both Ca and dimethonium are present, $\alpha = 2([Ca] + [dimethonium])/[Cl]$ and Eq. (A5) would give the total charge compensation due to divalent cations. The relative compensation due to Ca and dimethonium would be as their relative bulk concentrations.

Note Added in Proof

While surface [Ca] does not appear to affect Na-Ca exchange, it does alter sarcolemmal Ca binding. Recently, Fintel, Langer, Rohloff and Jung (1985) reported that 10 mM dimethonium decreases both surface Ca and tension development by rabbit ventricular muscle only at low ionic strength (and not high ionic strength). The present study indicates that Ca binding to the sarcolemma is related to surface [Ca] in a predictable manner. Two explanations for the correlations reported between sarcolemmal Ca binding and tension (i.e., Bers & Langer, 1979; Bers et al., 1981; Philipson et al., 1984*a*,*b*; Fintel et al., 1985) are (i) bound Ca may provide a local sink for Ca entry via Ca channels (Bers & Peskoff, 1984), (ii) surface [Ca] may be important in the regulation of Ca influx and sarcolemmal Ca binding, with the Ca binding change being incidental.