HIGH AND LOW AFFINITY Ca^{2+} BINDING TO THE SARCOPLASMIC RETICULUM

USE OF A HIGH-AFFINITY FLUORESCENT CALCIUM INDICATOR

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ABSTRACT The fluorescent calcium indicator, calcein, has been used as a high-affinity indicator of Ca^{2+} in the aqueous phase at physiological pH in the study of high-affinity calcium binding to sarcoplasmic reticulum (SR). The binding constant of the indicator at physiological pH is 10^3–10^4 M^{-1} and increases with increasing pH. The binding mechanism of the indicator with Ca^{2+} and Mg^{2+} is described. Application of calcein as an aqueous indicator of Ca^{2+} binding to the SR at room temperature has revealed two classes of binding sites: one with high capacity and low affinity (ca. 820 nmol/mg protein, K_d = 1.9 mM), and another with low capacity and higher affinity (ca. 35 nmol/mg protein, K_d = 17.5 μM). The divalent cation specificity of the low-affinity site is low and Ca^{2+}/Mg^{2+} specificity of the high-affinity site is high. Quantitative studies of the bindings indicate that the high-affinity site resides in the Ca^{2+} ATPase (carrier) protein and represents complexation in the active site of the carrier and that the low-affinity site resides in the nonspecific acidic binding proteins. The contribution of Donnan equilibrium effects to the measured binding is shown to be insignificant.

Stopped flow kinetic studies of Ca^{2+} passive binding with calcein and arsenazo III dyes have demonstrated that the binding to high-affinity site is very fast and that the overall binding reaction with the low-affinity site is slow, with a time-course of about 4 s. Our analysis has shown that at least part of the low-affinity acidic proteins are within the SR matrix and that Ca^{2+} can reach them only by transversing the membrane via the Ca^{2+} carrier (Ca^{2+} ATPase). A model of the SR is proposed that accounts for several functional properties of the organelle in terms of its known protein composition and topological organization.

INTRODUCTION

The release and uptake of Ca^{2+} by the sarcoplasmic reticulum are responsible for the contraction and relaxation of cardiac and skeletal muscle. Many in vitro studies of preparations of this organelle have aimed at an understanding of these functions on a more detailed molecular level. There are no in vitro studies that can account for sufficient and sufficiently rapid Ca^{2+} release to explain the triggering of skeletal muscle contraction. Studies of the active transport of Ca^{2+} with the isolated sarcoplasmic reticulum (SR) have demonstrated that the initial rate under physiological conditions is sufficient to account for muscle relaxation (1). Two important experimental results

were the determinations that two Ca^{2+} are taken up per ATP split (2, 3) and that the SR is capable of reducing the external Ca^{2+} to submicromolar concentrations.

Recent work on the phospholipid and protein composition of the skeletal SR reveals a simplicity that makes this organelle a prime candidate for biophysical investigation. The SR protein consists of only four major components (4): (*a*) The Ca^{2+}-activated ATPase (mol wt 102,000) that serves as the Ca^{2+} pump, (*b*) a protein (mol wt 55,000) with one high-affinity Ca^{2+} binding site and a large number of nonspecific low-affinity Ca^{2+} binding sites; (*c*) a highly acidic low-affinity Ca^{2+} binding protein with comparable molecular weight, whose high-Ca^{2+} binding capacity led the cited investigators to dub it "calsequestrin"; and (*d*) three fractions of lower molecular weight acidic proteins with binding capacities and affinities comparable to that of calsequestrin. These authors proposed that the function of calsequestrin is to increase the Ca^{2+} storage capacity of the SR. Recent work of Meissner (5) has shown that the microsomal fraction is heterogeneous with respect to the distribution of these acidic proteins and Ca^{2+} pump protein. Meissner (5) used isopycnic centrifugation to show that skeletal muscle microsomes range in bouyant density between that of 25 and 45% sucrose (wt/wt) and that in the lighter vesicles the Ca^{2+} pump protein constitutes up to 90% of the protein, as compared to 55–65% for the heavier vesicles. Furthermore, his electron microscope studies on these fractions showed electron-dense material referable to the Ca^{2+} binding proteins inside the heavy vesicles but not in the light vesicles. Comparison with electron micrographs of muscle led him to suggest that the light and heavy vesicles were derived from the longitudinal sections and the terminal cisternae of the SR, respectively. An important implication of these findings is that this heterogeneity may cause some difficulty in defining the precise mechanism of action of the intact SR in vivo by conducting detailed in vitro experiments with fragmented SR.

From the voluminous literature on Ca^{2+} "uptake" by SR fragments, a few papers can be singled out on the basis of their relevance to the molecular mechanism of Ca^{2+} transport and net uptake. The early work of Carvalho and Leo (6, 7), not complicated by the effects of precipitating anions, showed that the SR preparation behaves as a cation exchanger. The total amount of bound Ca^{2+}, Mg^{2+}, and K^+ was shown to be constant and equivalent to about 350 μeq/g SR protein over a wide range of ionic composition of the medium, indicating a stoichiometric exchange of these cations. Addition of ATP to the medium in absence of precipitating anions had the effect of increasing the apparent affinity of these binding sites about 1,000-fold for Ca^{2+}. Subsequent experimentation, in which Ca^{2+} was released from the SR by treatment with diethyl ether (8) and by Ca^{2+} ionophores (9), demonstrated that the Ca^{2+} was indeed transported against a concentration gradient. From these results one may hypothesize that the acidic ion exchange binding proteins are probably located within the SR matrix and that their function is to lower the free concentration of the Ca^{2+} transported into the SR matrix. This would lower the gradient against which the Ca^{2+} pump must operate.

The equilibrium dialysis experiments of Chevallier and Butow (10) showed evidence

for a small number of high-affinity Ca^{2+} binding sites on the isolated SR. In addition to finding nonspecific sites of the type measured by Carvalho and Leo (7), they found about 90 nmol/mg SR protein of Ca^{2+}-specific sites with $K_d = 3.2 \times 10^{-5}$M and about 10 nmol/mg SR protein high-affinity sites with $K_d = 1.3 \times 10^{-6}$M. The equilibrium dialysis experiments on Meissner (5) on purified Ca^{2+} pump protein showed specific Ca^{2+} binding of 10–15 nmol/mg (1.0–1.5 mol/mol ATPase) with an affinity constant of 0.5–1.5 $\times 10^6$ M^{-1}. Combination of these two results suggests that the high-affinity sites of SR represent binding sites on the Ca^{2+} pump protein involved in Ca^{2+} transport.

The object of the present study was to develop spectroscopic methods for the rapid and continuous readout of Ca^{2+} binding to the SR and to apply these, together with rapid kinetic methods, to the problem of elucidating the partial steps in the overall transport sequence. Our first efforts were devoted to the screening of indicators in hopes of finding one with the requisite high affinity and large spectroscopic change. Murexide, which we used in a previous study of Ca^{2+} binding to phospholipid membranes (11), was unsuitable because of its low affinity (log $K_a = 2.8$ at pH 7.8) at neutral pH. Based on the evidence cited above and on the K_m for active transport, we expected the binding constants for Ca^{2+} on the ATPase would be of the order of $10^5 - 10^6$ M^{-1}. The first section of the paper thus describes our efforts to characterize the fluorescent properties of the indicator calcein and to develop methods for its use as a Ca^{2+} indicator at neutral pH. Previous literature reported the applications of this dye only at pH > 12. The remainder of the paper reports our use of the fluorescent indicator in the study of the passive binding of Ca^{2+} to the SR.

METHODS

Sarcoplasmic reticulum vesicles, referred to as SR in the remainder of the text, were prepared from rabbit back muscle by the method of Martonosi and Feretos (12). Calcein dye was purchased from the G. Frederick Smith Chemical Co. (Columbus, Ohio). Other chemicals used in the study were reagent grade.

Fluorescence titrations were performed with a Hitachi-Perkin Elmer MPF3 spectrofluorometer (Perkin-Elmer Corp., Hitachi-Perkin Elmer Instruments, Mountain View, Calif.). We were prepared to evaluate the influence of light scattering changes on the titration behavior of the indicator by monitoring light scattering throughout the titration and by determining empirical relationships between light scattering, turbidity, optical density, and fluorescence at the excitation wavelength. However, the efficient collecting optics of the Perkin-Elmer instrument and our choice of 5-mm cells and the small size of the light scattering changes made such corrections unnecessary. An Aminco stopped flow spectrometer (Cat. No. 4-8409, American Instrument Co., Travenal Laboratories, Inc., Silver Spring, Md.) was used in the fluorescence model with a 495 nm Schott cutoff filter (Schott Optical Glass, Inc., Daryea, Pa.) in the kinetic studies. All the experiments were carried out at room temperature. In the experiments involving divalent cation titrations of calcein fluorescence, back titrations with EDTA were performed to show that the results were not affected by possible contamination of our water, reagents, or SR by Ca^{2+}.

RESULTS AND DISCUSSION

Indicator Properties of Calcein

Calcein was first described as a fluorochromic cation indicator by Diehl and Ellingboe (13). The original application was as an end point indicator in EDTA titration of Ca^{2+} at pH 12. Wallach et al. (14) published a slightly different method of preparation and proposed the structure given in Fig. 1. The pK values and their assignments are also taken from that paper. The effect of pH on the fluorescence of the dye with alkaline earth (M^{2+}) complexation was reported by Wallach and Steck (15). These authors found that the fluorescence intensity of calcein excited around 490 nm and measured around 520 nm is fairly constant between pH 5 and 9, but drops off rapidly for pH values less than 4 or greater than 10. Complexation with Ca^{2+} or Mg^{2+} extended this plateau to about pH 13. Thus large increases in fluorescence could be measured upon complexation at pH 12 whereas only small (<12%) changes in fluorescence could be produced by complexation at neutral pH. The indicator would thus seem to be sensitive only under highly alkaline conditions.

Wallach and Steck (15) carried out quantitative studies of the Ca^{2+} complexation of calcein at pH 12.5. On the basis of biphasic titration behavior of the fluorescence with Ca^{2+} addition, they reported that two types of complex were formed: A 1:1 complex with a formation constant

$$K' = [M\text{-}I]/([M][I]), \tag{1}$$

where M represents the cation and I represents the indicator calcein, and a 2:1 complex with a formation constant

$$K'' = [M_2\text{-}I]/([M][M\text{-}I]) \tag{2}$$

To the first complexation they attributed high binding affinity and a relatively small increase in the indicator fluorescence, and to the second complexation reaction they attributed a smaller binding affinity but larger fluorescence increase. The authors reported the following values of log (K'') for the alkaline earths at pH $\cong$ 12.5: Ba^{2+}, 5.57; Sr^{2+}, 5.86; Ca^{2+}, 6.63; Mg^{2+}, 7.90. The complexation of calcein was also inves-

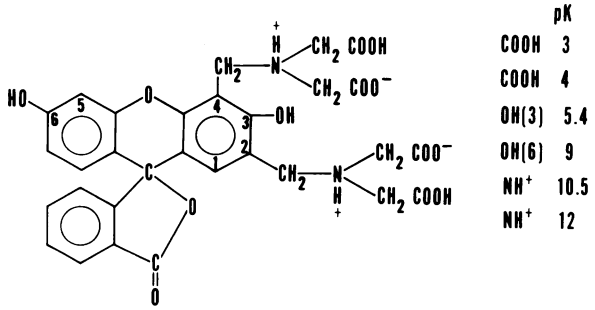

FIGURE 1 Structure and pK values of calcein dye (ref. 14).

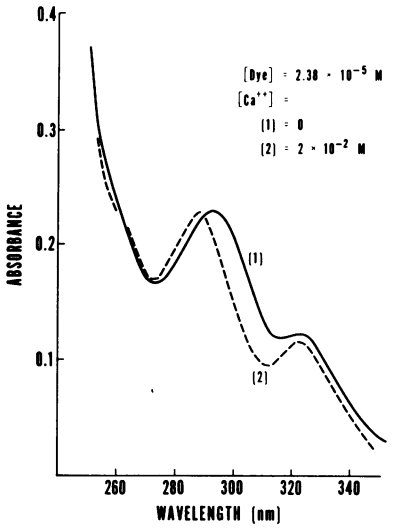

FIGURE 2 Absorption spectra of free dye and Ca^{2+}-complexed dye. Buffer medium: 0.1M Tris-HCl, pH 7.35; 1 = cm cell.

tigated by Bozhevolnov and Kreingold (16), who found evidence only for 1:1 complexation and determined the Ca^{2+} binding constant (K). They reported log (K) = 6.8 ± 0.3 for Ca^{2+}, in good agreement with the log (K'') value reported by Wallach and Steck (15).

In our own complexation experiments with a commercial sample of calcein, we found evidence for two types of complexation, as judged from the change in the fluorescence upon titration with Ca^{2+}. Electrophoresis at pH 12 indicated that the sample was an approximately 50:50 mixture of two fluorescent materials, with one having a mobility 37 ± 10% higher than the other. Since this difference was substantially reduced at pH 8.5, we conclude that we are dealing with two materials that differ either in the pK or in the presence of a titratable group. The difference could be explained as the difference in the pK of one of the NH^+ groups. These could be due to differences in the positioning of the methyl amino diacetic acid groups (2–4 vs. 4–5 isomerization, for example). As is discussed below, these differences in structure and electrophoretic mobility are also reflected in differences in complexation behavior. While Wallach and Steck found calcein dye sensitive to divalent cation complexation at pH 12.5 when excited at 495 nm wavelength, we reported here that calcein dye can also be used as Ca^{2+} indicator at physiological pH when excited at 305 nm.

Fig. 2 shows that the absorption spectrum of the mixture changes upon Ca^{2+} complexation at pH 7.3. The 293 nm peak of the aromatic OH is shifted to 285 nm, giving an appreciable change in extinction coefficients at 305 nm from 7.06 × $10^3 cm^{-1}M^{-1}$ to 4.75 × $10^3 cm^{-1}M^{-1}$. Figs. 3 and 4 show that this change upon complexation is also manifested in the fluorescence of the indicator excited at 305 nm. The fluorescence intensity decreases about 40%, whereas with excitation at other wavelengths

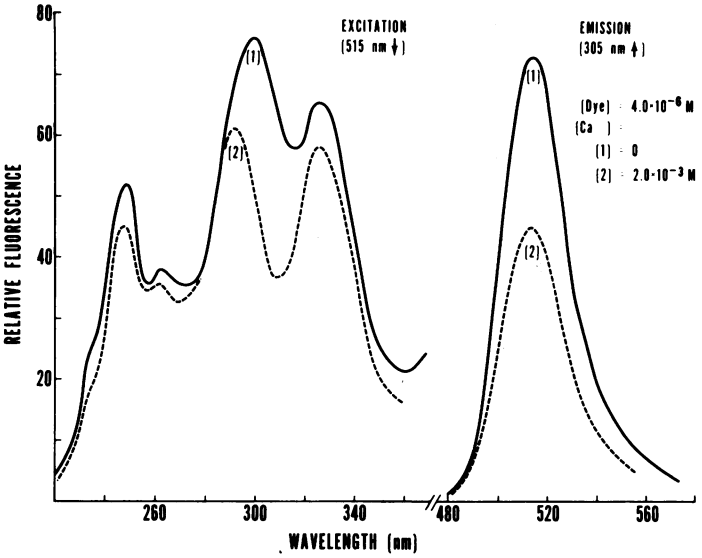

FIGURE 3 Uncorrected fluorescence excitation and emission spectra of free dye and Ca^{2+} complexed dye. Buffer medium: 0.1 M Tris-HCl, pH 7.35.

only a small difference is noted. Similar behavior was observed for Mg^{2+}. We have determined the affinity of the two components of the mixture by analyzing the dependence of the fluorescence signal on the concentration of added Ca^{2+}. Scatchard plots of the fractional decrease in fluorescence ($\Delta Fl/\Delta Fl_0$) at pH 7.35 and the Ca^{2+} concentration added showed biphasic behavior. The first phase represented about 42% of the total change of fluorescence and gave a binding constant of 2.26×10^4 M^{-1}. The second phase, which accounted for the remaining 58% of the maximal change in fluorescence, gave a binding constant of 5.04×10^3 M^{-1}. The mixture thus demonstrates about equal fluorescent changes for the high-affinity binding and the lower-affinity

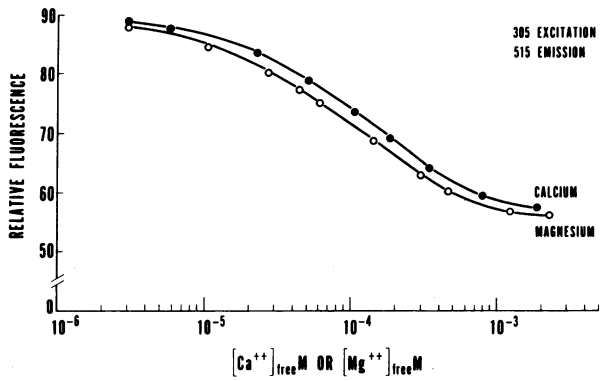

FIGURE 4 Change of fluorescence as a function of calcium or magnesium concentration. Buffer medium: 10 mM histidine, 1.06×10^{-6}M calcein dye, pH 7.3.

binding. Further experimentation was directed towards characterizing the lower-affinity form.

The measured binding constant, K_{app}, was found to increase with increasing pH, suggesting to us that we were dealing with multiple binding and protonation equilibria of the form:

$$\begin{array}{ccccc} M^{2+} + H_3D^{3-} & \xrightleftharpoons{K_1} & MH_3D^- & & \\ \Big\uparrow\downarrow K_2 & & \Big\uparrow\downarrow K_3 & & \\ M^{2+} + H_2D^{4-} + H^+ & \xrightleftharpoons[K_4]{} & MH_2D^{2-} + H^+ & & \end{array} \qquad (3)$$

where K values are the equilibrium constants for complexation and protonation reactions. The pK_2 is the pK value of the more basic aromatic OH group (cf. Fig. 1). For the limiting case where $K_2 \gg K_3$, $K_4 \gg K_1$ the cation binding will be accompanied by H^+ dissociation in an obligatory fashion, and the net reaction will be described as:

$$M^{2+} + H_3D^{3-} \overset{K_{2,4}}{\rightleftharpoons} MH_2D^{2-} + H^+, \qquad (4)$$

with

$$K_{2,4} \equiv ([MH_2D^{2-}][H^+])/([M^{2+}][H_3D^{3-}]) = K_4/K_2 \qquad (pH < pK_2). \qquad (5)$$

This would hold for pH values substantially below the fourth pK of 9.0 (i.e. pK_2 value). Since under this condition the spectrochemically determined apparent binding constant K_{app} would be equal to $[MH_2D^{2-}]/([M^{2+}][H_3D^{3-}])$, we would expect:

$$\log (K_{app}) = \log (K_{2,4}) + pH \qquad (pH < pK_2) \qquad (6)$$

Fig. 5 shows this type of linearity, indicating that the complexation of Ca^{2+} and Mg^{2+} does indeed proceed according to the mechanism of Eq. 4 and that a change of one pH unit increases K_{app} 10-fold.

The description of the interaction of Mn^{2+} with the dye is more complex. At high concentrations Mn^{2+} quenches the fluorescence of the dye completely. At low concentrations, the quenching is greater than can be accounted for by complexation alone. This indicates that Stern-Volmer quenching makes a significant contribution to the total fluorescence decrease.

In summary, the Ca^{2+}/Mg^{2+} specificity of the calcein dye for both low affinity and high affinity bindings is small. The commercial preparations of calcein dye seem to be an approximately 50:50 mixture of two isomers which give nearly equal fluorescence changes and which differ by a factor of four in complexation constant. In the next section, we describe experiments that make use of these properties of the mixture to determine the degree of binding of Ca^{2+} to the sarcoplasmic reticulum.

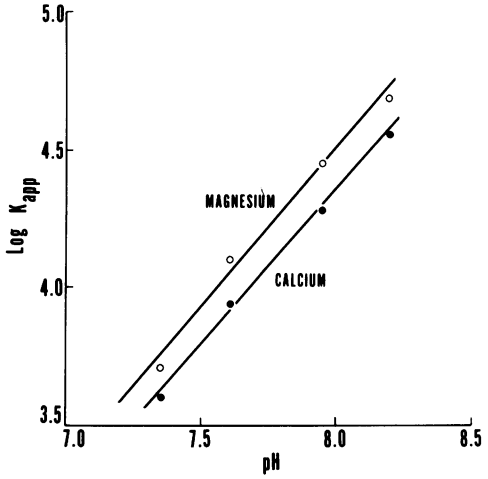

FIGURE 5 Log K_{app} of calcein dye as a function of pH.

Divalent Cation Binding to SR

Centrifugation experiments showed that calcein does not bind to the SR; the indicator is thus competent to report Ca^{2+} binding to the SR. Experimentally, the amount of divalent metal ions bound to sarcoplasmic reticulum was obtained by subtracting the fluorometric titration curves with and without sarcoplasmic reticulum. The indicator

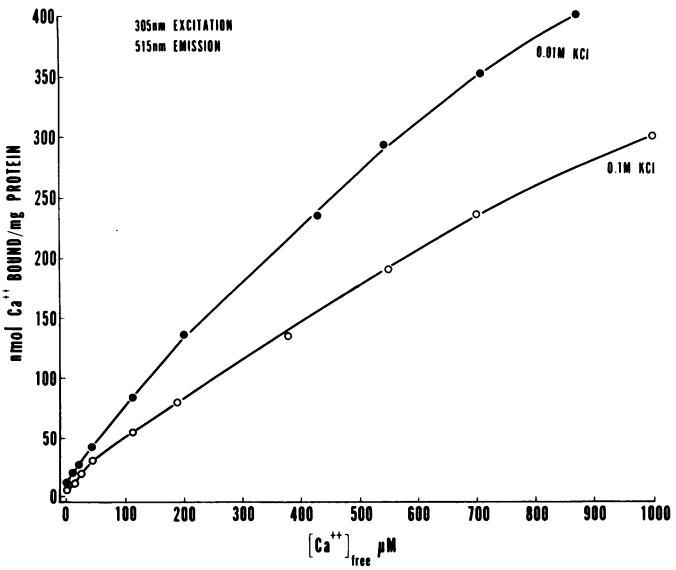

FIGURE 6 Passive calcium binding by the sarcoplasmic reticulum at 25°C as a function of the free $[Ca^{2+}]$. Medium contains 1.79×10^{-6} calcein dye, 10 mM histidine (pH 7.4), 0.8 mg/ml protein, and KCl concentration as indicated in the figure.

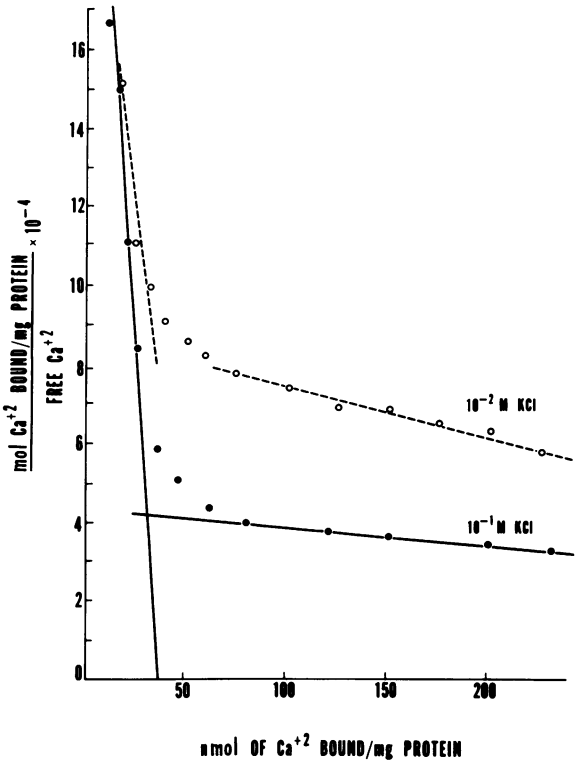

FIGURE 7 Scatchard plot for the binding of Ca^{2+} by sarcoplasmic reticulum. The medium was identical to that of Fig. 6.

concentration was low enough to not influence the free Ca^{2+} concentration, and under our conditions $\Delta Fl/\Delta Fl_{max}$ was free from extraneous influences such as light scattering changes. Fig. 6 gives the binding isotherm of Ca^{2+} to SR. Figs. 7 and 8 are Scatchard plots of Ca^{2+} and Mg^{2+} binding data, obtained in this manner, resolving high and low affinity binding sites. The numbers of sites in each category and their dissociation constants, K_d, are given in Table I. The significance of these data is discussed in a later section.

It occurred to us that a portion of this binding might represent a Donnan effect (cf. ref. 18) of the fixed negative charges of the acidic proteins within the SR matrix rather than actual binding. A Donnan potential could preferentially concentrate Ca^{2+} within the matrix and give the appearance of binding. The ion distributions in a Donnan equilibrium are governed by distribution constant r where:

$$r \equiv ([M^{+z}]_i/[M^{+z}]_o)^{1/z} = ([A^{-z}]_o/[A^{-z}]_i)^{1/z} \quad (7)$$

The subscripts i and o represent the inside and outside of SR. For the case of a fixed concentration of negative sites $[X^-]_i$ within the matrix and variable $[K^+]_o$ and $[Ca^{2+}]_o$

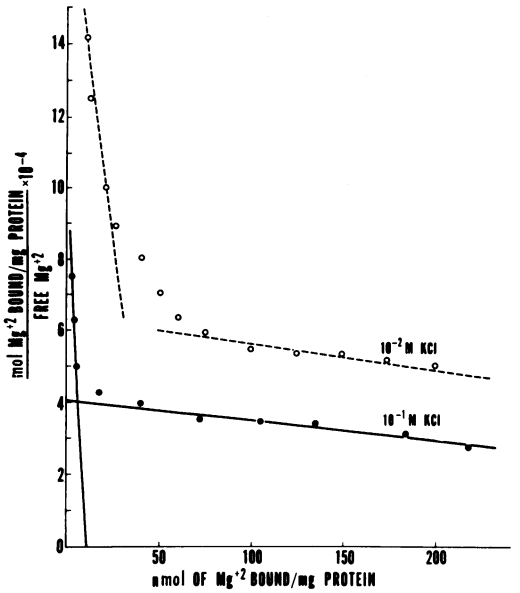

FIGURE 8 Scatchard plot for the binding of Mg^{2+} by sarcoplasmic reticulum. The medium was identical to that of Fig. 6.

TABLE I

COMPARISONS OF CALCIUM, MAGNESIUM BINDING SITES

Cation bound	Ionic strength	High affinity		Low affinity	
		n*	K_d‡	n	K_d
	M KCl	*nmol/mg SR protein*	*μM*		*mM*
Ca^{2+}	0.01	45	17.5	890	1.09
		(58.7)§		(633)	
Ca^{2+}	0.1	35	17.5	820	1.90
		(1.02)§		(88.4)‖	
Mg^{2+}	0.01	33	10.0	880	1.41
		(58.7)§		(633)‖	
Mg^{2+}	0.1	13	14.7	720	1.71
		(1.02)§		(88.4)‖	

*Amount of cations bound, evaluated from Scatchard plots.

‡Dissociation constants for each binding site, evaluated from Scatchard plots.

§Maximal Donnon equilibrium contribution to binding for $1.0 \times 10^{5-}$M Ca^{2+}, taking the fixed negative charge as 2×885 nmol/mg protein and matrix water as 5.0 μl/mg protein (37). The value of $[x^-]$ becomes 0.354 M.

‖ Maximal Donnan equilibrium contribution to binding for $1.0 \times 10^{3-}$M Ca^{2+}, taking the fixed negative charge as 2×885 nmol/mg protein and matrix water as 5.0 μl/mg protein (37). The value of $[x^-]$ becomes 0.354 M.

in the medium one obtains:

$$r = \frac{\{[K^+]_o^2 + 8[Ca^{2+}]_o([K^+]_o + 2[Ca^{2+}]_o + [X^-]_i)\}^{1/2} - [K^+]_o}{4[Ca^{2+}]_o} \quad (8)$$

The total concentration of Ca^{2+} held in Donnan equilibrium within the matrix, Ca_{de}^{2+} in nanomoles per milligram SR protein, will be given as:

$$Ca_{de}^{2+} = [Ca^{2+}]_i V_{matrix} = [Ca^{2+}]_o r^2 V_{matrix} \quad (9)$$

The maximal contributions of Donnan equilibrium to each class of binding site were evaluated with our estimates of fixed negative charge and literature values of the volume of matrix matter. Those estimates are given in parentheses in Table I. Except for the case of low ionic strength, the contributions are minor (maximum contribution 12%), indicating that both Ca^{2+} and Mg^{2+} are physically bound to the acidic proteins within the SR matrix. The small effect of [KCl] on the K_d values is probably the result of an ionic strength effect on the activity coefficients of the Ca^{2+} and the binding sites. The calculated values of r were between 4.2 and 34, suggesting that under our experimental conditions the Donnan potential is less than −90 mV.

Interpretation of Divalent Cation Binding to SR

Chevallier and Butow (10)[1] performed Ca^{2+} binding studies of SR at 4°C using equilibrium dialysis. In addition to finding 850 nmol/mg protein SR of nonspecific binding sites, with a dissociation constant of 0.32 mM, they found evidence for two types of high-affinity Ca^{2+} binding sites in the presence of 0.6 M KCl and 1 mM $MgCl_2$ at pH 6.5. The sites have the following capacities, n, and dissociation constants, K_d: n = 10 nmol/mg protein, K_d = 1.3 μM; n = 90 nmol/mg, K_d = 32 μM. Considering the differences in temperature and ionic composition and possible differences in the preparation, we found no serious discrepancy between their and our data. Meissner (19)[1] determined the Ca^{2+} binding of purified Ca^{2+} pump protein in the presence of 1 and 5 mM Mg^{2+} at 0°C using a dialysis technique. He determined a binding capacity of about 14 nmol/mg pump protein with a K_d value between 3.8 and 5.6 μM. The binding capacity corresponds to 1.4 mol Ca^{2+}/mol of pump protein. Using a dialysis technique on purified ATPase, Ikemoto (20) under similar conditions found three classes of binding sites with the following stoichiometries, n for the number of moles of Ca^{2+} per mole of ATPase protein, and K_d values for dissociation constant: n = 0.7, K_d = 0.26 μM; n = 1.0, K_d = 26 μM; n = 1.3, K_d = 1 mM. Since the enzyme has been shown to transport two Ca^{2+} for every ATP split (2, 3), a minimal high-affinity binding capacity of two Ca^{2+} per molecule of enzyme is expected if the latter functions according to a simple Wilbrandt-Rosenberg carrier mechanism (22).

[1] Experiments of Chevallier and Butow and of Meissner were carried out by equilibrium dialysis lasting up to 24 h. With our method, the measurement of the binding required only a few seconds. Fiehn and Migala presented only an amount of Ca^{2+} bound at a fixed Ca^{2+} concentration. Neither number of binding sites nor dissociation constants was reported. The results were obtained in the presence of high Mg^{2+} concentrations (5 mM).

TABLE II

CALCULATION OF THE CONCENTRATIONS OF Ca^{2+} BINDING SITES

Types of Ca^{2+} binding sites	K_d	Binding capacity	
		Lower limit	Upper limit
		nmol Ca^{2+} bound/mg protein	
ATPase	1–15 μM	4.0[a]	13.2[b]
Acidic proteins	0.78–5.0 mM	162.5[c]	836.[d]
Phospholipids	0.1–1.0 mM	45.0[e]	

Total sarcoplasmic reticulum binding capacity: lower limit, 221 nmol/mg SR protein (addition of *b*, *c*, and *e*); upper limit, 885 nmol/mg SR protein (addition of *a*, *d*, and *e*).

a: based on 20% ATPase/mg SR protein (27, 28); two Ca^{2+} bind to one ATPase (3, 2).

b: based on 66% ATPase/mg SR protein (37); two Ca^{2+} bind to one ATPase (3, 2). ATPase mol wt = 10^5.

c: based on the lowest binding capacity of acidic protein, M55 protein. mol wt = 55,000 (4). Assume 34% of this protein and 66% of ATPase.

d: based on the maximum binding capacity of acidic protein, mol wt = 33,000–38,000 (4). Assume 80% of this protein and 20% of ATPase.

e: based on one Ca^{2+} bound per two negatively charged lipids (11) and reported lipid composition of the SR (30).

For a simple carrier model in which complexation and decomplexation do not represent the rate-limiting steps in the net transport reaction, the K_m value of the carrier system is identical to the dissociation constant of the permeant. A study of Duggan (23) shows that an external Ca^{2+} concentration of about 15 μM gives half-maximal rate of ATP-supported Ca^{2+} uptake in the presence of 0.1 M K^+. This value can be compared with the K_m value of 12 μM reported for rat SR by Worsfold and Peter (24). Kanazawa et al. (25) have proposed that the mechanism of Ca^{2+}-catalyzed increase in rate of phosphorylation of the pump protein involves the same Ca^{2+} binding process that leads to transport. They found that the rate of E-P formation had a second-power dependence on the external Ca^{2+}, with the concentration for half-maximal effect ranging between 0.93 and 16.7 μM. The uncertainty as to the exact value was due to their uncertainty as to the exact value of the dissociation constant for EGTA (0.1 – 2.0 μM), which they used to control the external Ca^{2+} concentration. Taken together, the above data indicate that the K_d value for the Ca^{2+} – ATPase complex should be in the range of 1–15 μM.[2] We would thus assign our observed 35 nmol/mg high-affinity Ca^{2+} binding sites with K_d = 17.5 μM to the Ca^{2+} pump protein. Table II shows that the capacity of this class of sites is in line with the reported concentrations of pump protein within the SR.

In the studies of Chevallier and Butow (10), Meissner (19), and Ikemoto (20) and Fiehn and Migala (8),[1] Ca^{2+} binding that occurred in the presence of millimolar Mg^{2+} concentrations was considered specific. In the present study, Mg^{2+} and Ca^{2+} binding were measured separately. Table I shows that there are 35 nmol/mg high-affinity sites

[2]Yamamoto and Tonomura (26) determined a K_m of 0.4 μM for Ca^{2+} activation of SR ATPase. However, this constant was derived from experiments using sub-millimolar concentrations of ATP and thus cannot be compared directly with the findings for Ca^{2+} transport cited above.

for Ca^{2+} and 13 nmol/mg high-affinity sites for Mg^{2+} in our SR preparations. It is possible that these sites overlap so that only 22 nmol/mg of them are Ca^{2+}/Mg^{2+} specific. This value would be quite close to the expected value for the simple carrier model discussed above. Meissner (19) found some Mg^{2+} binding on the purified ATPase in the presence of 0.13–0.65 mM Ca^{2+}. Kanazawa et al. (25) showed that Mg^{2+} catalyzes the dephosphorylation of the pump protein and reported a K_m of 51 μM for this effect. This can be taken as evidence for high-affinity binding of at least one Mg^{2+} on the phosphoenzyme.[3] We suggest that the high-affinity Mg^{2+} binding we observed here has significance to the Ca^{2+} for Mg^{2+} exchange for the deenergized form of the carrier (the form studied here) and will be of importance to kinetics of the overall exchange reaction. Such competition is an important property of the carrier mechanism postulated by Kanazawa et al. (25) to explain the kinetic behavior of the ATPase phosphorylation and dephosphorylation (Yamada and Tonomura [31]). Our high-affinity Ca^{2+} binding constant in the absence of Mg^{2+} is at least a factor of three smaller than these values reported in the presence of Mg^{2+}. This is probably due to the absence of Mg^{2+} under our conditions and temperature difference, and may indicate allosteric interation between the Ca^{2+} and Mg^{2+} binding sites. This problem is under investigation presently with the ATPase-rich fraction.

Ca^{2+} Binding on Acidic Proteins and Lipids

As described in the introduction, the SR contains three major protein components in addition to the ATPase. Ostwald and Maclennan (32) have shown that these components in the isolated form can bind between 478 and 1,045 nmol/Ca^{2+} per mg of isolated protein, with dissociation constants ranging from 0.78 to 5.0 mM.[4] Considering these proteins to represent the remainder of the non-ATPase protein and using the above upper and lower limits for their binding capacity, we have estimated their expected contribution to the total nonspecific binding capacity. Table II estimates the lower and upper limit for the contribution of these acidic proteins as between 162.5 and 836 nmol/mg SR protein, respectively.[5] Our finding of 820 nmol/mg protein and the

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[3]In a subsequent study, Yamada and Tonomura (31) found that in purified ATPase the Mg^{2+} effect was completely inhibited by Ca^{2+} and that the ratio of the Mg^{2+} K_m to the Ca^{2+} K_m was 2.45. If it is proper to combine the results on fragmented SR with those of the purified ATPase, we estimate that the inhibitory dissociation constant for the Ca^{2+} is 2.08 μM on the phosphoenzyme. However, since the measured rate constant of phosphoenzyme breakdown (0.12 s^{-1}), a partial reaction of the overall transport process, is much lower than the measured turnover number, caution must be exercised in comparing these results.

[4]Meissner et al. (29) studied the "nonspecific" Ca^{2+} binding to the acidic binding protein using an equilibrium dialysis technique, and determined a capacity of 900–1,000 nmol-Ca^{2+}/mg protein and a dissociation constant between 3.3 and 5.0 μM. Since the experiment was carried out at low ionic strength (1 mM HEPES buffer) the K_d value could have been influenced by the Donnan effects. We have therefore not used this value to define the lower limit of the K_d.

[5]It is possible that the binding studies in solution underestimate the total binding capacity of the same proteins in the SR. The maximal Ca^{2+} binding reported for calsequestrin at high ionic strength by Ostwald and MacLennan (32) was 40 Ca^{2+}/molecule. However, total number of free carboxyl groups (taken as the sum of the glutamic and aspartic acid residues) was 146. If all of the carboxyl groups could be used in 2:1 complexation, the number of binding sites would be 73.

finding of Chevallier and Butow (10) of 850 nmol/mg SR protein represent the upper end of this range, while the binding capacities determined by Carvalho and Leo (7) represent the lower end. The recent work of Meissner (5) on compositional heterogeneity of the SR indicates to us that the high and low affinity binding capacities will have to be determined for isopycnically separated fractions.

Acidic phospholipids probably make only a small contribution to the total low-affinity binding. Meissner and Fleischer reported the phospholipid content of the SR as 72.7% phosphatidyl choline, 13.5% phosphatidyl ethanolamine, 8.7% phosphatidyl inositol, 1.8% phosphatidyl serine, 0.3% cardiolipin, 0.2% phosphatidic acid, and 1.8% unidentified phospholipids (33). These authors gave a phospholipid-to-protein ratio of 0.65 mg/mg. Assuming an average mol wt of 700, we would expect that the concentration of phosphatidyl inositol plus phosphatidyl serine to be 90 nmol/mg SR protein. Using the binding of Ca^{2+} to phosphatidic acid (11) as an example, we would

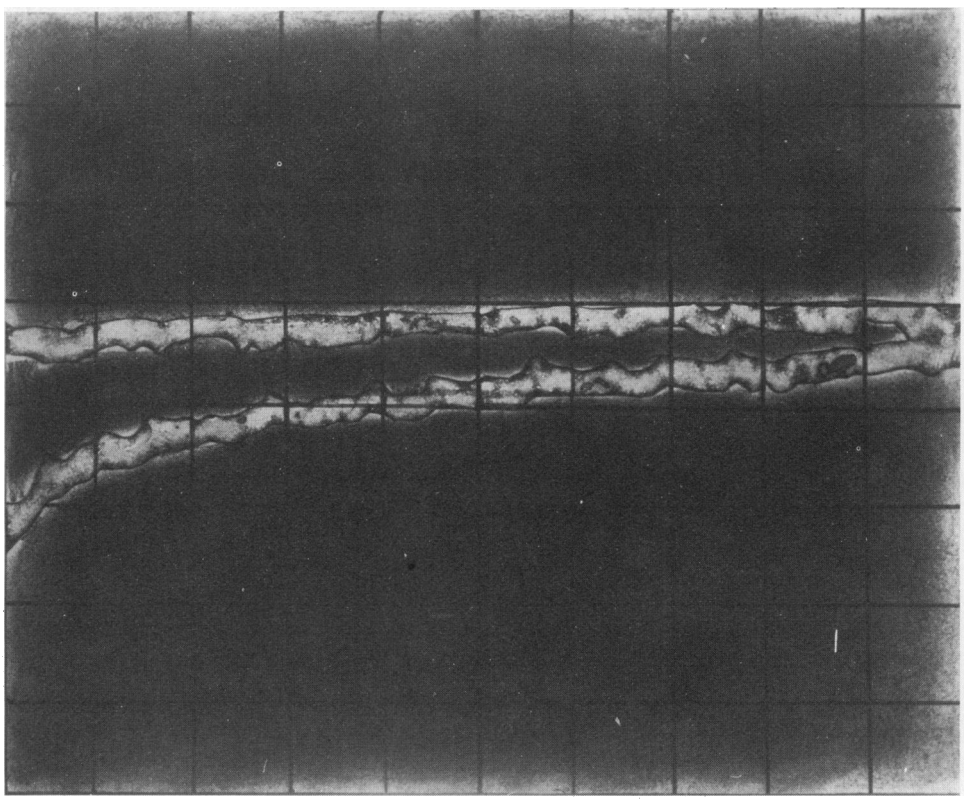

FIGURE 9 Stopped flow trace of passive Ca^{2+} binding at 25°C with calcein as calcium fluorescent indicator. Reservoir A contained 2.0 mg/ml of sarcoplasmic reticulum protein, 0.25 M sucrose and 10 mM KCl, adjusted pH 6.65. Reservoir B contained 2×10^{-4}M Ca^{2+}, 0.25 M sucrose, 30 mM histidine, pH = 7.0, 10 mM KCl, 10 μM calcein dye. The trace has been retouched. The horizontal sensitivity is 1 s/division. The vertical scale is relative fluorescence intensity with 305-nm excitation and 515-nm emission.

expect a maximal binding stoichiometry of one Ca^{2+} per two acidic phospholipids and an apparent K_d value between 10^{-4} and 10^{-3}M. The maximal contribution of the phospholipids to the nonspecific binding would be 45 nmol/mg SR protein accounting for about 5% of the total nonspecific binding.

Kinetics of the Passive Binding Reaction

The equilibrium binding studies described above were carried out as a preliminary to the study of the kinetics of Ca^{2+} transport via the Ca^{2+} pump protein and Ca^{2+} binding to the acidic proteins. Fig. 9 shows a stopped flow experiment measuring the time-course of passive Ca^{2+} uptake by the SR, with the calcein mixture as an indicator. The $t_{1/2}$ of maximal initial fluorescence change of the indicator was 2.2 s. Under the condition of Fig. 9, this corresponds to an initial rate of Ca^{2+} binding of 34 ± 2 nmol Ca^{2+} mg^{-1} s^{-1}. Comparison with equilibrium experiments under the same condition shows that most of the fluorescence change corresponds to Ca^{2+} binding to the low-affinity sites. The reaction goes to completion within 4 s.

To check the above results, we also used arsenazo III as an absorption indicator of calcium in aqueous phase. Complexation of this dye with Ca^{2+} results in a considerable increase in extinction coefficient in 595 nm and 650 nm. ($\Delta\epsilon_{595}$ = 4.00 × 10^3 M^{-1} cm^{-1}, $\Delta\epsilon_{650}$ = 2.40 × 10^3 M^{-1} cm^{-1}) The dye forms a 1:1 complex with calcium at physiological pH (21, 34). We have determined the binding constant to be 9.19 × 10^3 M^{-1} in the presence of 0.25 M sucrose, 0.1 M Tris-HCl at pH 7.8. Centrifugation experiments showed that the dye does not bind to the SR. Fig. 10 shows a stopped flow experiment using arsenazo III to report the passive binding of Ca^{2+} by SR. Under the conditions described in Fig. 10, we found the change of amplitude corresponds to 172 nmol Ca^{2+} bound/mg SR protein, in good agreement with the results of Fig. 6 obtained using calcein as indicator. The initial rate of Ca^{2+} binding with 5 × 10^{-4}M external Ca^{2+} is 39 ± 2 nmol s^{-1}/mg SR protein. Further analysis indicated arsenazo III dye reports the same calcium binding and transport rates as calcein dye.

We have carried out experiments similar to those reported in Fig. 6 as a function of pH and found H^+ competes with Mg^{2+} and Ca^{2+} for binding (7). It was of interest to study and compare the rate of proton permeation with Ca^{2+} permeation. In the absence of Ca^{2+}, arsenazo III can be used as a pH indicator. The absorbance of the dye decreases at 540 nm and increases at 650 nm as a function of increasing pH. We have carried out experiments (analogous to the Ca^{2+} permeation experiments) in which the internal binding sites were reacted with H^+ rather than Ca^{2+}. The stopped flow experiments indicate that H^+ is transported at rates comparable to Ca^{2+} transport.

The experiments described above allow us to draw two important conclusions: (*a*) that the low-affinity binding sites are located behind a permeability barrier, and (*b*) that the rate of movement of Ca^{2+} across this permeability barrier is comparable to that of active transport. The former conclusion is the result of a consideration of the rate constants for simple Ca^{2+} binding reactions. In the absence of any cooperative or strongly anticooperative effects on the negatively charged proteins, the binding reac-

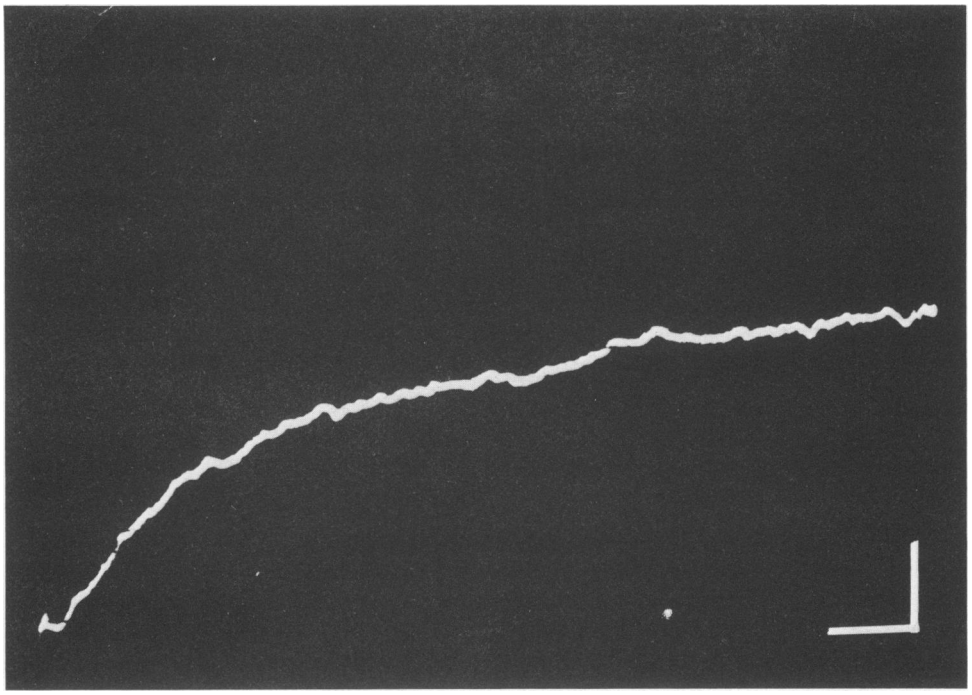

FIGURE 10 Stopped flow trace of passive Ca^{2+} binding at 25°C with arsenazo III as a Ca^{2+} absorption indicator. Reservoir A contained 1.41 mg/ml SR protein, 0.25 M sucrose, 10 mM KCl, 0.1 M Tris-HCl, pH = 7.8. Reservoir B contained 1 mM Ca^{2+}, 0.25 M sucrose 10 mM KCl, 0.1 M Tris-HCl, pH = 7.8, and 1.0×10^{-4}M arsenazo III dye. The horizontal sensitivity is s/division. The vertical scale is sample transmission, 0.43 %T (20 mV) per scale division. Absorption wavelength is 650 nm.

tion would be identical to the sum of the reactions of the individual binding sites, B. The reactions would thus be described as

$$Ca^{2+} + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ca^{2+} - B \tag{10}$$

The reciprocal lifetime ($1/\tau$) of the unoccupied binding site under irreversible conditions (with an excess of Ca^{2+}) would be given by:

$$1/\tau = k_1[Ca^{2+}] \tag{11}$$

Characteristic values of k_1 for the simple liganding reactions of Ca^{2+} in absence of slow conformational changes in the liganding molecule are of the order of 10^8 M^{-1} s^{-1} (17). This predicts that the lifetime of the unoccupied low-affinity binding site under the conditions of our stopped flow experiment would be about 10^{-4} s, a value four orders of magnitude smaller than that actually observed (about 4 s). The obvious explanation for this discrepancy is that the low-affinity binding sites are located within the SR matrix behind a permeability barrier.

Our conclusion that the Ca^{2+} enters via the ATPase is based on a quantitative comparison of rate data. Our measurement of the initial rate of Ca^{2+} binding (34 ± 2 nmol mg^{-1} s^{-1}) for the passive reaction is close to the initial rate of the transport reaction (60–70 nmol mg^{-1} s^{-1}) measured by Inesi and Scarpa (1), using murexide as a Ca^{2+} indicator in a specially designed stopped flow mixing apparatus. The obvious conclusion is that under both conditions, the Ca^{2+} is transported across the membrane by the same carrier (permease) system, viz. the Ca^{2+} ATPase molecule. Our observation of comparable rates of Ca^{2+} and H^+ permeation can be taken as evidence that H^+ also permeates via the same carrier mechanism. Further conclusions are that the maximal rate of turnover of the ATPase is relatively independent of whether the permease is operating in the passive or in the active transport mode, and that if the ATPase is indeed engaged in counter-transport, the rate of turnover is not extremely dependent of the nature of the counter-transported ion (Mg^{2+}, K^+, H^+). Using the upper and lower estimates of the membrane ATPase concentration cited in a previous section, we calculate the average value of the turnover number of the ATPase as between 3 and 9 Ca^{2+}/s. Artifacts such as membrane leakage, which may by coincidence give passive Ca^{2+} binding rate like that of active transport, are ruled out by three experiments: (*a*) Calcium active uptake experiment with a chlorotetracycline membrane probe (35) indicated that back reaction (i.e. calcium leakage) after maximal uptake does not occur until about 7 min later. (*b*) Active and passive calcium uptake processes can be enhanced by oxalate. (*c*) The rate of ANS^- permeation of SR vesicles from the outside membrane surface to the inside membrane surface is comparable to the rate performed on closed phospholipid vesicles.[6] We note that our SR preparation represents a collection of vesicles with a distribution of ratios of ATPase to low-affinity binding proteins (5), and that this heterogeneity will affect the time-course of the later phases of both the passive and active uptake.

Carvalho (36) has reported Millipore filtration experiments that showed that the bulk of the passive Ca^{2+} binding occurs rapidly and that a small passive uptake of about 40 nmol/mg Ca^{2+} can be entrapped or bound with a half-time of about 14 h. In his experiments, binding was determined by an assay involving preincubation with 20 mM Ca^{2+}, 20-fold dilution to a final concentration of 2 mM Mg^{2+}, 1 mM Ca^{2+}, followed by Millipore filtration and two washes with 0.24 M sucrose. This technique does not afford time resolution comparable to that of the present study, and the finding of fast exchange under his conditions is not proof of his conclusion (36) that the bulk of the passive binding sites are located on the outside surface of the SR membrane. We suggest that the small amount of slowly exchanging Ca^{2+} (about 40 nmol/mg) results either from entrapment of Ca^{2+} by a small number of vesicular structures lacking a Ca^{2+} permeability mechanism or is the result of a Ca^{2+}-induced aggregation of the SR under his preincubation conditions (20 mM Ca^{2+}). Our measurements of

[6]Haynes, D. H., and P. Simkowitz. 1-Anilino-8-naphthalene sulfonate. A fluorescent probe of ion and ionophore transport kinetics and transmembrane asymetry. In preparation.

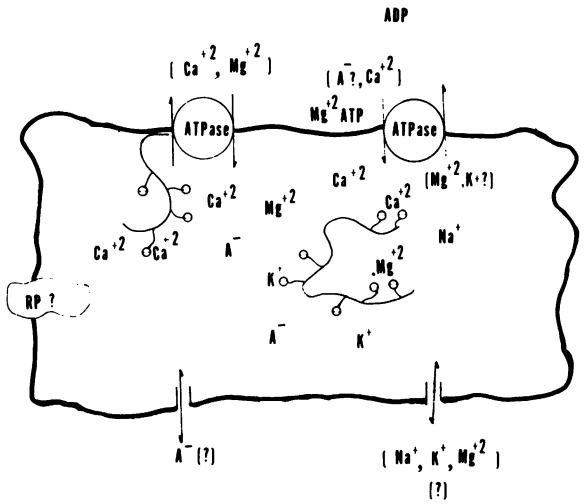

FIGURE 11 Schematic representation of SR permeability and nonspecific binding function. The boundary is a lipid bilayer, and the lines drawn in the boundary represent acidic proteins that may serve as storage sites for various cations (ie. nonspecific sites). A, anion channel, RP, calcium release protein. Ca^{2+}, actively transported against a concentration gradient and competing with other divalent cations such as Mg^{2+} and K^+, can either be counter-transported via the ATPase (25) or enter and exit from the matrix via their own permeability system. Similarly, permeating anions could enter (*a*) via the ATPase or (*b*) by a separate permeability system. In case (*b*) the various membrane permeabilities would be coupled through a SR membrane potential. Our review of the literature reveals no definite evidence on this question.

90° light scattering (not shown) indicate that aggregation of SR can be induced by increasing the Ca^{2+} concentration above 3 mM.

Our kinetic data, when taken together with the Ca^{2+} binding studies described in the previous sections, form the basis for our working model of the SR depicted in Fig. 11. The most important predictions of this minimal hypothesis deal with the permeability and compartmentation properties of the SR. Further embellishment of this model will require answers to whole new sets of specific questions about the permeability systems of the SR.

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REFERENCES

1. INESI, G., and A. SCARPA. 1972. Fast kinetics of adenosine triphosphate dependent Ca^{2+} uptake by fragmented sarcoplasmic reticulum. *Biochemistry.* **11**:356.
2. HASSELBACH, W., and M. MAKINOSE. 1963. Über den Mechanisms des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339**:94.

3. WEBER, A., R. HERZ, and I. REISS. 1966. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* **345**:329.
4. MACLENNAN, D. H., and P. C. HOLLAND. 1975. Calcium transport in sarcoplasmic reticulum. *Ann. Rev. Biophys. Bioeng.* **4**:377.
5. MEISSNER, G. 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta.* **389**:51.
6. CARVALHO, A. P. 1966. Binding of cations by microsomes from rabbit skeletal muscle. *J. Cell. Physiol.* **67**:73.
7. CARVALHO, A., and B. LEO. 1967. Effects of ATP on the interaction of Ca^{2+}, Mg^{2+}, and K^+ with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. Gen. Physiol.* **50**:1327.
8. FIEHN, W., and A. MIGALA. 1971. Calcium binding to sarcoplasmic reticulum. *Eur. J. Biochem.* **20**: 245.
9. CASWELL, A. H., and B. C. PRESSMAN. 1972. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292.
10. CHEVALLIER, J., and R. A. BUTOW. 1971. Calcium binding to the sarcoplasmic reticulum of rabbit skeletal muscle. *Biochemistry.* **10**:2733.
11. HAYNES, D. H. 1974. 1-anilino-8-naphthalenesulfonate: a fluorescent indicator of ion binding and electrostatic potential on the membrane surface. *J. Membr. Biol.* **17**:341.
12. MARTONOSI, A., and R. FERETOS. 1964. Sarcoplasmic reticulum the uptake of Ca^{2+} by sarcoplasmic reticulum fragments. *J. Biol. Chem.* **239**:648.
13. DIEHL, H., and J. L. ELLINGBOE. 1956. Indicator for titration of calcium in presence of magnesium with disodium dihydrogen ethylenediaminetetraacetate. *Anal. Chem.* **28**:882.
14. WALLACH, D. F. H., D. M. SURGENOR, J. SODERBERG, and E. DELANO. 1959. Preparation and properties of 3,6-dihydroxy-2,4-bis-[N, N'-di-(Carboxymethyl)-aminomethyl] fluoran. *Anal. Chem.* **31**: 456.
15. WALLACH, D. F. H., and T. L. STECK. 1963. Fluorescence techniques in the microdetermination of metals in biological materials. *Anal. Chem.* **35**:1035.
16. BOZHEVOLNOV, E. A., and S. U. KREINGOLD. 1962. Fluorescent complexometric determination of microamounts of calcium. *Zh. Anal. Khim.* **17**:560; *J. Anal. Chem.* **31**:456.
17. CZERLINSKI, G. H., H. DIEBLER, and M. EIGEN. 1959. Relaxation investigation on the kinetics of metal complexes formation. *Z. Physik. Chem.* **19**:246.
18. DAWES, E. A. 1965. Quantitative problems in biochemistry. The Williams and Wilkins Company, Baltimore, Md. 61.
19. MEISSNER, G. 1973. ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum. *Biochim. Biophys. Acta.* **298**:906.
20. IKEMOTO, N. 1974. The calcium binding sites involved in the regulation of the purified adenosine triphosphatase of the sarcoplasmic reticulum. *J. Biol. Chem.* **249**:649.
21. BUDESINSKY, B. 1969. *Chelates Anal. Chem.* **2**:1.
22. WILBRANDT, W., and T. ROSENBERG. 1961. The concept of carrier transport and its corollaries in pharmacology. *Pharmacol. Rev.* **13**:109.
23. DUGGAN, P. F. 1974. The role of potassium ion in the calcium-transport system of sarcoplasmic reticulum. *Biochem Soc. Trans.* **2**:1380.
24. WORSFELD, M., and J. B. PETER. 1970. Kinetics of calcium transport by fragmented sarcoplasmic reticulum. *J. Biol. Chem.* **245**:5545.
25. KANAZAWA, T., S. YAMADA, T. YAMAMOTO, and Y. TONOMURA. 1971. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biochem. (Tokyo).* **70**: 95.
26. YAMAMOTO, T., and Y. TONOMURA. 1967. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biochem. (Tokyo).* **62**:558.
27. IKEMOTO, N., G. M. BHATNAGAR, and J. GERGELY. 1971. Fractionation of solubilized sacroplasmic reticulum. *Biochem. Biophys. Res. Commun.* **44**:1510.
28. MARTONOSI, A., and R. A. HALPIN. 1971. Sarcoplasmic reticulum. The protein composition of sarcoplasmic reticulum membranes. *Arch. Biochem. Biophys.* **144**:66.
29. MEISSNER, G., G. E. CONNER, and S. FLEISCHER. 1973. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} pump and Ca^{2+}-binding proteins. *Biochim. Biophys. Acta.* **298**:246.

30. MEISSNER, G., and S. FLEISCHER. 1971. Characterization of sarcoplasmic reticulum from skeletal muscle. *Biochem. Biophys. Acta.* **241**:356.
31. YAMADA, S., and Y. TONOMURA. 1972. Reaction mechanism of the Ca^{2+} dependent ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biochem. (Tokyo).* **72**:417.
32. OSTWALD, T. J., and D. H. MACLENNAN. 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* **249**:974.
33. MEISSNER, G., and S. FLEISCHER. 1971. Characterization of sarcoplasmic reticulum from skeletal muscle. *Biochim. Biophys. Acta.* **241**:356.
34. MICHAYLOVA, V., and P. ILKOVA. 1971. Photometric determination of micro amounts of calcium with arsenazo III. *Anal. Chim. Acta.* **53**:194.
35. CASWELL, A. H. 1972. The migration of divalent cations in mitochondria visualized by a fluorescent chelate probe. *J. Membr. Biol.* **7**:345.
36. VALE, M. G. P., and A. P. CARVALHO. 1975. Utilization of X-537A to distinguish between intravesicular and membrane-bound calcium ions in sarcoplasmic reticulum. *Biochim. Biophys. Acta.* **413**:202.
37. DUGGAN, P. F., and A. MARTONOSI. 1970. Sarcoplasmic reticulum. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol.* **56**:147.