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Ca^{2+}-Binding Activity of Protein Isolated from Sarcotubular Membranes*

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ABSTRACT: The Ca^{2+}-binding activity of the protein fraction of rat skeletal muscle sarcotubular membranes, termed fraction 2, and accounting for 90% of the membrane protein has been investigated. Using a dual-wavelength spectrophotometer and murexide, a metallochromic indicator sensitive to Ca^{2+}, fraction 2 has been shown to have binding sites of both a high and low affinity for Ca^{2+}. The sites of fraction 2 with a high affinity for Ca^{2+} are characterized by an association constant greater than 10^5 M^{-1} and the low-affinity sites by an association constant of 10^4 M^{-1}. The high affinity of fraction 2 for Ca^{2+} is not markedly affected by protein or murexide concentrations nor by the temperature of the assay system. However, the high Ca^{2+} affinity of the membrane protein is affected by both pH and buffer concentration. Further, the Ca^{2+}-binding activity of fraction 2 is inhibited noncompetitively by the monovalent cations, Na^+ and K^+, and competitively by the divalent cation, Mg^{2+}. The Ca^{2+}-binding activity of fraction 2 is not enhanced in the presence of ATP. The similarity of Ca^{2+}-binding characteristics of fraction 2 and fragmented sarcoplasmic reticulum is discussed.

Sarcoplasmic reticulum, a complex tubular intracellular membrane system, is believed to play an important part in both the excitation–contraction coupling and the relaxation activities of skeletal muscle (Ebashi and Endo, 1968). The release of Ca^{2+} from its membranous structure or tubular lumen to the myoplasm (Winegrad, 1968, 1970), in response to excitation of the muscle, is the way the sarcoplasmic reticulum is envisioned to function in excitation–contraction coupling. This Ca^{2+} interacts with troponin (Fuchs and Briggs, 1968) to unfasten the troponin–tropomyosin molecular lock thus enabling the contractile interaction of actin with myosin to occur. The sequestering of Ca^{2+} by the sarcoplasmic reticulum either within its membranous structure or tubular lumen or both from the myoplasm depletes myoplasm and troponin of Ca^{2+} thus returning the muscle to the relaxed state

Muscle microsomes prepared from skeletal muscle homogenates by differential centrifugation contain membranous fragments of sarcoplasmic reticulum (which will be called sarcotubular membranes) along with other cellular structures (Hasselbach, 1964). The sarcotublar membranes are present in the form of closed vesicular structures (Hasselbach, 1964) which exhibit several biochemical activities believed to be related to their *in vivo* function such as the ATP-dependent, oxalate-enhanced active transport of large amounts of Ca^{2+} from the external medium into the aqueous spaces of the vesicular structure (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961), the $(Ca^{2+} + Mg^{2+})$-ATPase activity believed to energize this Ca^{2+} transport (Martonosi and Feretos, 1964), the capacity to bind Ca^{2+} to their membranous structure in the absence of ATP (ATP-independent Ca^{2+} binding) and the enhancement of this binding by ATP (ATP-dependent Ca^{2+} binding) (Carvalho and Leo, 1967).

Mommaerts (1967) suggested that sarcotubular membranes are highly specialized for the task of ejecting and resorbing massive amounts of Ca^{2+} and that they contain little protein not involved in this transport function. It is this high specialization which led us to postulate that these membranes might be of a simple enough molecular nature to provide experimental material ideally suited for the study of the molecular aspects of membrane structure and function.

A method was developed for the preparation of a highly purified population of sarcotubular membranes (Yu *et al.*, 1968a). This preparation as modified by Yu and Masoro (1970) provides sarcotubular membranes that appear to be almost entirely free of other cellular components (Deamer and Baskin, 1969; Yu and Masoro, 1970).

Solubilization of this purified sarbotubular membrane preparation (which we call SF_1) by treatment with sodium dodecyl sulfate permits more than 90% of the sarcotubular membrane protein to be recovered as a single fraction from Sepharose 4B column chromatography (Yu and Masoro, 1970). This fraction called fraction 2 is lipid poor, sodium dodecyl sulfate free, and composed of high molecular weight aggregates of low molecular weight species of polypeptide chains.

Of course, it is of interest to know if fraction 2 has any of the functional activities of the intact sarcotubular membranes. Obviously soluble proteins cannot transport Ca^{2+} across a membranous barrier. Moreover on the basis of other studies

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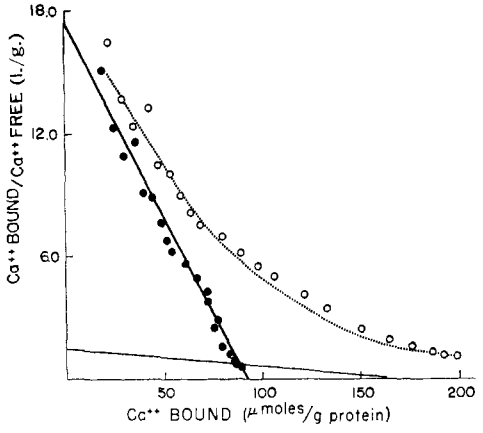

FIGURE 1: Scatchard plot of the Ca^{2+}-binding activity of fraction 2. Open circles, Scatchard plot of the Ca^{2+}-binding activity prior to any further analytical treatment; solid lines, computer breakdown of Scatchard plot by method of Danchin and Gueron; closed circles, Scatchard plot data points processed by computer according to the method of Danchin and Gueron; dotted line, theoretical Scatchard curve derived by the addition of the breakdown plots (solid lines) as processed by the computer program. The 2-ml assay incubation contained 0.96 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.8) at 25°. The temperature of assay was 30° and the $[Ca^{2+}]$ increased from 0 to 300 μM.

(Yu *et al.*, 1968b) it is clear that an extensive delipidation of the SF_1 membranes causes the total loss of their ATPase activity; since the protein of fraction 2 is almost lipid free it would not be expected to have ATPase activity. We therefore decided to study the Ca^{2+}-binding characteristics of fraction 2; as shown below fraction 2 contains high-affinity sites for the binding of Ca^{2+} which may well play an important role in the *in vivo* functional activity of the sarcoplasmic reticulum.

Materials and Methods

Materials. All chemicals were reagent grade and solutions were prepared in deionized-distilled H_2O. Hepes,[1] a buffer which does not bind Ca^{2+}, was purchased from Calbiochem, Los Angeles, Calif., and murexide from Dojinyaku Chemical Co. of Kumamoto, Japan. The $CaCl_2$ standard was purchased from Orion Research Inc. of Cambridge, Massachusetts.

Preparation of Fraction 2, the Ca^{2+}-Binding Protein. The major protein component of rat sarcotubular membranes called fraction 2 was prepared according to the method of Yu and Masoro (1970). The protein in fraction 2 was brought to the desired concentration by the commercially available S & S collodion bag filter process. The protein was then treated with 0.15 M KCl at neutral pH; this treatment frees the protein of bound Ca^{2+}. The KCl is then removed by dialyzing fraction 2 against a Ca^{2+}-free solution.

Determination of the Ca^{2+} Binding by Fraction 2. The Ca^{2+}-binding activity of fraction 2 is measured by the dual-wavelength spectrophotometric assay of Ohnishi and Ebashi (1963). The 1-cm light-path glass cuvet contains a 2-ml volume, 0.28 μmoles of murexide, 8 μmoles of Hepes–Tris (exact pH is presented in legends to figures), and 0.7–1.1 mg of fraction 2 protein (protein concentration estimated by the method of Lowry *et al.* (1951). The system is equilibrated at 30° with constant stirring prior to the stepwise addition of $CaCl_2$ (volume added did not exceed 22 μl and amount of Ca^{2+} did not exceed 0.6 μmole).

Following calibration of the dual-wavelength spectrophotometer by the addition of 2 nmoles of $CaCl_2$ to a system containing only murexide and buffer, quantitative corrections were made for the amount of contaminating Ca^{2+}. The Ca^{2+} contamination in the solutions used was determined by spectrophotometrically assaying the effect of the addition of 50 μmoles of EDTA to a system containing all components. The amount of contaminating Ca^{2+} bound to fraction 2 was assayed spectrophotometrically by measuring the effect of the addition of 5 μmoles of $MgCl_2$ (an amount of Mg^{2+} capable of completely releasing bound Ca^{2+}) to a system containing all components.

These corrections permitted the calculation of the free $[Ca^{2+}]$ and the bound $[Ca^{2+}]$ present in the assay system to which any Ca^{2+} additions are to be made. It also permits accurate determinations of both throughout the Ca^{2+}-binding assay procedure.

Determination of the Ca^{2+} Association Constants of Fraction 2. The relationship between the micromolar concentration of Ca^{2+} (both free and bound) and the ΔOD of the murexide absorbance was graphed for systems containing no fraction 2 and for those containing a known concentration of fraction 2. Graphic analysis of these plots permitted an estimation of the amount of Ca^{2+} bound by fraction 2 relative to the free $[Ca^{2+}]$.

The relationship between the ratio of bound $[Ca^{2+}]$/free $[Ca^{2+}]$ and the bound $[Ca^{2+}]$ is plotted by the procedure of Scatchard (1949). This resulted in a complex curvilinear relationship which could be analyzed further by the graphic procedures of Danchin and Gueron (1970) to yield classes of binding sites, association constants of the binding sites, and the number of such sites. A mathematical basis for the breakdown of a nonlinear Scatchard plot by the method of Danchin and Gueron is presented in the Appendix. A computer program was developed for these analyses and the data presented in the Results section were obtained by computer analysis.

Results

The binding characteristics of fraction 2 dissolved in 4 mM Hepes buffer at an approximately neutral pH are presented in the form of a Scatchard plot represented by the open circles in Figure 1. This plot is a complex curvilinear relationship which indicates either that the protein contains classes of binding sites which differ in their affinity for Ca^{2+} or that the binding of Ca^{2+} so affects the protein as to change the affinity of unfilled binding sites for Ca^{2+} or a combination of these properties. If it is assumed that fraction 2 is composed of two classes of binding sites functioning independently, then the affinity constant for each class of binding sites and the number of such sites in a given mass of protein can be estimated from two straight lines which may be drawn through the open points in Figure 1 (see Table I for the results of this analysis).

Recently Danchin and Gueron (1970) have published a graphic analysis of the Scatchard plot which enables the resolution of a complex curve such as that in Figure 1 into its primary components. Analysis of the Scatchard plot of Figure 1 by the method of Danchin and Gueron was carried out and the Ca^{2+} binding by fraction 2 is resolved into two straight lines indicating the presence of only two classes of binding sites. The association constants and number of binding sites from such analysis are reported in Table I.

[1] The abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-'tetraacetic acid.

TABLE I: Ca^{2+}-Binding Parameters of Fraction 2 Obtained from Three Correlated Analytical Procedures.[a]

Analytical Procedure	Binding Parameters: Ca^{2+} Association Constants (M^{-1}) High	Low	μmoles of Ca^{2+} Bound/g of Protein at High-Affinity Site	Low-Affinity Site
Scatchard plot	1.73×10^5	1.5×10^4	100	192
Danchin and Gueron analysis	1.92×10^5	1.5×10^4	93	135
Computer analysis	1.99×10^5	9×10^3	92	164

[a] The 2-ml assay incubation contained 0.96 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.8) at 25°. The temperature of the assay was 30°, and the $[Ca^{2+}]$ was increased from 0 to 300 μM.

TABLE II: Effect of Murexide Concentration on the High Ca^{2+} Association Constant of Fraction 2.[a]

Expt	Murexide Concn (mM)	High Ca^{2+} Association Constant (M^{-1})	μmoles of Ca^{2+} Bound/g of Protein at the High-Affinity Site
1	0.035	1.29×10^5	76
2	0.07	1.50×10^5	78
3	0.14	1.85×10^5	88

[a] The 2-ml assay incubation contained 0.96 mg of fraction 2 protein and 8 μmoles of Hepes–Tris (pH 6.9) at 25°. The temperature of the assay was 30°, and the $[Ca^{2+}]$ was increased from 0 to 300 μM.

Since the graphic method of Danchin and Gueron involves several mechanical steps each of which is subject to human error, it was clearly desirable to develop a computer program for this analysis. Such a program was developed and its use in the analysis of Ca^{2+} binding by fraction 2 is presented in Figure 1. The solid lines represent the breakdown of these data by the computer into two linear plots, one for each class of binding site. The dotted line represents the synthesis of the Scatchard plot by the computer from the two linear plots. The association constants and number of binding sites for each class obtained by this computer analysis are reported in Table I.

All three methods of analyses indicate an apparent association constant for the high-affinity site of approximately 2×10^5 M^{-1} and for the low-affinity site of approximately 10^4 M^{-1}. If the number of binding sites are considered in terms of the amount of protein required to bind 1 mole of Ca^{2+}, we find that approximately 1 mole of Ca^{2+} is bound to the high-affinity site per each 10,000 g of protein and the protein appears to contain about twice the number of low-affinity binding sites as high-affinity ones.

All of the above work was carried out with a murexide concentration of 0.14 mM. Since murexide binds Ca^{2+} and is bound to some extent to most protein, it is of importance to learn the effect of changing murexide concentrations on the estimation of the Ca^{2+}-binding parameters of fraction 2. Data on this point are reported in Table II. Reducing the concentration of murexide fourfold does not increase the apparent association constant of the high-affinity site for Ca^{2+} but rather somewhat decreases it. Also there appears to be some reduction in the amount of Ca^{2+} bound per gram of protein at this site as the murexide concentration falls; the reason for this is not clear but may relate to some interaction of murexide and protein. Nevertheless these effects are small which indicates that the murexide assay as we are using it provides good approximations of the apparent association constants and the number of binding sites.

If protein concentration influences the state of aggregation and if the state of aggregation of the protein in fraction 2 affects the Ca^{2+}-binding properties, it would be anticipated that the association constants and the Ca^{2+}-binding capacity would be influenced by the protein concentration of the assay system. It was found that as much as a twofold change in protein concentration had little effect on the values of the apparent association constants and number of binding sites.

The effect of the Hepes–Tris buffer concentration on the apparent association constant for Ca^{2+} binding by fraction 2 were studied (Figure 2). The association constant of the high-affinity site decreases as the buffer concentration increases while that of the low-affinity site is not affected by the buffer concentration. The lowest buffer concentration used was 1.25 mM because the protein of fraction 2 is insoluble at lower solute concentrations. In order to be certain that the protein remains soluble, a 4 mM Hepes–Tris buffer concentration was used for most of the work reported in this paper.

The effect of varying the pH from 6.5 to 8.0 on the Ca^{2+}-binding association constants of fraction 2 was investigated and the results are reported in Figure 3. The apparent association constant for the high-affinity site has a maximum value

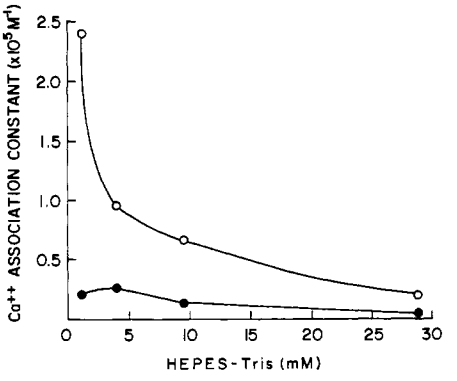

FIGURE 2: The effect of buffer concentration on the high and low Ca^{2+} association constants of fraction 2. Open circles, high Ca^{2+} association constant; closed circles, low Ca^{2+} association constant. Each 2-ml Ca^{2+}-binding assay incubation contained 0.84 mg of fraction 2 protein and 0.28 μmole of murexide. The temperature of incubation was 30° and the pH of the Ca^{2+}-binding assays ranged from 6.7 to 7.0 at 25°. The $[Ca^{2+}]$ was increased from 0 to 300 μM in each Ca^{2+}-binding assay.

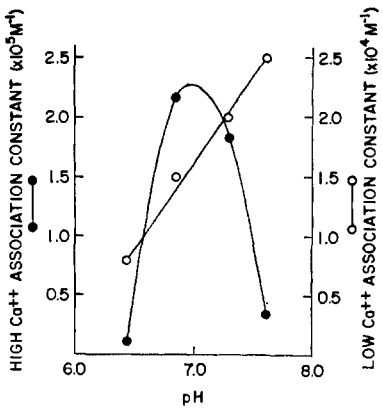

FIGURE 3: The effect of pH on the high and low Ca^{2+} association constants of fraction 2. Each 2-ml Ca^{2+}-binding assay contained 1.05 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris. The incubation temperature was 30° and the pH of each Ca^{2+}-binding assay was read following the addition of $CaCl_2$ which increased the $[Ca^{2+}]$ from 0 to 300 μM.

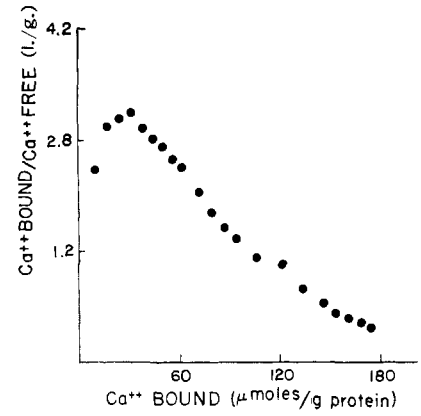

FIGURE 5: A Scatchard plot of the Ca^{2+}-binding activity of fraction 2 in the presence of 10 μM $MgCl_2$. The 2-ml Ca^{2+}-binding assay incubation contained 0.73 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.7) at 25°. The temperature of assay was 30° and the $[Ca^{2+}]$ was increased from 0 to 300 μM. This plot was made from computer analyzed data.

at or near pH 7.0. In contrast the apparent association constant for the low-affinity site increases linearly as the pH is increased from 6.5 to 8.0.

The effect of changing the temperature from 25 to 30° on the apparent association constant of the high-affinity site was studied. No effect of temperature was noted.

The binding studies reported to this point have involved systems containing Hepes–Tris buffer at low ionic strength and no other cations. Since muscle cells contain Mg^{2+} and K^+ as their major cations and low but not inappreciable concentrations of Na^+, it is clearly of interest to study the effects of such cations on Ca^{2+} binding by fraction 2.

The effect of Mg^{2+} on the binding of Ca^{2+} by fraction 2 was studied at 10 and 100 μM $MgCl_2$ and the data analyzed by the Lineweaver–Burk plot (Figure 4). Mg^{2+} inhibits the binding of Ca^{2+} by fraction 2 and least-squares analysis of these data show that this inhibition is primarily competitive in nature. When the Scatchard plot is used to analyze the effect of Mg^{2+} on Ca^{2+} binding (Figure 5), an interesting phenomenon is observed at a $MgCl_2$ concentration of 10 μM; namely at low Ca^{2+} concentrations the curve has a positive slope and a downward curvature. Cook and Koshland (1970) observed a similar phenomenon when studying the binding of NAD by glycerophosphate dehydrogenase as did Danchin and Gueron (1970) when studying the binding of Mn^{2+} by bacterial RNA. Both groups attribute this phenomenon to binding with positive cooperativity. In the case of fraction 2, there appears to be cooperativity of Ca^{2+} binding in the presence of $[MgCl_2]$ greater than or equal to 10 μM. Possibly Ca^{2+} replaces Mg^{2+} bound to fraction 2 which results in a conformational change that facilitates further binding of Ca^{2+} by the protein.

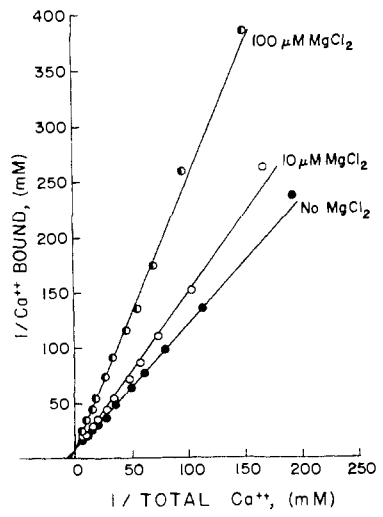

FIGURE 4: A Lineweaver–Burk analysis of the Ca^{2+}-binding activity of fraction 2 in the presence of $MgCl_2$. The 2-ml Ca^{2+}-binding assay incubation contained 0.73 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.7) at 25°. The temperature of assay was 30° and the $[Ca^{2+}]$ was increased from 0 to 300 μM at each concentration of $MgCl_2$. A least-squares determination of the ordinate intercept gave a value of 6 mM^{-1} for each concentration of $MgCl_2$. Evaluation of the abscissa intercept using the slopes and ordinate intercept derived by the method of least squares gave −5.0, −4.2, and −2.4 mM^{-1} for 0, 10, and 100 μM $MgCl_2$, respectively.

The effect of KCl at 1 and 10 mM concentrations on Ca^{2+} binding by fraction 2 was studied and a Lineweaver–Burk plot (Figure 6) of the Ca^{2+}-binding data suggests that K^+ inhibits Ca^{2+} binding apparently by a noncompetitive mode. Least-squares analysis of the Lineweaver–Burk plot demonstrates that K^+ inhibition is certainly not competitive but does not substantiate noncompetitive inhibition. Analysis of these data by the Scatchard plot technique revealed no evidence for positive cooperativity in this case.

The effect of NaCl at 10 and 100 mM concentrations on the Ca^{2+}-binding activity of fraction 2 was also examined. A Lineweaver–Burk analysis of these data (Figure 7) suggests that the Ca^{2+}-binding activity of fraction 2 is noncompetitively inhibited by Na^+. Again least-squares analysis rules out competitive inhibition by Na^+ but does not substantiate noncompetitive inhibition. Likewise Scatchard plot analysis of this inhibition does not suggest positive cooperativity in this case. It should be noted that although Na^+ and K^+ exhibit the same approximate mode of inhibition it requires a ten times higher concentration of Na^+ to exert the same amount of inhibition.

Carvalho and Leo (1967) found that over a given free $[Ca^{2+}]$ range the presence of ATP markedly increased the ability of intact sarcotubular membranes to bind Ca^{2+}. An attempt was made to study the effect of ATP on the binding of Ca^{2+} by

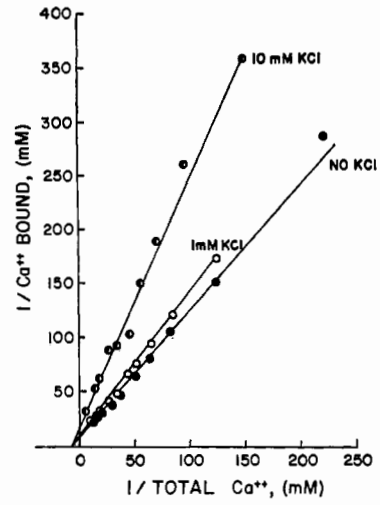

FIGURE 6: A Lineweaver–Burk plot of the Ca^{2+}-binding activity of fraction 2 in the presence of KCl. Each 2-ml Ca^{2+}-binding assay incubation contained 0.99 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.7) at 25°. The temperature of assay was 30° and the $[Ca^{2+}]$ was increased from 0 to 300 μM for each concentration of KCl. A least-squares determination of the ordinate intercept gave 7, 9, and 13 mM^{-1} at 0, 1, and 10 mM KCl, respectively. Evaluation of the abscissa intercepts using the slopes and ordinate intercepts derived by the method of least squares gave -5.7, -6.9, and -5.4 mM^{-1} for 0, 1, and 10 mM KCl, respectively.

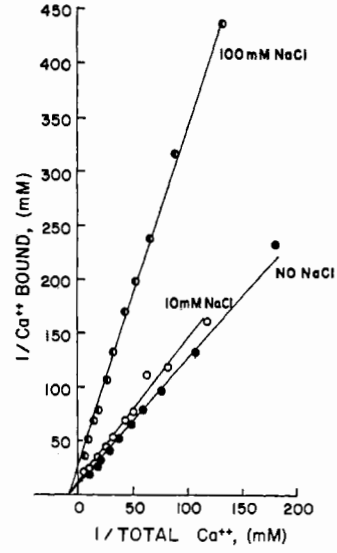

FIGURE 7: A Lineweaver–Burk plot of the Ca^{2+}-binding activity of fraction 2 in the presence of NaCl. Each 2-ml Ca^{2+}-binding assay incubation contained 0.93 mg of fraction 2 protein, 0.28 μmole of murexide, and 8 μmoles of Hepes–Tris (pH 6.7) at 25°. The temperature of assay was 30° and the $[Ca^{2+}]$ was increased from 0 to 300 μM for each concentration of NaCl. A least-squares determination of the ordinate intercept gave 9, 10, and 18 mM^{-1} at 0, 10, and 100 mM NaCl, respectively. Evaluation of the abscissa intercept using the slopes and ordinate intercepts derived by the method of least squares gave -7.4, -7.5, and -5.5 mM^{-1} for 0, 10, and 100 mM NaCl, respectively.

fraction 2. This presented a formidable technical problem with the murexide system since ATP binds Ca^{2+}. To minimize this problem Mg^{2+} at appropriate concentrations is added. The effect of Mg^{2+} and Mg^{2+} + ATP concentrations varying from 5 to 20 μM were studied and, while these additions had variable effects on the value of the apparent association constant of the high-affinity site, the changes noted are so small that it is not likely that they relate to the findings of Carvalho and Leo with intact sarcotubular membranes. In unpublished studies, we found that partial delipidation of sarcotubular membranes causes no loss in their capacity to bind Ca^{2+} but a marked loss in the ability of ATP to enhance this binding. Fraction 2 is a lipid-poor protein which may well account for the inability of ATP to enhance its affinity for Ca^{2+}.

Discussion

Fraction 2 contains more than 90% of the sarcotubular membrane protein present in the highly purified SF_1 preparation of this cellular structure (Yu and Masoro, 1970). This protein is in the form of large molecular weight structures comprised of low molecular weight polypeptide subunits. In the present paper it is established that fraction 2 has a great ability to bind Ca^{2+} and that two classes of binding sites are present, one with an apparent association constant of 1.46 ($\pm$0.15[2]) $\times$ 10^5 M^{-1} and the other with an apparent association constant of 1.80 ($\pm$0.24[2]) $\times$ 10^4 M^{-1}. These findings are most provocative since the major functional activities of the sarcotubular membranes involves the release and the sequestration of Ca^{2+} in relation to excitation–contraction coupling and relaxation of skeletal muscle (Ebashi and Endo, 1968).

Although the present studies provide much quantitative information on the binding of Ca^{2+} by fraction 2 and on the interplay of other physiologically important cations on this binding activity, the work sheds little light on the chemical nature of the binding sites. The findings, that pH affects binding in such a way as to cause a rather sharp maximum at a pH of approximately 7 and that there is positive cooperativity of Mg^{2+} relative to Ca^{2+} binding by fraction 2, do indicate that the Ca^{2+}-binding process is quite a complex phenomenon.

The molecular weight of the polypeptide subunits of fraction 2 was estimated to be somewhere between 6000 and 10,000 (Yu and Masoro, 1970). If it be assumed that fraction 2 contains only one species of polypeptide subunit, it would then appear by converting the data presented above from the moles of Ca^{2+} bound per g of fraction 2 to the g of fraction 2 required to bind 1 mole of Ca^{2+} that each subunit contains one high-affinity binding site and two of the low-affinity sites. This need not mean, however, that such polypeptide subunits existing as monomers would bind Ca^{2+} because on the basis of the gel filtration, gel electrophoresis, and analytical ultracentrifuge studies (Yu and Masoro, 1970) fraction 2 was shown to be composed of aggregates of 20 or more polypeptide subunits. It is quite possible that a state of aggregation of the polypeptide is needed for Ca^{2+}-binding activity.

Recently MacLennan and Wong (1971) reported the isolation and Ca^{2+}-binding properties of a specific Ca^{2+}-binding protein from sarcoplasmic reticulum. Although this membrane protein maximally binds more than three times the amount of Ca^{2+} that fraction 2 does, it does so with a Ca^{2+} association constant approximately one order of magnitude lower than the high-affinity Ca^{2+} binding of fraction 2.

How does the Ca^{2+}-binding activity of fraction 2 compare to that of intact sarcotubular membranes? The work of Cohen and Selinger (1969) provides information on this point. They found the membranes to have two classes of binding sites, both with the same apparent association constant. One of

[2] Means of 13 determinations ($\pm$SEM).

these classes is inactivated by trypsin proteolysis but is insensitive to its ionic environment and is called the specific Ca^{2+} binder. The other class is insensitive to trypsin proteolysis but its Ca^{2+}-binding activity is inhibited by Mg^{2+}, K^+, and Na^+. This latter class which comprises 90% of the binding sites is termed the nonspecific Ca^{2+} binder. Fraction 2 is similar to the nonspecific Ca^{2+} binder with regard to the ionic sensitivity of its Ca^{2+}-binding activity. Although the mode of inhibition of sarcotubular membrane nonspecific Ca^{2+} binding by Mg^{2+} and Na^+ was not reported, the K^+ inhibition was found to be noncompetitive as is the case with fraction 2. The apparent association constant of these binding sites is $2.5 \times 10^4\ M^{-1}$ which is an order of magnitude lower than that of the high-affinity site of fraction 2. The total binding capacity of the intact sarcotubular membranes was found to be 50 nmoles/mg of protein while that of fraction 2 is several times that amount. Although differences certainly exist between the Ca^{2+}-binding properties of the intact sarcotubular membrane structure and those of the solubilized protein of fraction 2, there is on the whole rather remarkable similarity between the Ca^{2+}-binding activities of these two very different systems containing sarcotubular membrane protein.

A great difference does exist between the intact membranes and fraction 2 in regard to their response to the addition of ATP. Carvalho and Leo (1967) have shown in the pCa range of 5–7 that the addition of ATP markedly enhances Ca^{2+} binding by sarcotubular membranes. This phenomenon could not be demonstrated with the soluble protein of fraction 2. It should be noted however that fraction 2 is almost lipid free. We have recently confirmed the enhancing effect of ATP on Ca^{2+} binding by sarcotubular membranes and further find that this action of ATP is almost abolished by the destruction of 60% of the phospholipid of the sarcotubular membranes by treatment with phospholipase C (unpublished observations). However in agreement with Cohen and Selinger this partial delipidation was not found to affect the ability of the membranes to bind Ca^{2+} in the absence of ATP.

It is only possible to speculate on the physiological meaning of the results of this investigation of Ca^{2+} binding by fraction 2. If the protein of fraction 2 has the same characteristics *in vivo* as have been demonstrated in the test tube with soluble protein it might at first glance be expected that the high intracellular concentrations of K^+ and Mg^{2+} would render the high-affinity site ineffective. However it should be noted that the intracellular compartment of the cell is markedly heterogeneous containing a large number of highly specialized subcompartments each with a different and specialized composition. It is quite possible therefore that in the cell this protein either is not in intimate contact with high concentrations of K^+ and Mg^{2+} at all times or that its interaction with the lipid of the membrane structure renders it capable of binding Ca^{2+} in the presence of K^+ and Mg^{2+}. Moreover it is important for the sarcotubular membrane system to release Ca^{2+} during excitation and as well as to bind it during relaxation. Since during excitation rapid migrations of Na^+ and K^+ occur, it may be of considerable significance that both Na^+ and K^+ inhibit Ca^{2+} binding by fraction 2 but require quite different concentrations in each case to cause the same level of inhibition.

The sarcoplasmic reticulum not only binds Ca^{2+} to its structure but also actively transports it into the intraluminal space of this tubular system (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961). There appears to be a specific ATPase involved in energizing this transport. This ATPase requires for its activity Mg^{2+} in millimolar concentrations and Ca^{2+} in micromolar concentrations. It is of obvious interest to learn if the Ca^{2+} binding protein of fraction 2 described here also functions as a component of this $(Ca^{2+} + Mg^{2+})$-ATPase. Since delipidation of the sarcotubular membranes destroys this $(Ca^{2+} + Mg^{2+})$-ATPase activity (Martonosi *et al.*, 1968; Yu *et al.*, 1968b) and since fraction 2 is lipid poor, fraction 2 would not be expected to have this ATPase activity and it has none. However in preliminary experiments, we were able to restore this ATPase activity to about 10% of that found with the intact membrane on a per mg of protein basis by interacting fraction 2 with sonic dispersions of phospholipids obtained from sarcotubular membranes. Much more work needs to be done on this reconstitution before a definitive statement can be made on the relationship between the Ca^{2+}-binding activity and this ATPase activity. Nevertheless, these preliminary studies are suggestive of the possibility that the polypeptide subunits of the Ca^{2+}-binding protein also function as part of this ATPase in conjunction with the appropriate phospholipid molecules.

Appendix

Mathematical basis for the breaking down of a nonlinear Scatchard plot discussed in terms of its use in the Ca^{2+}-binding studies.

Assume that the Ca^{2+} binding at each site is independent. Consider a system which contains two classes of Ca^{2+}-binding sites, we may write

$$k_i = \frac{x_i}{c(n_i - x_i)} \quad (i = 1, 2) \tag{1}$$

where k_i's are the association constants for site i, x_i's the amount of Ca^{2+} bound at site i, c the concentration of free Ca^{2+}, and n_i's the number of Ca^{2+}-binding site in class i.

The relationship between the total Ca^{2+} concentration (c_t) and the total Ca^{2+} concentration bound (x) is given by

$$c_t = c + x \tag{2}$$

$$x = \sum x_i \tag{3}$$

By defining y and y_i's by

$$y = x/c \tag{4}$$

$$y_i = x_i/c \tag{5}$$

we can derive the total Scatchard plot (plot y *vs.* x) and two partial plots (y_i *vs.* x_i) each representing a class of binding sites. From eq 1 and 5, we can derive two linear lines, *i.e.*, partial plots

$$y_i = k_i(n_i - x_i) \tag{6}$$

where the slope gives the association constant and the intersection with the abscissa the number of binding sites in a given class.

When $k_1 \neq k_2$, the total plot is not linear, but can be resolved into partial plots. Take a point R on the total plot and draw a straight line connecting this point to the origin. The slope of the line is equal to the inverse of free Ca^{2+} concentration (see eq 4). Let intersections of the line with two partial

plots be R_i's, and distances from the origin to R and R_i's be r and r_i's. We then have

$$r = x \sec \theta$$
$$r_i = x_i \sec \theta \quad (7)$$

where θ is the angle between the straight line and the abscissa. From eq 3 and 7, we can readily derive the following basic equation

$$r = \sum r_i \quad (8)$$

Knowing two partial plots, we can easily construct the total plot by changing θ step by step from 0 to 90°, and plotting a point each time according to eq 8.

Inversely, breaking down of the total plot into two partial plots may be done as follows. By assuming certain values for k_2 and n_2 we draw a line (partial plot) for $i = 2$. From the total plot and the partial plot, we can derive a partial plot for $i = 1$ according to the same principle described above. This new plot is not necessarily linear. Therefore, we take another combination of k_2 and n_2 and continue the process until we obtain satisfactory linear plot for $i = 1$.

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