Ca\textsuperscript{2+} Binding Effects on Protein Conformation and Protein Interactions of Canine Cardiac Calsequestrin*

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Calsequestrin is a Ca\textsuperscript{2+}-binding protein located intraluminally in the junctional sarcoplasmic reticulum (SR) of striated muscle. In this study, Ca\textsuperscript{2+} binding to cardiac calsequestrin was assessed directly by equilibrium dialysis and correlated with effects on protein conformation and calsequestrin's ability to interact with other SR proteins. Cardiac calsequestrin bound 800-900 nmol of Ca\textsuperscript{2+}/mg of protein (35-40 mol of Ca\textsuperscript{2+}/mol of calsequestrin). Associated with Ca\textsuperscript{2+} binding to cardiac calsequestrin was a loss in protein hydrophobicity, as revealed with use of absorbance difference spectroscopy, fluorescence emission spectroscopy, and photoaffinity labeling with the hydrophobic probe 3-(trifluoromethyl)-3-(\textsuperscript{125}I)iodophenyl)diazirine. Ca\textsuperscript{2+} binding to cardiac calsequestrin also caused a large change in its hydrodynamic character, almost doubling the sedimentation coefficient. We observed that cardiac calsequestrin was very resistant to several proteases after binding Ca\textsuperscript{2+}, consistent with a global effect of Ca\textsuperscript{2+} on protein conformation. Moreover, Ca\textsuperscript{2+} binding to cardiac calsequestrin completely prevented its interaction with several calsequestrin-binding proteins, which we identified in cardiac junctional SR vesicles for the first time. The principal calsequestrin-binding protein identified in junctional SR vesicles exhibited an apparent $M_r$ of 26,000 in sodium dodecyl sulfate-polyacrylamide gels. This 26-kDa calsequestrin-binding protein was greatly reduced in free SR vesicles and absent from sarcosomal vesicles and was different from phospholamban, an SR regulatory protein exhibiting a similar molecular weight. Our results suggest that the specific interaction of calsequestrin with this 26-kDa protein may be regulated by Ca\textsuperscript{2+} concentration in intact cardiac muscle, when the Ca\textsuperscript{2+} concentration inside the junctional SR falls to submillimolar levels during coupling of excitation to contraction.

Calsequestrin is a Ca\textsuperscript{2+}-binding protein localized inside the junctional SR of cardiac muscle and skeletal muscle which binds Ca\textsuperscript{2+} with high capacity and moderate affinity (1-4) and has been shown to interact with the luminal face of the junctional SR membrane (5-8). Although the Ca\textsuperscript{2+}-binding properties and associated structural changes of skeletal muscle calsequestrin have been extensively characterized (9), little is known about the Ca\textsuperscript{2+}-binding properties of cardiac calsequestrin. Indeed, the protein was not conclusively identified and purified from cardiac SR until relatively recently (3, 4). The carbocyanine dye Stains All has proven very useful for detecting the various tissue forms of calsequestrin (10, 11) due to the dye’s ability to stain calsequestrin an intense blue after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 4). Use of this protein stain along with antibodies specific for the cardiac form of calsequestrin has recently allowed detection of a similar protein in SR vesicles isolated from certain types of smooth muscle (12).

Skeletal muscle and cardiac calsequestrins have recently been sequenced by recombinant DNA methods; and although both proteins are found to be highly acidic, the sequence homology (approximately 60%) is not particularly striking (13, 14). However, both proteins are readily purified by Ca\textsuperscript{2+}-dependent elution from phenyl-Sepharose (4), a hydrophobic matrix, suggesting that in addition to the high density of negatively charged residues, hydrophobic regions on calsequestrin may be important for overall function. In view of the probable participation of cardiac calsequestrin in the Ca\textsuperscript{2+} release process at the junctional SR of cardiac muscle, this study was undertaken to quantitate Ca\textsuperscript{2+} binding to the protein and to characterize the associated structural changes which account for calsequestrin’s interaction with phenyl-Sepharose and the SR membrane. For the first time, a set of proteins localized to the junctional SR of cardiac muscle are identified which bind cardiac calsequestrin specifically with high affinity and in a Ca\textsuperscript{2+}-sensitive fashion.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

In this study, we have assessed Ca\textsuperscript{2+} binding to cardiac calsequestrin by five independent methods including equilibrium dialysis, spectrophotometric analysis, hydrophobic probe labeling, proteolysis susceptibility, and measurement of the ability of the protein to bind to receptor proteins in native membranes and on nitrocellulose blots. All methods are in general agreement, suggesting that cardiac calsequestrin binds Ca\textsuperscript{2+} with high capacity and moderate affinity and that there is an apparent specificity for Ca\textsuperscript{2+} relative to Mg\textsuperscript{2+}. The latter

*Portions of this paper (including "Experimental Procedures," and "Results," Figs. 1-11, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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four methods and the hydrodynamic studies in particular suggest a rather large conformational change in cardiac calsequestrin upon binding Ca\(^{2+}\), which is associated with the burying of hydrophobic amino acid side chains and a resultant decrease in calsequestrin’s ability to interact with other proteins. These findings are consistent with our earlier results (4) on purification of cardiac calsequestrin by phenyl-Sepharose chromatography. The Ca\(^{2+}\) concentration dependence for effects on structural parameters in all cases closely paralleled Ca\(^{2+}\) binding to the protein measured directly, suggesting that Ca\(^{2+}\) binding and resultant conformational changes occurred in a closely coordinated fashion.

The number of Ca\(^{2+}\)-binding sites calculated for cardiac calsequestrin (35-40 mol/mol) appears to be very similar to that measured for the skeletal muscle protein (40-50 mol/mol, Refs. 1, 2, and 40-42). The half-maximal Ca\(^{2+}\) concentrations required for binding were also approximately the same: 400-600 \(\mu\text{M}\) Ca\(^{2+}\) when measured in the presence of 150 mM KCl and 100 \(\mu\text{M}\) Ca\(^{2+}\) when measured in 20 mM KCl. The complete amino acid sequences of both rabbit skeletal muscle and canine cardiac calsequestrins have now been deduced by recombinant DNA methods. Skeletal muscle calsequestrin contains 103 acidic amino acid residues (13), with an excess of 73 negatively charged residues over basic residues; whereas cardiac calsequestrin contains 109 acidic amino acid residues, with an excess of 69 negatively charged residues over basic residues (14). The many acidic residues found for both cardiac and skeletal muscle calsequestrins are undoubtedly important in determining their large Ca\(^{2+}\)-binding capacities, and the similar numbers of these residues for both proteins are consistent with the two proteins binding similar amounts of Ca\(^{2+}\). Campbell et al. (3) earlier reported that cardiac calsequestrin bound one-half to one-third as much Ca\(^{2+}\) as that reported here. However, in this earlier study, only one Ca\(^{2+}\) concentration was tested; and it is not clear if correction was made for protein loss during dialysis.

Earlier fluorescence studies of skeletal muscle calsequestrin (43) and our more recent observation of the interaction of the protein with phenyl-Sepharose (4) suggested that a hydrophobic patch was present on the protein’s surface and that Ca\(^{2+}\) caused this hydrophobic site to internalize. In the work described here on cardiac calsequestrin, internalization of tryptophan residues was clearly indicated by the absorbance difference spectrum and the blue-shifted fluorescence spectrum obtained when Ca\(^{2+}\) was bound to the protein. Likewise, labeling of cardiac calsequestrin with the hydrophobic probe \(^{125}\text{I}\)TID was substantially decreased when Ca\(^{2+}\) was bound to the protein. In a preliminary study to determine the site(s) labeled by \(^{125}\text{I}\)TID, calsequestrin was cleaved with trypsin, and the products were fractionated by reverse-phase high performance liquid chromatography (data not shown). Only one peptide was observed to contain \(^{125}\text{I}\)TID. These results obtained with cardiac calsequestrin are in agreement with the recent observations of Fliegel et al. (13), who identified a single hydrophobic site on skeletal muscle calsequestrin with the use of the hydrophobic probe \(^{3}\text{H}\)trifluoromethyl chloroformate.

Sedimentation analysis demonstrated that cardiac calsequestrin underwent a dramatic shift in conformation upon binding Ca\(^{2+}\), as evidenced by a doubling of the sedimentation coefficient and a reduction in the partial specific volume. Our observations appeared consistent with those previously reported for skeletal muscle calsequestrin, which were attributed to an extended molecule becoming more compact upon binding Ca\(^{2+}\) (9, 34). With this in mind, we predicted that the Stokes radius (measured by gel filtration) of cardiac calsequestrin would decrease when Ca\(^{2+}\) was bound to the protein. This has been noted previously for the skeletal muscle protein (34). We found, however, that the apparent Stokes radius of the molecule actually increased slightly in Ca\(^{2+}\)-containing buffer, yielding data which were consistent with protein dimerization upon binding Ca\(^{2+}\). Since both aggregation and crystallization of skeletal muscle calsequestrin are well-known to occur in Ca\(^{2+}\)-containing or low ionic strength buffers (9, 44), we were sensitive to this point. Sedimentation studies on cardiac calsequestrin were also performed in which the KCl concentration was varied from 15 to 500 mM in either the absence or presence of Ca\(^{2+}\) (data not shown). The results clearly indicated that although a small degree of aggregation did occur at low KCl concentrations, the predominant effect of the addition of Ca\(^{2+}\) was always a doubling of the sedimentation coefficient, again consistent with dimerization of the molecule. However, we detected no binding of \(^{125}\text{I}\)-calsequestrin to nonradioactive calsequestrin in the protein blotting experiments. Therefore, if calsequestrin does dimerize under native conditions, it has probably lost this ability after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose.

Of particular interest was the finding that cardiac calsequestrin in the Ca\(^{2+}\)-bound state was highly protected from proteolytic digestion. This protective effect may have been due to one of several alterations in calsequestrin’s physical properties upon binding Ca\(^{2+}\). As shown by our hydrophobic localization and hydrodynamic studies, in the presence of Ca\(^{2+}\), the protein evidently underwent a conformational change which 1) internalized hydrophobic residues, 2) resulted in a more compact structure, and 3) perhaps resulted in dimerization of the molecule. Since the proteases tested varied widely in their specificities, the loss in protease sensitivity associated with Ca\(^{2+}\) binding to calsequestrin suggested that the conformational change was global in nature and/or that charge alterations may have obscured protease specificity requirements. Thus, in the Ca\(^{2+}\)-bound state, calsequestrin has apparently lost its ability to interact with proteins other than itself. This conclusion was substantiated by measuring \(^{125}\text{I}\)-calsequestrin binding to junctional SR vesicles and to junctional SR proteins transferred to nitrocellulose panels.

The specific association of calsequestrin with junctional SR proteins causing it to anchor to the membrane has long been postulated, both by ourselves (4) and by others (5-8, 35). In this study, we identified for the first time a major calsequestrin-binding protein of apparent \(M_{r} = 26,000\) which was localized to junctional SR vesicles isolated from both cardiac and skeletal muscle. Calsequestrin bound to this 26-kDa protein with high affinity (nanomolar concentrations of calsequestrin were used) and in a Ca\(^{2+}\)-regulated fashion. Our in vitro results suggest that when Ca\(^{2+}\) is stored inside the junctional SR of intact muscle, calsequestrin would not be able to bind to this protein. When Ca\(^{2+}\) is released from inside the junctional SR during muscle contraction, calsequestrin binding to the 26-kDa protein (as well as to the other minor proteins detected) could occur. Whether the 26-kDa protein is involved in the Ca\(^{2+}\) release process is presently unknown, but is an attractive hypothesis for future testing. Also, whether this protein is related to the 30-kDa protein previously localized to skeletal muscle junctional SR vesicles by Campbell et al. (7) is also presently unknown. Work in our laboratory is currently being directed toward purification and biochemical characterization of this 26-kDa calsequestrin-binding protein.

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Ca²⁺ Binding Effects on Protein Conformation and Cardiac Calsequestrin Interactions of Calcium Calsequestrin

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EXPERIMENTAL PROCEDURES

Preparation of Calcium-binding Protein and Calsequestrin Partial Purification of proteins from the heart of the rabbit. Calcium Calsequestrin is a prominent component of the sarcoplasmic reticulum of cardiac muscle. Calcium Calsequestrin is isolated from cardiac muscle by differential centrifugation and subsequent precipitation. Calcium Calsequestrin is isolated from cardiac muscle by differential centrifugation and subsequent precipitation. Calcium Calsequestrin is isolated from cardiac muscle by differential centrifugation and subsequent precipitation. Calcium Calsequestrin is isolated from cardiac muscle by differential centrifugation and subsequent precipitation.
Ca\textsuperscript{2+} Effects on Cardiac Calsequestrin

Controlled gradients, calsequestrin was prevented by protein assay and by measuring radioactivity. No difference was noted between the migration of \textsuperscript{125}I-labeled and unlabeled calsequestrin proteins prepared with \textsuperscript{125}I-labeled and unlabeled calsequestrin proteins were centrifuged at 45,000 rpm for 17 h using a Beckman SW-60 rotor and a Model L8-75 ultracentrifuge. Gradients prepared with \textsuperscript{125}I-labeled were centrifuged for 30 h.

Protein migration by centrifugation using SDS-macro and an ion exchange HPLC gradient fractionator. 15% of the gradients were collected and analyzed for radioactivity. The percentage of total radioactivity in each fraction was determined using a Beckman and Lum 5-B fluorometer.

Proteins were also analyzed by SDS-PAGE and autoradiography in order to differentiate \textsuperscript{125}I-labeled calsequestrin from the SPS of higher molecular weight. Using SDS-PAGE, a protein with an apparent molecular weight of 100,000 daltons was observed. The protein was isolated and analyzed by SDS-PAGE and autoradiography. The slow-moving form of the protein was determined to be \textsuperscript{125}I-labeled calsequestrin, and it was used as the standard for further studies.

Filtration assay of calsequestrin binding to ocean water sediments - Binding of \textsuperscript{125}I-labeled calsequestrin to ocean water sediments, 30 mg of sediments was suspended in 150 ml of buffer containing 1 M NaCl (pH 7.4) and 10 mM Tris-HCl, or 10 mM NaCl and 10 mM Tris-HCl, or 10 mM NaCl and 10 mM Tris-HCl, or 10 mM NaCl and 10 mM Tris-HCl, or 10 mM NaCl and 10 mM Tris-HCl.

In order to identify potential calsequestrin binding proteins in ocean water sediments, a Western blotting assay was used. 60 mg of membrane proteins were first electrophoresed and transferred to nitrocellulose membranes. The transfer time was 2 h at 100 V. The blots were incubated with \textsuperscript{125}I-labeled calsequestrin (200,000 cpm) at 4°C for 1 h and washed twice with 20 ml of 0.1 M Tris buffer containing 100 mM NaCl, 10 mM NaCl, and 100 mM NaCl, or 10 mM NaCl and 10 mM NaCl, or 10 mM NaCl and 10 mM NaCl, or 10 mM NaCl and 10 mM NaCl, or 10 mM NaCl and 10 mM NaCl.

Blank subtractions were performed by adding an additional 0.1 M NaCl solution to a filter paper and a filter paper alone. Filter paper was washed with an additional 0.1 M NaCl solution, and the filters were air-dried before autoradiography.

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Ca²⁺ Effects on Cardiac Calsequestrin

Calsequestrin is a protein that binds calcium ions, playing a crucial role in regulating calcium levels in the heart. Changes in cardiac calsequestrin can affect cardiac function and are involved in various cardiac conditions. This section discusses the effects of Ca²⁺ on cardiac calsequestrin, with a focus on its concentration dependence and the implications for cardiac physiology.

**Protein Labeling of Cardiac Calsequestrin**

The concentration dependence of Ca²⁺ binding to cardiac calsequestrin was studied using a specific method. Samples were prepared, and the concentration of Ca²⁺ was varied. The binding of Ca²⁺ was measured using a sensitive technique, allowing for the determination of the concentration range in which Ca²⁺ bound to the protein. This revealed the concentration range over which Ca²⁺ binding to cardiac calsequestrin occurs, providing insights into the regulation of calcium levels in the heart.

**Hydrodynamic Properties of Calf Cardiac Calsequestrin**

Hydrodynamic measurements were performed to investigate the molecular properties of calsequestrin. The results showed that the molecular weight of cardiac calsequestrin under native conditions is consistent with the expected value. The hydrodynamic analysis provided additional information about the protein's structure and interactions.

**Results and Discussion**

The results from these experiments highlight the importance of Ca²⁺ in the regulation of cardiac calsequestrin. Changes in Ca²⁺ concentration can significantly affect the protein's structure and function, impacting cardiac physiology. Understanding these interactions is crucial for the development of therapies aimed at managing cardiac conditions associated with altered calcium homeostasis.

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**Table 1: Hydrodynamic Properties of Calf Cardiac Calsequestrin**

| Parameter       | d (kDa) | c (kDa) |
|-----------------|---------|---------|
| S₀,₅₀ (10⁻⁵ M)  | 3.5     | 3.4     |
| v /[ mg ]      | 0.73    | 0.71    |
| S₀,₅₀ (10⁻⁵ M)  | 3.5     | 3.3     |
| v /[ mg ]      | 0.75    | 0.70    |
| R₉ (kDa)       | 85      | 80      |
| R₉ (kDa)       | 61,000  | 120,000 |
| R₉ (kDa)       | 1.63    | 1.67    |

Note: Values are determined according to Refs. [1, 2], utilizing protein standards. Values are calculated according to the equations: (d) = 10⁵ × [mg] / [v], where [mg] is the mass of protein, and [v] is the volume of buffer. The density of R₉ is calculated from the measured values.

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**Figures and Tables**

- **Fig. 1:** Photograph of cardiac calsequestrin at low and high magnification, showing the typical appearance of the protein under different conditions.
- **Fig. 2:** Gel electrophoresis of cardiac calsequestrin, illustrating the molecular size and charge properties.
- **Fig. 3:** Measurement of Ca²⁺ concentration dependence, demonstrating the concentration range over which Ca²⁺ binding occurs.
- **Fig. 4:** Hydrodynamic properties of cardiac calsequestrin, highlighting the molecular weight and its distribution.

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**References**

These results are supported by previous studies conducted by other researchers, which have contributed to our understanding of cardiac calsequestrin and its role in cardiac function. Further research is needed to fully elucidate the mechanisms underlying these observations and to develop targeted interventions for managing cardiac conditions associated with altered calcium homeostasis.
In order to identify which protein bound 125I-alkaline phosphatase in the cardiac junctional SR fraction, the junctional SR proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with 125I-cardiac calcequinin. This type of approach has been very useful for identifying a variety of different kinds of calcium-mediated protein interactions (28,29). We observed that cardiac calcequinin bound to six major proteins (band 1) of apparent Mr ≥ 29,000 in the junctional SR fraction, but only when incubations were conducted in the presence of BSA (Fig. 11, left). The major 125I-calcequinin binding protein (Mr 29,000) represented the majority of the bound 125I-calcequinin (Fig. 11, left). Interestingly, labelled bands 5 and 6 (Fig. 11, left) ran with mobility identical to proteins previously identified as interactors of the Ca2+/calmodulin-binding subdomain in cardiac junctional SR vesicles [13,23]. All of the proteins bound by 125I-calcequinin depicted in Fig. 11 were not altered in significant amounts in cardiac sequestration or from SR fractions (data not shown). Nonetheless, a 43-kDa protein of identical mobility could also be identified in rabbit skeletal SR vesicle fractions that contained cardiac calcequinin. This 43-kDa protein was absent from free vesicles prepared from rabbit skeletal muscle (data not shown).

In Fig. 10, sequestration of calcequinin into sucrose gradients was accomplished by using sucrose gradients (40 to 0%) from which the SR vesicles were isolated. The vesicle pellets were then resuspended in 0.5 M sucrose, sonicated, and centrifuged at 100,000 g for 1 h. The 125I-calcequinin binding protein was eluted from the Sepharose CL-4B column using 0.5 M sucrose (100 ml) (25). The 125I-calcequinin binding protein was not dissociated by incineration in SDS as is known to occur for phosphatase (27). The 125I-calcequinin binding protein was electrophoresed on SDS-polyacrylamide gels, and its apparent Mr was determined to be 29,000. The 125I-calcequinin binding protein was further characterized by treating the 125I-calcequinin binding protein with thrombin (Sigma 1:500:1). The thrombin-treated 125I-calcequinin binding protein was eluted from the Sepharose CL-4B column (data not shown). The thrombin-treated 125I-calcequinin binding protein was then tested for kinase activity in the presence of SR vesicles and Ca2+ (data not shown). The results of these experiments suggest that the protein is a member of the protein kinase C family. The role of this protein in cardiac function and/or calcium homeostasis remains to be determined.