

# Frog Cardiac Calsequestrin

## Identification, Characterization, and Subcellular Distribution in Two Structurally Distinct Regions of Peripheral Sarcoplasmic Reticulum in Frog Ventricular Myocardium

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Calsequestrin is a calcium-binding protein known to sequester calcium accumulated in the sarcoplasmic reticulum (SR) of muscle cells during relaxation. In the present study, we used affinity-purified antibodies to chicken cardiac calsequestrin to identify a 60,000-Da calsequestrin in frog myocardium. Like previously identified cardiac calsequestrins, it is enriched in cardiac microsomes, it is enriched by biochemical procedures previously used to purify cardiac and skeletal calsequestrins, and it exhibits a pH-dependent shift in its apparent  $M_r$  on a two-dimensional gel system. Finally, the  $\text{NH}_2$ -terminal amino acid sequence of this 60,000-Da immunoreactive protein purified by fast protein liquid chromatography was identical to that of rabbit skeletal and canine cardiac calsequestrin. Thus, we conclude that this protein corresponds to the calsequestrin isoform in frog ventricular muscle. Frog calsequestrin was localized in discrete foci present at the periphery but absent from the central regions of frog ventricular myocytes as determined by immunofluorescence labeling. Immunoelectron microscopic labeling demonstrated that calsequestrin was confined to the lumen of two structurally distinct regions of the SR, where it was localized in the subsarcolemmal region of the myofibers. One of these appeared to correspond to the terminal SR previously reported to be closely apposed to the sarcolemma of frog myofibers. The other region, although close to the sarcolemma, was not physically joined to it and appeared to correspond to corbular SR. It generally is believed that frog cardiac SR does not provide activator  $\text{Ca}^{2+}$  required for excitation-contraction coupling. However, the identification of a calsequestrin isoform very similar to mammalian cardiac calsequestrin that is confined to specialized regions of frog cardiac SR lends support to the idea that frog cardiac SR has the ability to store  $\text{Ca}^{2+}$  and thus function in some capacity in frog cardiac muscle contraction. (*Circulation Research* 1991;69:344-359)

Sarcoplasmic reticulum (SR) is known to be an essential source of activator calcium required for contraction in mammalian and avian cardiac muscle. In contrast, physiological studies by Morad and Cleeman<sup>1</sup> and Fabiato<sup>2</sup> have suggested that SR does not provide activator calcium required for excitation-contraction coupling in frog ventricular muscle, although some evidence to the contrary has been presented.<sup>3,4</sup> Nonetheless, electron microscopic studies of frog ventricular muscle have dem-

onstrated the presence of a sparsely distributed SR<sup>5,6</sup> composed of two structurally distinct but continuous regions called tubular and terminal SR.<sup>5</sup> A major portion of the tubular SR encircles in part the myofibrils at the Z-line level. The remaining tubular SR is oriented more or less parallel to the longitudinal axis of the myofiber and interconnects the transversely oriented tubular SR in both the interior and peripheral regions of the myofiber. The diameter of the tubular SR varies between 35 and 60 nm.

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A.O.J. is a Scientist of the Medical Research Council of Canada and a recipient of a grant-in-aid T455 from the Heart and Stroke Foundation of Ontario. K.P.C. is an Investigator of the Howard

Hughes Medical Institute and a recipient of National Institutes of Health grant HL-14388. M.M. is a Scholar of the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research, as well as a recipient of a grant-in-aid from the Alberta Heart and Stroke Foundation. A.G.M. is a recipient of a Research Traineeship Award from the Canadian Heart Foundation (1987-88).

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Received November 28, 1990; accepted March 8, 1991.

Branches of tubular SR in the subsarcolemmal region of the myofibers terminate in flat, saclike structures called terminal SR. These specialized regions of the SR are closely apposed to the sarcolemma in the Z-line region of the myofibers.

Comparison between the ultrastructural features of frog and mammalian cardiac SR suggests that the terminal and tubular SR of the frog cardiac SR are analogous to the junctional and network SR of mammalian cardiac SR, respectively; the specialized region of the mammalian cardiac SR, however, called corbular SR, has not been observed so far in frog heart.

Biochemical studies have shown that canine cardiac SR vesicles are composed of at least two subpopulations. Whereas both populations contain the  $\text{Ca}^{2+}$ -ATPase,<sup>7</sup> only one contains a 55,000-Da protein<sup>7</sup> identified as the  $\text{Ca}^{2+}$ -binding protein calsequestrin.<sup>8</sup> Immunoelectron microscopic determination of the subcellular distribution of SR proteins in mammalian and avian cardiac muscle has shown that  $\text{Ca}^{2+}$ -ATPase<sup>9</sup> and phospholamban<sup>10</sup> are distributed fairly uniformly in the network SR but are absent from the nonjunctional region of the junctional SR. In contrast, calsequestrin is confined to the lumen of the junctional SR<sup>11-13</sup> and is absent from the lumen of the network SR.

Comparison of biochemical and immunocytochemical studies with electron probe analysis of the subcellular distribution of calcium in rabbit<sup>14</sup> and rat<sup>13</sup> cardiac muscle suggested that the corbular<sup>13</sup> and junctional<sup>13,14</sup> SR contain relatively high calcium concentrations, as well as calsequestrin,<sup>11,13</sup> whereas the network SR has a relatively low calcium concentration and lacks calsequestrin.<sup>11,13</sup> These results support the idea that the junctional and corbular SR containing calsequestrin sequesters  $\text{Ca}^{2+}$  accumulated during relaxation, thus being potential sources of  $\text{Ca}^{2+}$  released into the cytosol during excitation-contraction coupling in mammalian and avian cardiac muscle.

So far, information obtained from biochemical studies on the function and protein composition of isolated frog cardiac SR is very sparse. A biochemical study comparing the oxalate-supported  $\text{Ca}^{2+}$  uptake in crude microsomal fractions from ventricular tissue of different species reported that  $\text{Ca}^{2+}$  uptake was not detectable in crude microsomes from frog ventricular muscle.<sup>15</sup>

To assess frog myocardium for potential calcium storage sites, we decided to investigate whether frog cardiac SR contains a protein or proteins similar to calsequestrin, previously reported to be present in mammalian and avian cardiac muscle. In the present study, we used immunochemical and biochemical techniques to identify and characterize a calsequestrinlike protein in frog myocardium. It has an apparent molecular mass of 60,000 and biochemical characteristics similar to those of other cardiac calsequestrins. Immunofluorescence and immunoelectron microscopic studies showed this calseques-

trinlike protein to be localized in the lumen of two structurally distinct regions of SR in the subsarcolemmal region of the myofibers corresponding to what appears to be the lumen of the terminal SR and a corbularlike SR region in frog ventricular myocytes.

## Materials and Methods

### *Preparation of Microsomal Fraction of Frog Ventricular Muscle*

Frog cardiac microsomes were prepared according to the method of Chamberlain et al,<sup>16</sup> except that the potassium chloride extraction of actomyosin was not carried out because of the very small volumes involved. Forty frog hearts with a total weight of 1.85 g were homogenized in 4 volumes of buffer. From this, 1.8 mg of microsomal membranes was recovered by ultracentrifugation of the postmitochondrial supernatant for 2 hours at 100,000g. The postmicrosomal supernatant also was recovered from frog ventricular muscle by this procedure.

### *Identification of Calsequestrin in Frog Ventricular Muscle*

Frog cardiac calsequestrin was partially purified according to the method originally developed by Krause et al<sup>17</sup> and Slupsky et al.<sup>18</sup> Forty frog hearts were homogenized and extracted by ammonium sulfate solubilization and acid precipitation. This procedure recovered 1 ml of extract (3.7  $\mu\text{g}/\text{ml}$ ), which was dialyzed overnight in buffer A (0.1 M potassium phosphate, 1 mM EDTA, pH 7.1). The dialyzed extract then was loaded onto a 1.5-ml diethylaminoethyl (DEAE)-cellulose column equilibrated with buffer A. Because of the very small column size, proteins were eluted from the column in one step with 0.7 M sodium chloride and not with a sodium chloride gradient (0.05–0.8 M).

### *Preparation and Affinity Purification of Antisera to Chicken Cardiac Calsequestrin*

Chicken cardiac calsequestrin was purified according to the method of Campbell et al<sup>8</sup> and was used to prepare rabbit antisera to chicken cardiac calsequestrin as described previously.<sup>19</sup> Affinity-purified antibodies were prepared from rabbit antiserum to chicken cardiac calsequestrin by affinity chromatography on a calsequestrin-nitrocellulose complex prepared as described by Fowler and Bennett.<sup>20</sup> To prepare a calsequestrin-nitrocellulose complex, chicken skeletal calsequestrin, purified according to Slupsky et al,<sup>18</sup> was separated from minor contaminants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)<sup>21</sup> and was transferred onto nitrocellulose.<sup>22</sup> The 55,000-Da chicken skeletal calsequestrin band identified on the nitrocellulose transfer by Ponceau S staining<sup>23</sup> was cut from the nitrocellulose membrane and used for affinity purification.

### *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

Proteins and subcellular fractions from chicken cardiac and skeletal muscle and frog cardiac muscle were separated by SDS-PAGE using the discontinuous buffer system of Laemmli<sup>21</sup> in 1.5-mm thick gradient gels (5–15% acrylamide). Two-dimensional gel electrophoresis was carried out according to Michalak et al.<sup>24</sup> Some SDS-PAGE gels of frog proteins were stained with either Coomassie blue<sup>25</sup> or Stains-all.<sup>26</sup> Others were transferred electrophoretically onto nitrocellulose membranes and immunoblotted according to the procedure of Towbin et al.<sup>22</sup> For immunoblots, the nitrocellulose transfers were blocked with 5% powdered milk in Tris-buffered saline (100 mM Tris [hydroxymethyl] aminomethane, 2.5 mM NaCl, pH 7.5) before incubation with the primary antibody.<sup>27</sup> Secondary antibodies were coupled to alkaline phosphatase and developed with *p*-nitroblue-tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate toluidine (NBT/BCIP) in carbonate buffer, pH 9.8.<sup>28</sup> Before immunoblotting, the position of proteins transelektrophoresed from SDS-PAGE gels into nitrocellulose was visualized by staining with Ponceau S.<sup>23</sup>

### *Calcium-45 Overlay*

Partially purified frog cardiac calsequestrin preparations were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose as described above. <sup>45</sup>Ca<sup>2+</sup> overlay of the nitrocellulose transfer then was carried out according to the method of Maruyama et al.<sup>29</sup>

The nitrocellulose membrane was immersed in a solution of 60 mM potassium chloride, 5 mM magnesium chloride, and 10 mM imidazole-HCl (pH 6.8) and was washed several times. The membrane then was incubated in 1 mCi/l <sup>45</sup>Ca<sup>2+</sup> diluted in the above buffer for 10 minutes. The membrane then was rinsed with distilled water and allowed to dry at room temperature for several hours, and was exposed to XAR-5 x-ray film (Eastman Kodak Co., Rochester, N.Y.), and left at -20°C for 14 days before developing. Subsequently, the membrane was rehydrated in the above solution and rinsed with EGTA to remove <sup>45</sup>Ca<sup>2+</sup>. Immunolabeling of the blot with affinity-purified antibodies to calsequestrin then was carried out as outlined above.

### *Protein Sequencing*

Frog calsequestrin was partially purified from the ammonium sulfate extract<sup>17,18</sup> followed by Mono Q fast protein liquid chromatography as described by Milner et al.<sup>30</sup> Briefly, a Mono Q 5/5 fast protein liquid chromatographic column (Pharmacia LKB Biotechnology, Quebec, Canada) was loaded with partially purified protein and eluted with a linear NaCl gradient (50–750 mM). The protein composition of the eluted fractions was assayed by Western blotting of SDS-PAGE-separated aliquots with anti-calsequestrin antibodies. The peak fractions contain-

ing the 60,000-Da band positively labeled with calsequestrin antibodies (450–500 mM NaCl) were pooled, dialyzed, and freeze-dried.

NH<sub>2</sub>-terminal sequence analysis of the 60,000-Da protein was carried out on electrophoretically purified protein obtained by transblotting SDS-PAGE-separated aliquots of the freeze-dried sample to Immobilon (polyvinylidene difluoride, Millipore Corp., Bedford, Mass.) membrane<sup>31</sup> and excising the band. Automated sequence analysis was performed with a Model 470A gas-liquid phase protein sequencer (Applied Biosystems, Inc., Foster City, Calif.) connected on-line to an Applied Biosystems Model 120A high-performance liquid chromatograph<sup>32</sup> using the current protocols of Applied Biosystems for both instruments. Two independent sequence analyses were performed with repetitive yields of approximately 94%.

### *Dissection and Fixation*

Adult *Rana pipiens*, with a body length of approximately 6.4–7.6 cm, were pithed, and hearts were removed and rinsed in phosphate buffered saline. Hearts to be used for preparation of cryosections and epoxy resin (Epon) sections were cut in half and fixed in 3.5% paraformaldehyde for 2 hours as described previously.<sup>33</sup> Myocardial tissue to be used for immunoelectron microscopic labeling was prepared as described previously (Procedure II, Reference 34).<sup>13</sup> Briefly, the myocardial tissue was cryofixed,<sup>35</sup> freeze-dried, osmicated, and embedded in Spurr resin.<sup>36,37</sup> Thin sections (60–80 nm) were collected on nickel grids coated with formvar.

### *Preparation of Cryosections*

After fixation, hearts were washed, infused for 1 hour with 0.6 M sucrose, and frozen as described previously.<sup>33</sup> Tissue blocks were stored in liquid nitrogen until cryosectioned. Cryosections (4–8 μm) were stored desiccated at -20°C for up to 6 months.

### *Preparation of Sections of Epon-Embedded Frog Ventricular Muscle*

Fixed hearts were treated with 10% polyvinylpyrrolidone in 0.2 M ethylacetimidate-HCl and then embedded in Epon as described by Kuhlmann and Krischan.<sup>38</sup> Semithin sections (1–2 μm) were cut with a glass knife on a Sorval MT2-B ultramicrotome (Du Pont Instruments).

### *Indirect Immunofluorescence Labeling*

Indirect immunofluorescence labeling of 4–8-μm cryosections and 1–2-μm sections of Epon-embedded fixed frog ventricular muscle for calsequestrin was carried out as described previously,<sup>33</sup> except that sections of Epon-embedded tissue were etched for 3 minutes with 3% sodium methoxide in a 1:1 mixture of methanol/benzene before immunolabeling.<sup>38</sup> Affinity-purified antibodies to chicken cardiac calsequestrin were used as the primary antibody at various dilutions. A fluorescein isothiocyanate (FITC)-la-

beled  $\gamma$ -globulin fraction of goat anti-rabbit serum was used as the secondary antibody (1:20 dilution).

Double labeling of cryosections and Epon-embedded sections was carried out by first labeling for calsequestrin by the indirect immunolabeling procedure outlined above and then labeling with wheat germ agglutinin conjugated to rhodamine (1:200 dilution) (Sigma Chemical Co., St. Louis). Fluorescently labeled sections were examined with a photomicroscope (Carl Zeiss, Inc., Thornwood, N.Y.) provided with an epifluorescence attachment with filter systems designed for double labeling with fluorescein and rhodamine fluorescence markers. The FITC fluorescence pictures were photographed with Kodak Tmax film, and rhodamine fluorescence pictures were photographed with Kodak 2415 film. Both films were developed with XPI-400 processing chemical (Ilford, England).

#### Indirect Immunoelectron Microscopic Labeling

Immunocolloidal-gold labeling of thin sections of cryofixed, freeze-dried, and Spurr-embedded frog ventricular muscle was carried out as described previously.<sup>13</sup> Sections first were labeled with affinity-purified antibodies to chicken cardiac calsequestrin (40  $\mu$ g/ml in phosphate buffered saline, pH 7.4) and then with affinity-purified goat anti-rabbit  $\gamma$ -globulin 5 nm colloidal gold conjugate (0.5 mg/ml phosphate buffered saline containing 3% bovine serum albumin; Janssen Pharmaceutica, Beerse, Belgium).

To assess the specificity of the immunolabeling for calsequestrin on thin sections of Spurr-embedded tissue, rabbit  $\gamma$ -globulin purified from preimmune serum (40  $\mu$ g/ml phosphate buffered saline) was substituted for affinity-purified chicken cardiac calsequestrin antibodies in the immunolabeling procedure. After immunolabeling, the sections first were stained for 15 seconds in saturated uranyl acetate in 50% ethanol<sup>39</sup> and then for 15 seconds in lead citrate. The sections were examined in a transmission electron microscope (model H-7000, Hitachi, Tokyo).

## Results

#### Identification and Characterization of Frog Cardiac Calsequestrin

To determine if frog cardiac SR contains a calsequestrinlike protein, the microsomal fraction containing SR vesicles and the postmicrosomal supernatant were isolated from frog ventricular muscle and subjected to SDS-PAGE followed by transelektrophoresis to nitrocellulose. Subsequent immunoblotting of these fractions showed that affinity-purified antibodies to chicken cardiac calsequestrin bound to a single band with an apparent molecular mass of 60,000 in frog cardiac microsomes (Figure 1A, lane 1). No bands were detected in the postmicrosomal supernatant from frog cardiac muscle (Figure 1A, lane 2). These results show that a 60,000-Da protein that is immunologically related to chicken cardiac and skeletal calsequestrin is present in the micro-

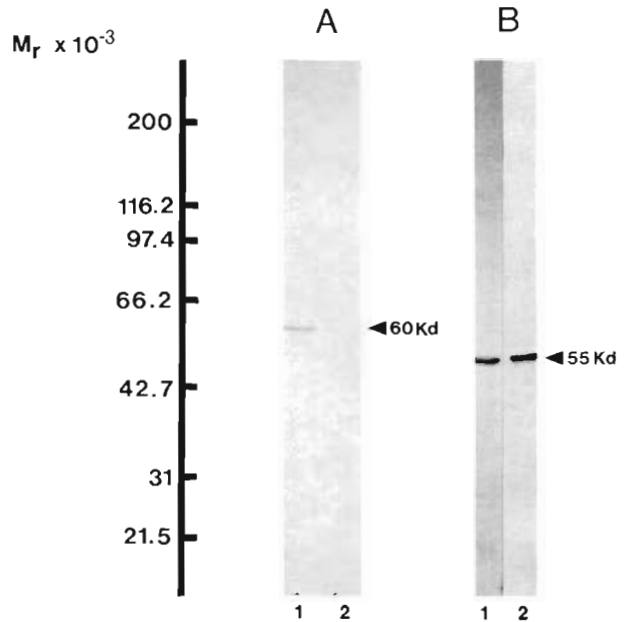


FIGURE 1. Identification of calsequestrin in frog cardiac sarcoplasmic reticulum. Panel A: Microsomal fraction (lane 1) (90  $\mu$ g) and postmicrosomal supernatant (lane 2) (90  $\mu$ g) from frog cardiac muscle prepared as described in "Materials and Methods" were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5–15% gradient gels), transferred onto nitrocellulose paper, and immunolabeled with affinity-purified chicken cardiac calsequestrin antibodies as described in "Materials and Methods." Antibodies to chicken cardiac calsequestrin specifically bind a 60-kDa component that is present in the frog cardiac microsomes (lane 1) but absent from the microsomal supernatant (lane 2). Panel B: Purified chicken skeletal calsequestrin (1  $\mu$ g) prepared as described in "Materials and Methods" was analyzed by SDS-PAGE (5–15% acrylamide gradient gels) and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose and immunolabeled with affinity-purified chicken cardiac calsequestrin antibodies (lane 2). Antiserum for chicken cardiac calsequestrin specifically binds a 55-kDa component (lane 2) that coelectrophoresed with chicken skeletal calsequestrin (lane 1) but has a distinctly lower electrophoretic mobility than that of the 60-kDa band present in frog cardiac microsomes (panel A, lane 1).

somal fraction containing frog ventricular SR and absent from the postmicrosomal supernatant of frog ventricular muscle. The finding that affinity-purified antibodies to chicken cardiac calsequestrin bound to only one band of 60,000 Da strongly supports the conclusion that this antibody is specific for a 60,000-Da calsequestrinlike protein in frog ventricular SR. However, the electrophoretic mobility of the 60,000-Da calsequestrinlike protein in frog cardiac microsomes is distinct from that of chick cardiac<sup>12</sup> and skeletal<sup>17</sup> calsequestrin (Figure 1B, lanes 1 and 2), both of which are 55,000 Da.

Cardiac calsequestrin previously has been extracted from whole-cell homogenate of mammalian myocardial tissue by ammonium sulfate solubilization

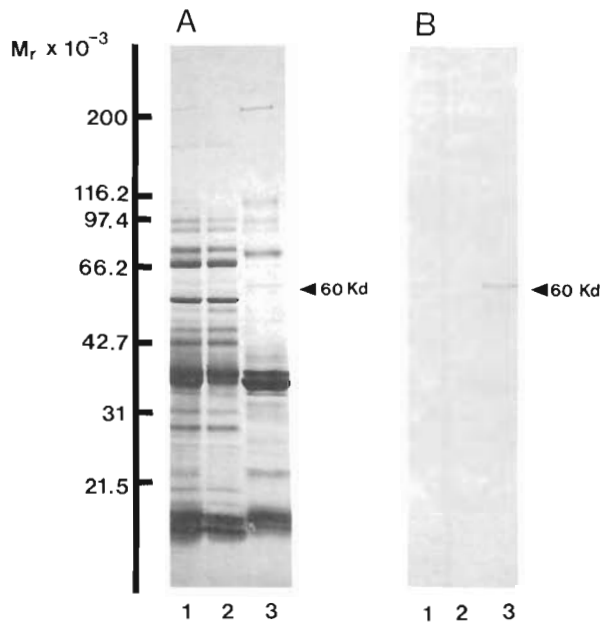


FIGURE 2. Partial purification of calsequestrin from frog ventricular muscle. Frog cardiac calsequestrin was extracted from homogenized whole hearts by ammonium sulfate solubilization and acid precipitation. This extract then was loaded onto a diethylaminoethyl (DEAE)-cellulose column, and a calsequestrin-enriched fraction eluted from the column with 0.7 M sodium chloride as described in "Materials and Methods." The various fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5–15% acrylamide gradient gels) followed by Coomassie blue staining (panel A) or were transferred onto nitrocellulose and immunolabeled with affinity-purified chicken cardiac calsequestrin antibodies (panel B). Lane 1, frog cardiac extract recovered after ammonium sulfate solubilization and acid precipitation (40  $\mu$ g). Lane 2, fraction of the frog cardiac extract that did not bind to the DEAE-cellulose column (40  $\mu$ g). Lane 3, fraction of the frog cardiac extract bound to the DEAE-cellulose column, from which it was subsequently eluted with 0.7 M sodium chloride (40  $\mu$ g).

and acid precipitation.<sup>18</sup> Subsequently, calsequestrin was partially purified from this extract by elution from a DEAE-cellulose column with 0.7 M sodium chloride.<sup>18</sup> To determine if the 60,000-Da protein in frog ventricular SR also could be partially purified by this procedure, frog ventricular muscle was subjected to the purification procedure described above. Coomassie blue staining of the cell extract loaded onto the DEAE-cellulose column (Figure 2A, lane 1), of the fraction of the extract that did not bind to the column (~90% of total protein) (Figure 2A, lane 2), and of the fraction of the cell extract that bound to and was subsequently eluted from the DEAE-cellulose column with 0.7 M sodium chloride (~7% of the total protein) (Figure 2A, lane 3) demonstrated the presence of many protein bands in each of these fractions.

The protein composition of the cell extract (Figure 2A, lane 1) was very similar to that of the unbound

fraction of the cell extract (Figure 2A, lane 2). In contrast, the protein composition of the fraction of the cell extract that bound to and was eluted from the column (Figure 2A, lane 3) was distinctly different from that of the cell extract and the unbound protein. Interestingly, a minor band with an apparent  $M_r$  of 60,000 was enriched in the fraction eluted from the DEAE column (Figure 2A, lane 3) and not discernible either in the cell extracts (Figure 2A, lane 1) or in the unbound fraction (Figure 2A, lane 2). Like other calsequestrins,<sup>40–43</sup> this protein also stains blue with Stains-all; however, the intensity of staining was too weak for photographic reproduction.

Immunoblotting of the same fractions as described above with affinity-purified antibodies to chicken cardiac calsequestrin showed that a single band with an apparent molecular mass of 60,000 was present in both the cell extract (Figure 2B, lane 1) and in the fraction eluted from the column (Figure 2B, lane 3). No band was detected in the unbound fraction, demonstrating that all of the 60,000-Da protein was bound to the DEAE-cellulose column (Figure 2B, lane 2). The 60,000-Da band in the fractions eluted from the DEAE column (Figure 2B, lane 3) was relatively strongly labeled compared with that in the cell extract (Figure 2B, lane 1). These results showed that the 60,000-Da protein from frog ventricular muscle binding the affinity-purified antibodies to chick cardiac calsequestrin can be enriched using the same procedure previously used to purify cardiac calsequestrins.<sup>18</sup>

Calsequestrins previously have been shown to bind  $^{45}\text{Ca}^{2+}$  on nitrocellulose transfers.<sup>17,44</sup> To determine if the 60,000-Da calsequestrinlike protein in frog myocardium also binds  $\text{Ca}^{2+}$ , nitrocellulose transfers of SDS-PAGE-separated partially purified frog cardiac calsequestrin (Figure 2A, lane 3) were double labeled first with  $^{45}\text{Ca}^{2+}$  and then immunoblotted with antibodies to chick cardiac calsequestrin. An autoradiogram of the nitrocellulose transfer showed that several protein bands bound  $^{45}\text{Ca}^{2+}$  (Figure 3, lane 2). Comparison between the autoradiogram (Figure 3, lane 2) and the immunoblot (Figure 3, lane 1) showed that the electrophoretic mobility of one of the  $^{45}\text{Ca}^{2+}$  bands corresponded to that of a 60,000-Da protein that bound calsequestrin antibodies (Figure 3, lane 1). These results strongly support the conclusion that the 60,000-Da calsequestrinlike protein from frog ventricular muscle shares with other cardiac calsequestrins the ability to bind calcium.

Calsequestrins from muscle cells also have been shown to undergo a change in apparent molecular mass with pH shifts as demonstrated by two-dimensional SDS-PAGE.<sup>24</sup> Thus, in a two-dimensional gel system with a first separation step at pH 7.0 on a Weber-Osborn gel system<sup>25</sup> and a second separation step at pH 8.7 in a Laemmli gel system,<sup>21</sup> calsequestrin from rabbit and rat skeletal muscle and canine cardiac muscle<sup>8</sup> exhibits a higher apparent molecular mass in the pH 8.7 system when compared with other proteins of a similar apparent molecular mass in the

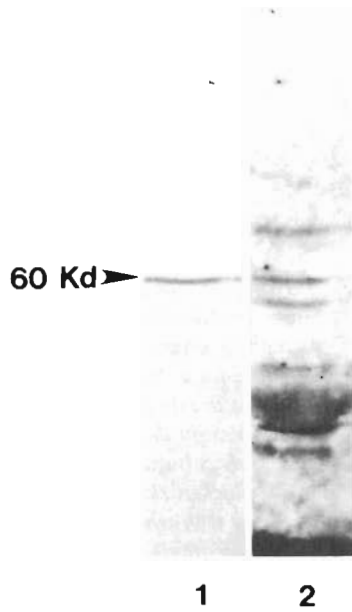


FIGURE 3.  $^{45}\text{Ca}^{2+}$  overlay and calsequestrin antibody immunolabeling of partially purified frog cardiac calsequestrin. Partially purified frog cardiac calsequestrin (40  $\mu\text{g}$ ) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5–15% gradient gels) and transferred to nitrocellulose as described in "Materials and Methods." The nitrocellulose membrane was incubated with  $^{45}\text{Ca}^{2+}$  and exposed to x-ray film (lane 2). After exposure, the membrane was washed to remove  $^{45}\text{Ca}^{2+}$  and then was immunolabeled with affinity-purified antibodies to chicken cardiac calsequestrin (lane 1).

pH 7.0 system. For example, canine cardiac calsequestrin (45,000 Da, as calculated on the basis of the amino acid sequence deduced from the sequence of the DNA)<sup>45</sup> has been shown to exhibit an apparent molecular mass of 44,000 when electrophoresed at pH 7.0 in the Weber-Osborn gel system and an apparent molecular mass of 54,000 at pH 8.7 in the Laemmli gel system.<sup>8</sup> Immunoblotting of partially purified frog cardiac calsequestrin with affinity-purified antibodies to chicken cardiac calsequestrin after two-dimensional gel electrophoresis identified a single protein (Figure 4). This protein exhibited an apparent molecular mass of 54,000 in the pH 7.0 gel

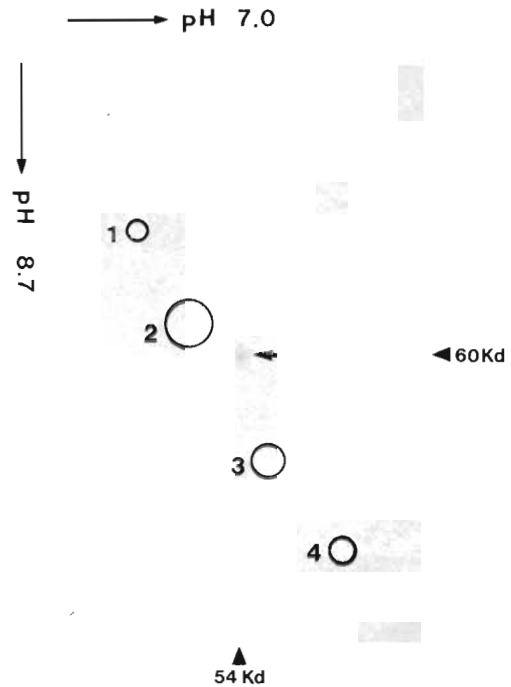


FIGURE 4. Immunoblotting and gel electrophoresis of partially purified frog cardiac calsequestrin separated by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Partially purified frog cardiac calsequestrin (40  $\mu\text{g}$ ) first was separated on a Weber-Osborn-type SDS-PAGE (10% acrylamide continuous gel, pH 7.0). Subsequently, a single lane was cut from the Weber-Osborn gel and applied to and separated on a Laemmli-type SDS-PAGE (5–15% acrylamide gradient gel, pH 8.7). Separated proteins then were transferred onto nitrocellulose and immunolabeled with affinity-purified antibodies to chicken cardiac calsequestrin as described in "Materials and Methods." Arrowhead indicates the position of the protein in the frog cardiac extract specifically labeled with antibodies to chicken cardiac calsequestrin. Molecular weight markers used were 1) rabbit muscle phosphorylase b: 97,000 Da; 2) bovine serum albumin: 66,200 Da; 3) hen ovalbumin: 42,699 Da; 4) bovine carbonic anhydrase: 31,000 Da. Position of the molecular weight markers on the immunoblot was determined by staining the nitrocellulose sheet with Ponceau S before immunoblotting.

system and a molecular mass of 60,000 in the pH 8.7 gel system and therefore was displaced from the diagonal formed by the marker proteins exhibiting the same molecular mass in both dimensions (Figure 4). Thus, the 60,000-Da calsequestrinlike protein in frog myocardium exhibits a shift in its apparent molecular mass on a two-dimensional gel system analogous to that of other calsequestrins.

#### *NH<sub>2</sub>-Terminal Amino Acid Sequence*

The amino acid sequence of canine cardiac calsequestrin previously has been determined by deduction from the nucleotide sequence of cDNA.<sup>45</sup> To assess more precisely the likeness of the 60,000-Da protein band positively labeled with affinity-purified antibodies to calsequestrin, its  $\text{NH}_2$ -terminal amino

TABLE 1. Comparison Between  $\text{NH}_2$ -Terminal Amino Acid Sequences of Calsequestrins and Calreticulins

Protein	$\text{NH}_2$ -Terminal amino acid sequence	Reference
Frog cardiac calsequestrin	EEGLNFPTY	Present publication
Canine cardiac calsequestrin	EEGLNFPTY	18, 45
Rabbit skeletal calsequestrin	EEGLDFPEY	46
Rabbit skeletal calreticulin	EPVVYFKEQ	47
Rabbit liver calreticulin	EPVVYFKEQ	48, 49

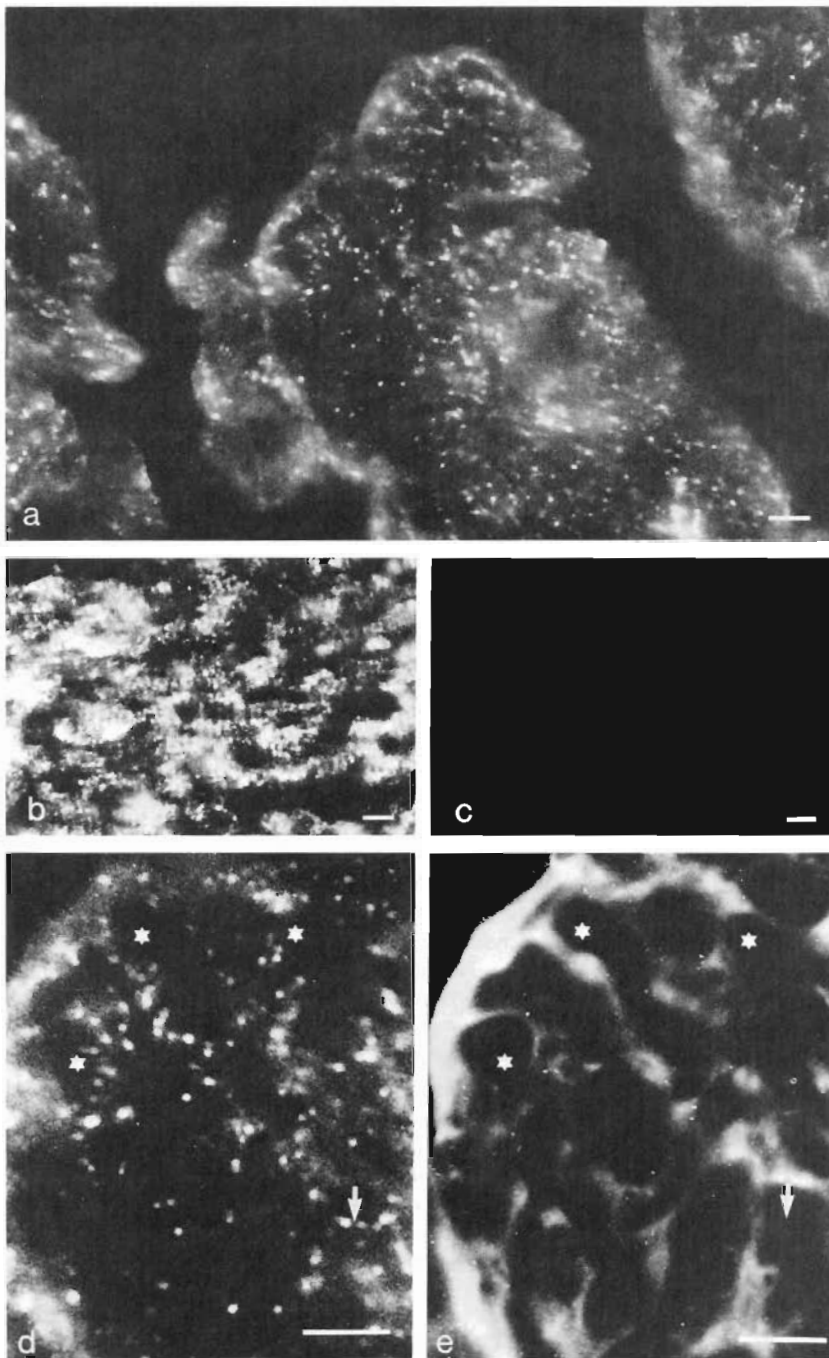


FIGURE 5. Immunofluorescent localization of cardiac calsequestrin in 4–8- $\mu\text{m}$  cryosections of frog ventricular muscle by immunofluorescence labeling. Light micrographs of cryosections of 3.5% paraformaldehyde-fixed frog ventricular muscle labeled with affinity-purified chicken cardiac calsequestrin antibodies (panels a, b, and d), with calsequestrin antibodies absorbed with chicken skeletal calsequestrin (panel c), and with wheat germ agglutinin conjugated to rhodamine (panel e). In some regions of the cryosection, immunolabeling for calsequestrin was distributed in discrete foci (panels a and d), whereas in other regions of the section, a cross-striated pattern was observed (panel b). Intensity of the fluorescent staining observed after immunolabeling with chicken cardiac calsequestrin antibodies (panel b) was greatly decreased when the antibodies to chicken cardiac calsequestrin were absorbed with chicken skeletal calsequestrin before immunolabeling (panel c). Comparison between the distribution of the two staining patterns in panels d and e shows that some of the discrete foci labeled for calsequestrin (stars; panel d) are distributed at the periphery of the ventricular cells (stars; panel e), whereas other foci (arrow; panel d) are not (arrow; panel e). Bar, 5  $\mu\text{m}$ .

acid sequence was determined by automatic sequence analysis.

The results presented in Table 1 show that the sequence of the first nine amino acids at the  $\text{NH}_2$ -terminal end of the 60,000-Da calsequestrinlike protein from frog ventricular myocardium was identical to that of both canine cardiac calsequestrin<sup>18,45</sup> and rabbit skeletal calsequestrin<sup>46</sup> but distinct from that of the  $\text{Ca}^{2+}$  binding protein calreticulin (earlier referred to as the high-affinity calcium binding protein) of skeletal muscle<sup>47</sup> and rabbit liver.<sup>48,49</sup> Thus, we conclude that the 60,000-Da protein antigenically related

to chicken cardiac calsequestrin indeed represents a calsequestrin isoform of frog ventricular muscle.

#### *Subcellular Distribution of Calsequestrin in Frog Myocardium In Situ*

**Immunofluorescence staining.** Examination of cryosections of frog ventricular myocardium labeled with affinity-purified antibodies to cardiac calsequestrin showed the presence of two distinct fluorescence staining patterns (Figures 5a and 5b). One of these appeared as discrete foci distributed throughout bundles of myocytes (Figures 5a and 5d). In other regions

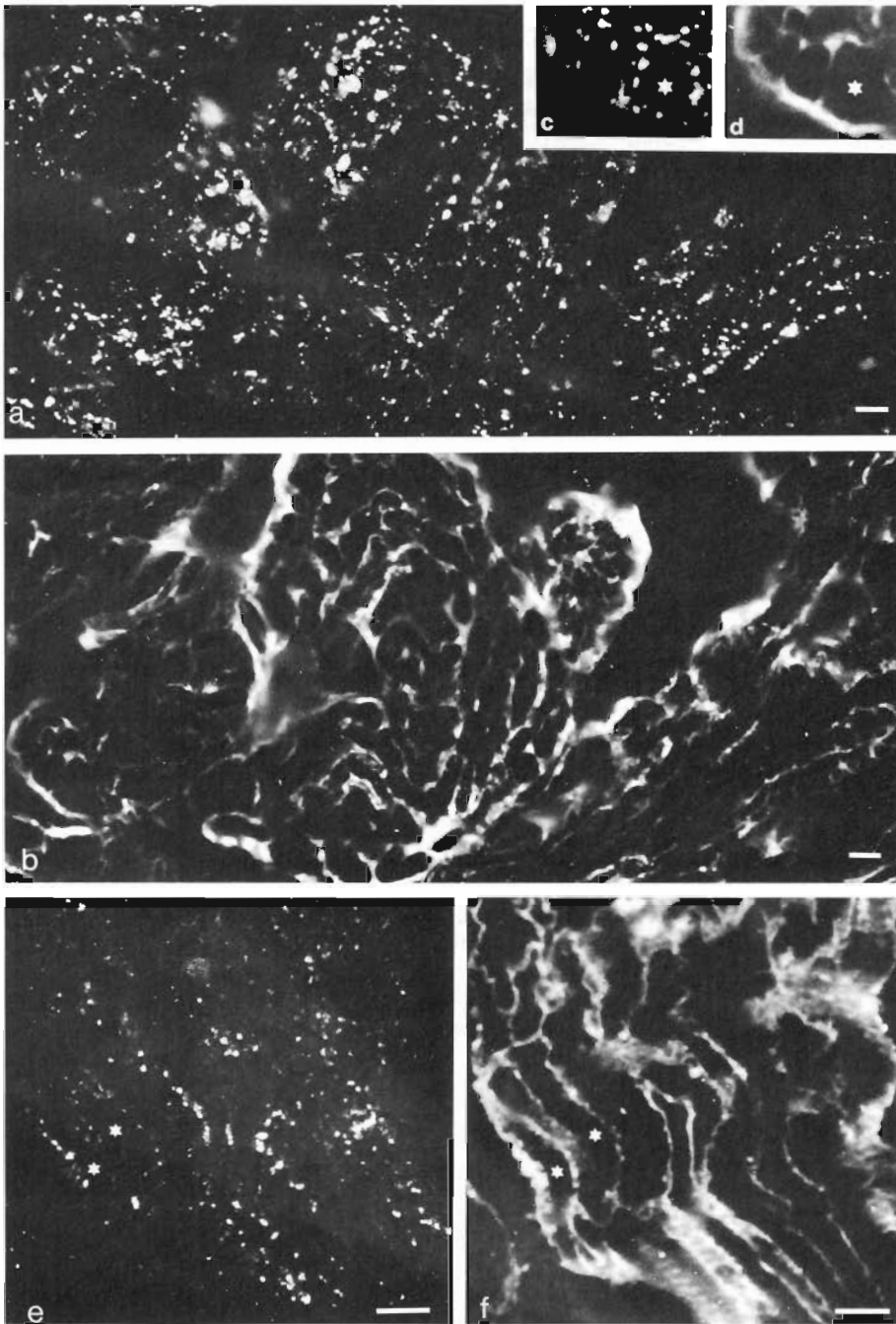
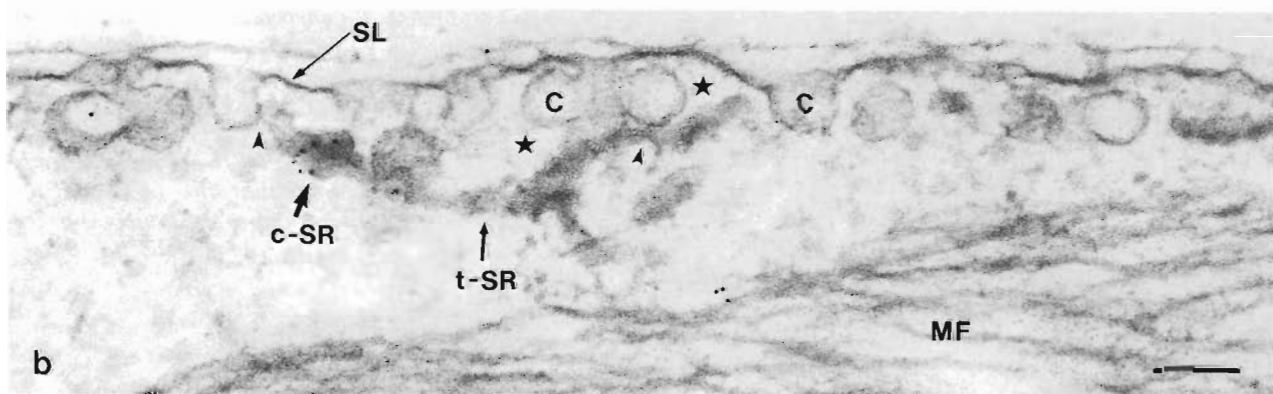
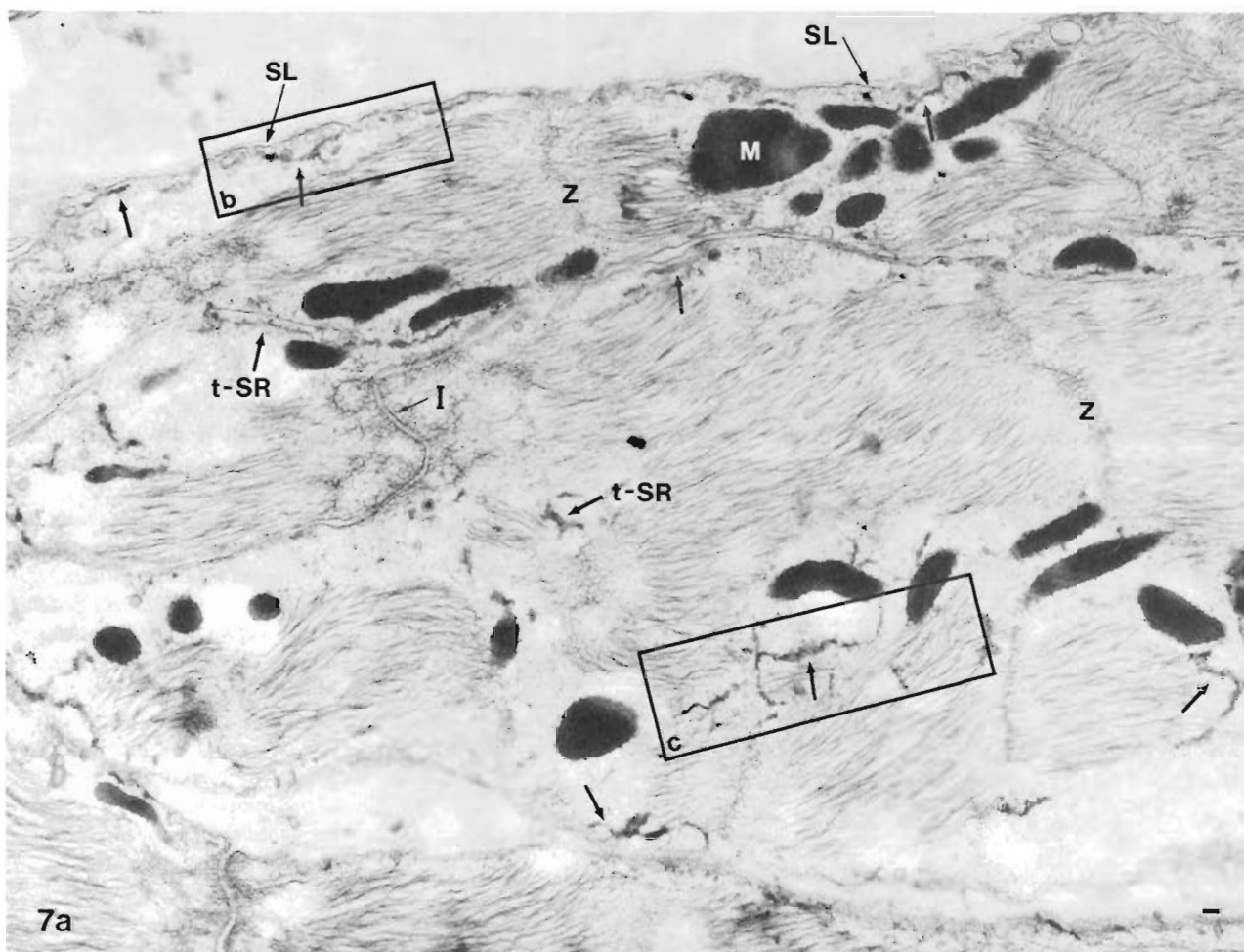


FIGURE 6. Localization of cardiac calsequestrin in 1–2- $\mu$ m plastic sections of frog ventricular muscle by immunofluorescence labeling. Semithin (1  $\mu$ m) sections of Epon-embedded frog myocardium were double labeled with affinity-purified antibodies to chicken cardiac calsequestrin (panels a, c, and e) and with wheat germ agglutinin, a marker of the sarcolemma (panels b, d, and f). At low power, a pattern of discrete calsequestrin-containing foci was observed throughout the section (panel a). The wheat germ agglutinin labeling pattern observed after double labeling of this same section (panel b) demonstrates that the frog ventricular muscle cells are arranged in bundles in which there is very extensive interdigitation and considerable variations in the orientation of the individual myocytes within the bundles (panel b), thus explaining why it is so difficult to discern a distinct pattern of calsequestrin labeling from the cryosections shown in Figure 4. Comparison between the distribution of the calsequestrin antibody-labeled foci (stars; panels c and e) and the wheat germ agglutinin labeling pattern (stars; panels d and f) in the same sections clearly show that the calsequestrin-labeled foci are distributed at the periphery of the cells. Bar, 5  $\mu$ m.

of the cryosections, a cross-striated fluorescent staining pattern was observed (Figure 5b). Comparison of the cross-striated fluorescent staining pattern with the phase-contrast view of the same field (not shown) also showed a cross-striated pattern, implying that this staining pattern was observed in longitudinally sectioned myocytes. In contrast, phase-contrast imaging (not shown) did not reveal either the orientation or the outline of the individual cells present in bundles of myocytes in areas showing a staining pattern of discrete fluorescent foci (Figures 5a and 5d). This is most likely due to the fact that myocardial cells in a bundle of frog ventricular myocytes are very

closely apposed, thus making it very difficult to determine the distribution of calsequestrin-containing foci in relation to the circumference of the myocytes.<sup>5</sup> To determine the subcellular distribution of these fluorescent foci (in relation to the circumference of the myocytes), cryosections were double labeled with calsequestrin antibodies (Figure 5d) and with wheat germ agglutinin (Figure 5e) by fluorescence labeling. Comparison between the distribution of the two staining patterns strongly suggests that several of the fluorescent foci (Figure 5d, stars) are located at the periphery of the frog myocardial cells as outlined by the labeling with wheat germ agglutinin





(Figure 5e, stars). However, the subcellular distribution of the fluorescent foci in other regions is more difficult to interpret (Figures 5d and 5e, arrows).

To determine more precisely the subcellular distribution of the fluorescent foci in frog myocardium, 1- $\mu$ m sections of Epon-embedded frog ventricular muscle were double labeled with antibodies to calsequestrin (Figures 6a, 6c, and 6e) and with wheat germ agglutinin (Figures 6b, 6d, and 6f). In these thin sections, it is very obvious that most of the calsequestrin antibody labeling is present in discrete foci confined to the periphery of both transversely (Figures 6c and 6f, stars) and obliquely and longitudinally (Figures 6e and 6f, stars) sectioned myocytes. The lack of a cross-striated calsequestrin labeling pattern in thin (1- $\mu$ m) longitudinal cryosections (Figures 6c and 6f) previously observed in some of the thick (4–8- $\mu$ m) longitudinal cryosections (Figure 5b) strongly suggests that most calsequestrin labeling is confined to the cell periphery. The cross-striated pattern seen in some thick (4–8- $\mu$ m) cryosections (Figure 5b) is most likely due to the fact that large portions of longitudinally cut frog myocardial cells (2–10- $\mu$ m diameter) may be present within the thickness of the section.

The specificity of the staining observed in the frog myocardium with antibodies to chicken cardiac calsequestrin was demonstrated by absorbing the affinity-purified antibodies to cardiac calsequestrin with chicken skeletal calsequestrin before the indirect immunofluorescence staining procedure. The results show that the intensity of the staining pattern described above (Figure 5b) was greatly diminished after absorption (Figure 5c).

*Immunoelectron microscopic localization of frog cardiac calsequestrin.* The immunofluorescence labeling studies presented above support the idea that frog cardiac calsequestrin is densely distributed in discrete regions of the SR in the subsarcolemmal region of frog ventricular myocardial cells and is generally

absent from SR in the interior regions of these fibers. To determine if this is indeed the case, the studies were extended to include immunoelectron microscopic localization of calsequestrin in frog ventricular muscle.

We previously have shown that cryofixed, freeze-dried, and osmicated rat cardiac muscle is very suitable for simultaneous visualization of the ultrastructural features of cardiac SR and of the distribution of cardiac calsequestrin by immunocolloidal-gold labeling.<sup>13</sup> Thus, thin sections of frog ventricular muscle prepared by this procedure were labeled with calsequestrin antibodies by the indirect immunocolloidal-gold staining technique.

Regarding the subcellular distribution of the SR in frog cardiac myofibers, Figures 7–9 show that most of the SR as previously reported by Page and Niedergecker<sup>5</sup> is composed of a loose network of continuous tubular structures sparsely distributed in the subsarcolemmal (t-SR and large arrows; Figures 7a, 7b, 8a, 9a, 9b, and 9e) and intermyofibrillar (t-SR and large arrows; Figures 7a and 7c) regions of the myofibers. Generally, transversely oriented tubular SR was observed to encircle the myofibrils at the level of the central region of the A-band (t-SR; Figures 8a and 8b). This is in contrast to the ultrastructural studies by Page and Niedergecker,<sup>5</sup> who reported transversely oriented tubular SR to encircle the myofibrils at the level of the Z-line. Furthermore, in the present study, terminal SR appeared to be either absent (Figures 7a, 7b, and 8a) or very sparsely distributed (T-SR; Figures 9a, 9b, and 9d) in most images of myocardial fibers although apparently densely distributed in a few myocardial fibers (T-SR; Figure 9e). In addition, terminal SR did not appear to be confined to the level of the Z-line (T-SR; Figure 9b).

Examination of the distribution of colloidal gold particles in thin sections of frog ventricular muscle showed that most of the calsequestrin labeling was confined to SR in the subsarcolemmal region of the myofibers, where it was localized over two structurally distinct specializations of the SR (Figures 7–9). Thus, calsequestrin labeling generally was confined to terminal SR (T-SR; Figures 9b, 9d, and 9e) and to discrete ovoid expansions (60 $\times$ 100 nm) on transversely (c-SR; Figures 8a and 8b) and longitudinally (c-SR; Figures 7b, 9a, 9b, and 9c) oriented tubular SR. Calsequestrin labeling apparently was absent from the narrow regions of tubular SR (Figures 7–9).

It is noteworthy that subsarcolemmal tubular SR containing calsequestrin-labeled ovoid expansions very frequently was observed to meander in close proximity to the short invaginations of the sarcolemma called caveolae (C and arrowheads; Figures 7b, 8a, 8b, 9a, 9b, and 9c). The calsequestrin-labeled ovoid structures of the tubular SR, although close to the sarcolemma as judged by their close proximity to caveolae (short arrows; Figure 7b), were, however, not closely apposed to the surface region of the sarcolemma (SL; Figure 7b). This is in contrast to calsequestrin-labeled terminal SR, which is closely apposed

FIGURE 7. Electron micrograph of an ultrathin section of cryofixed, freeze-dried, and Spurr-embedded frog ventricular myocardium labeled with affinity-purified antibodies to calsequestrin by the indirect immunocolloidal-gold labeling technique (see "Materials and Methods"). Panel a: Low-magnification view of frog myocardial fibers shows that sarcoplasmic reticulum (t-SR and arrows) is sparsely distributed in the subsarcolemmal and interior regions of the myofibers. The enclosed areas of a peripheral and interior region of two myocardial cells in panel a are shown at higher magnifications in panels b and c, respectively. Panel b: Colloidal gold particles are observed in ovoid expansions (c-SR) on tubular SR (t-SR) in the subsarcolemmal region. It is noteworthy that t-SR at the cell periphery frequently is observed to be closely apposed to caveolae (C) (arrowhead) but not particularly closely apposed to the sarcolemma (star). Panel c: Generally, colloidal gold particles are not associated with t-SR in the interior regions of the myofibers. Z, Z-line; MF, myofibrils; M, mitochondria; I, intercalated disc; SL, sarcolemma. Bar, 0.1  $\mu$ m.



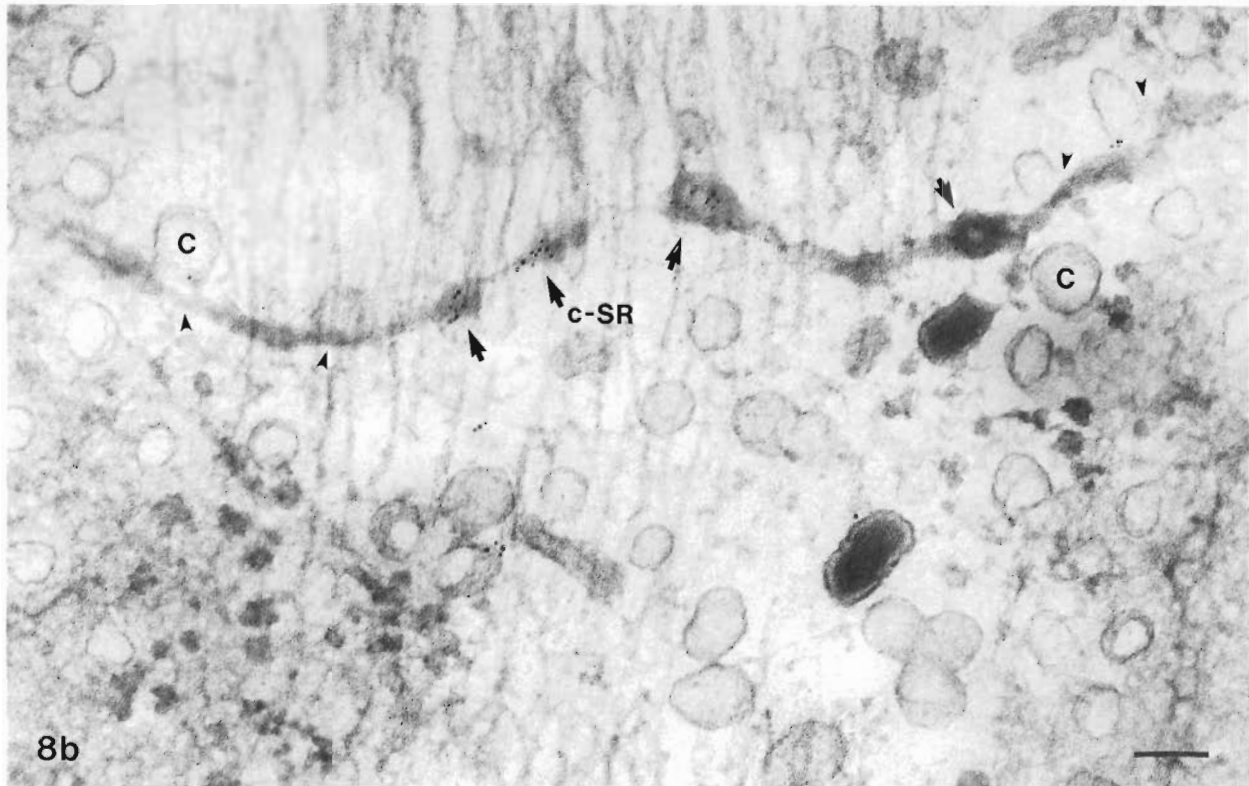


FIGURE 8. Panel a: Electron micrograph of longitudinal section of parts of two frog ventricular myofibers (labeled I and II) immunolabeled for calsequestrin (see "Materials and Methods" for details). Panel b: Higher magnification of upper portion of myofiber I. Dense distribution of caveolae (stars; panel a) in portions of the A-band regions imply that myofiber I is sectioned longitudinally through the subsarcolemma in these regions of the myofiber. Tubular SR (t-SR) continuous with ovoid expansions (c-SR and large arrows) are observed to be transversely oriented in the central region of two of the A-bands in myofiber I. Colloidal gold particles are densely and specifically distributed over approximately half of the ovoid expansions (c-SR and large arrows) of the transversely oriented t-SR (panels a and b). The t-SR appears to meander in very close proximity to caveolae (C) also present in the subsarcolemmal region of the myofiber (arrowheads; panels a and b). Networklike t-SR (double arrow; panel a) oriented parallel to the longitudinal axis of the myofibers also is present in both fibers I and II. A, A-band; Z, Z-line; SL, sarcolemma. Bar, 0.1  $\mu\text{m}$ .

to the sarcolemma (T-SR; Figures 9b and 9d). Electron-dense structures bridging the terminal SR and the sarcolemma as previously reported<sup>5</sup> were rarely observed (small arrows; Figure 9d). Because these structures were not periodic, it is uncertain whether they represent the bridging structures previously reported to be characteristic of terminal SR.<sup>5</sup> Calsequestrin-labeled SR only very rarely was observed in the interior regions of the myofibers (not shown).

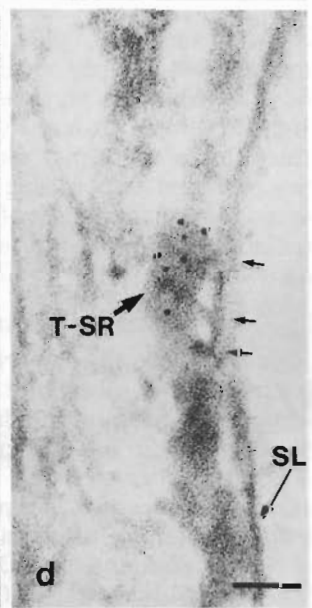
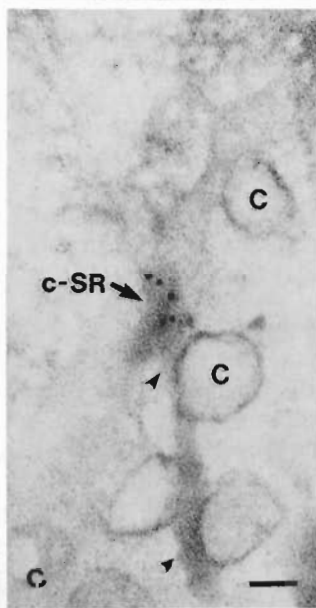
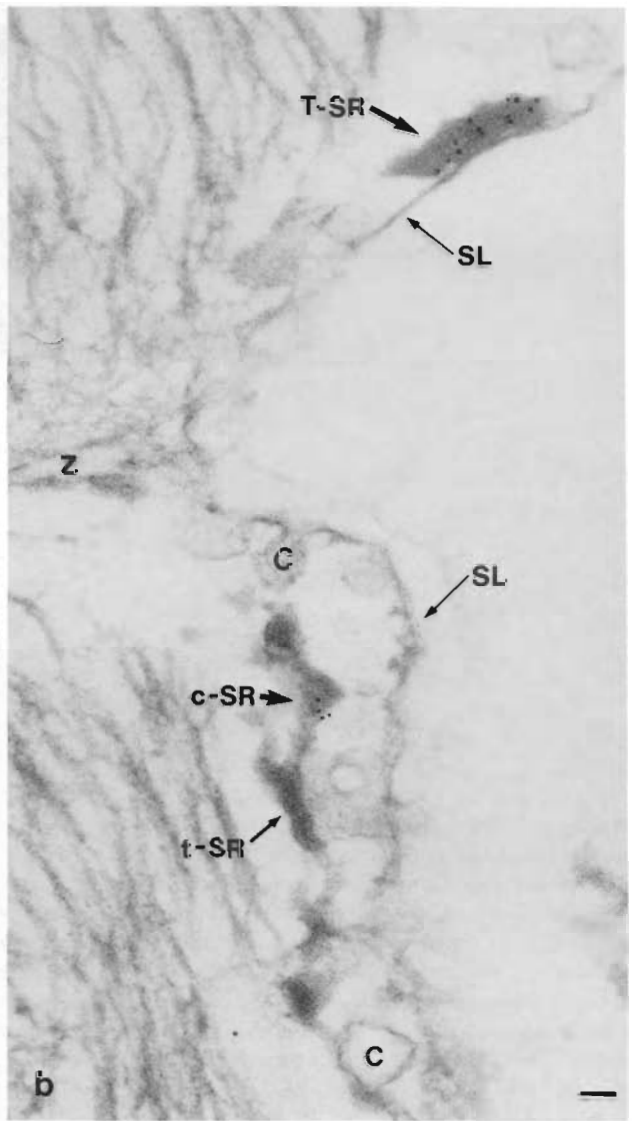
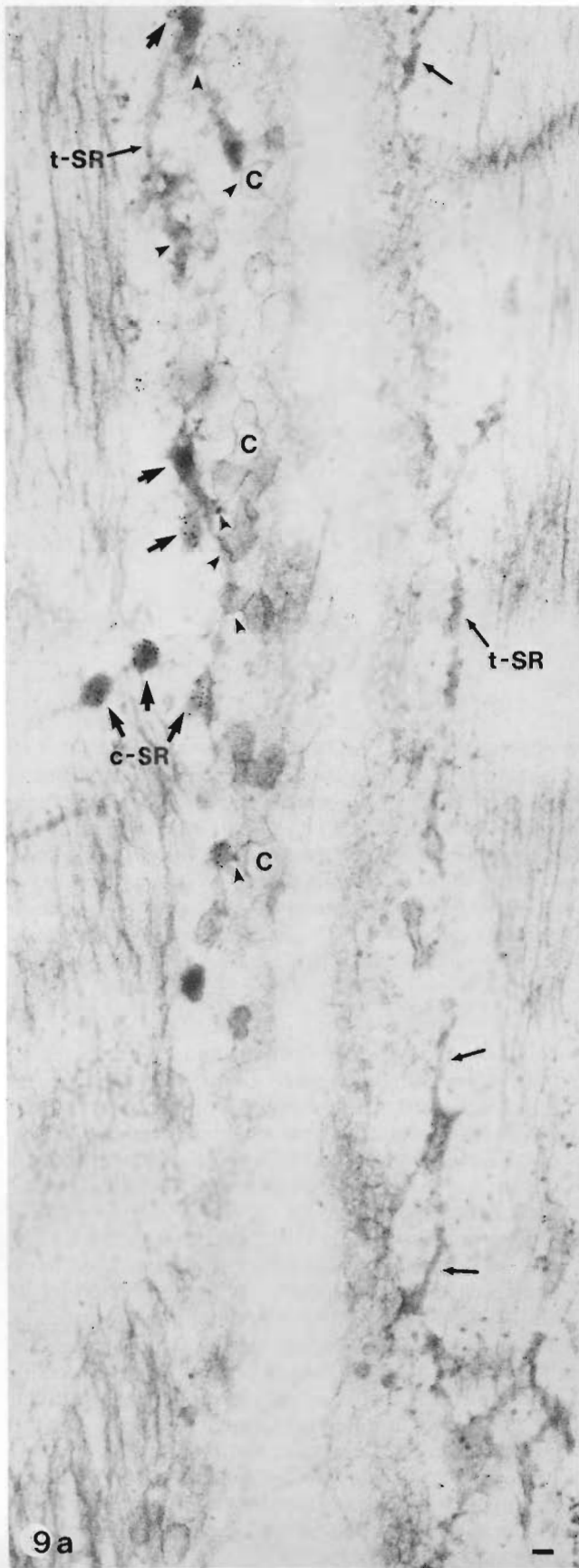
### Discussion

We have identified, partially characterized, and determined the subcellular distribution in situ of a calsequestrin isoform in adult frog ventricular muscle. This protein has the following biochemical and biophysical characteristics in common with other muscle calsequestrins: It is present in the microsomal fraction containing SR membrane vesicles. Its apparent  $M_r$ , as determined by SDS-PAGE shifts with pH. Thus, its  $M_r$  is higher (60,000 Da) in the Laemmli system (pH 8.7) than in the Weber-Osborn system (54,000 Da; pH 7.0). Its ability to bind to DEAE

cellulose at low ionic strength and to be eluted at high ionic strength suggests that it, like other calsequestrins, is an acidic protein. This characteristic of frog cardiac calsequestrin enabled partial purification of this protein, according to a procedure previously used to purify both cardiac<sup>17,18</sup> and skeletal muscle<sup>18</sup> calsequestrins.

This protein is antigenically related to chick skeletal and cardiac muscle calsequestrins as well as to canine cardiac calsequestrin as demonstrated by its ability to bind affinity-purified antibodies to chick and canine cardiac calsequestrins. The finding that <sup>45</sup>Ca<sup>2+</sup> binds to a 60,000-Da protein on a nitrocellulose blot of SDS-PAGE-separated partially purified frog cardiac calsequestrin strongly suggests that the 60,000-Da calsequestrinlike protein is a Ca<sup>2+</sup> binding protein. The NH<sub>2</sub>-terminal sequence of the first nine amino acids of frog ventricular calsequestrin is indistinguishable from that of canine cardiac<sup>45</sup> and rabbit skeletal<sup>46</sup> calsequestrin.

Immunofluorescence and immunoelectron microscopic localization showed that frog calsequestrin is



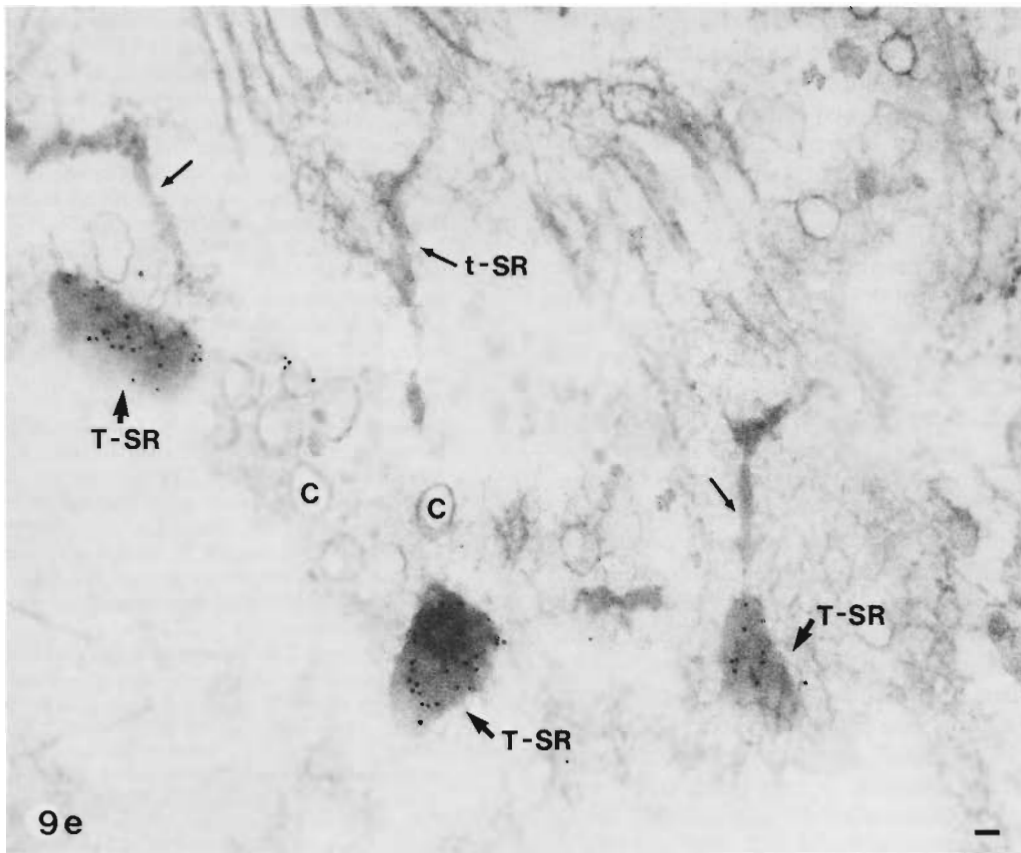


FIGURE 9. Electron micrographs of thin sections of frog myocardial fibers immunolabeled with antibodies to calsequestrin. Peripheral regions of two frog ventricular myofibers show that tubular SR (t-SR and small arrows) with ovoid expansions labeled for calsequestrin (c-SR) generally are closely apposed to caveolae (C) (arrowheads; panels a and c). By contrast, tubular SR lacking ovoid expansions labeled with calsequestrin antibodies in the subsarcolemmal region do not appear to be closely apposed to caveolae (arrows; panel a). Terminal SR (T-SR and large arrows) is densely labeled for calsequestrin (T-SR; panels b, d, and e) and may be present in a fiber containing calsequestrin-labeled ovoid expansions (c-SR) on the tubular SR (panel b). Occasionally, electron-dense structures were observed to bridge terminal SR and the sarcolemma (small arrows; panel d). Terminal SR (T-SR and large arrows) occasionally are observed to be densely distributed in a few myofibers (panel e). Z, Z-line; SL, sarcolemma. Bar, 0.05  $\mu\text{m}$ .

confined to myocardial cells and is not observed in fibroblasts or endothelial cells also present in this tissue. Within the myocardial cells, the calsequestrinlike protein generally was confined to the subsarcolemmal region, where it was present over the lumen of two distinct membrane-bound structures. One of these probably corresponds to the lumen of the terminal SR previously reported to be closely apposed to the sarcolemma.<sup>5,6</sup> The other one corresponds to the lumen of ovoid expansions (60 $\times$ 100 nm) on transversely and longitudinally oriented tubular SR. The diameter of these structures corresponds closely to that of calsequestrin-containing bulbous expansions on the network SR in rat myocardial fibers<sup>8,13</sup> previously suggested to represent corbular SR. In contrast, very few membrane-bound vesicles in the interior regions of the cells were labeled, suggesting that the sparse network SR surrounding the myofibrils does not contain calsequestrin. Because the calsequestrin-containing ovoid expansions on the tubular frog heart SR clearly represent a structurally specialized region of

nonjunctional SR and have diameters of approximately 60 $\times$ 100 nm, we tentatively refer to these structures as corbular SR-like structures. The finding that frog calsequestrin is confined to terminal SR and corbular SR-like structures but apparently is absent from the network SR is consistent with our previous immunoelectron microscopic studies of the distribution of calsequestrin in mammalian and avian cardiac SR.<sup>11,12</sup> These studies showed that cardiac calsequestrins are confined to the lumen of junctional and corbular SR but are absent from the network SR. An obvious difference between the SR of frog myocardium and mammalian and avian SR is that frog SR lacks calsequestrin-containing corbular SR-like structures in the interior regions of the frog ventricular myofibers.

Regarding excitation-contraction coupling, it generally is believed that the SR in frog myocardium<sup>1,2</sup> does not provide activator  $\text{Ca}^{2+}$  for this process. Nonetheless, studies by Chapman<sup>3</sup> and Anderson et al<sup>4</sup> support the idea that frog SR might indeed play a role in the  $\text{Ca}^{2+}$  recycling proposed to be required

under special conditions for the excitation-contraction-relaxation cycle in frog cardiac muscle. The identification of a calsequestrin isoform very similar to mammalian cardiac calsequestrin that is confined to specialized regions of frog cardiac SR strongly suggests that cardiac SR has the ability to store and possibly release  $\text{Ca}^{2+}$ , thus lending support to the idea that cardiac SR functions in some capacity in frog cardiac muscle contraction.

Although  $\text{Ca}^{2+}$  uptake into crude frog cardiac microsomes was not detectable in a previous study,<sup>15</sup> thus implying that frog cardiac SR does not accumulate  $\text{Ca}^{2+}$  during relaxation, it is quite possible that the sparseness of SR in frog myocardium would require further purification of the frog cardiac SR to reasonably assess its ability to accumulate  $\text{Ca}^{2+}$ .

A recent study of frog atrial cells with caged  $\text{Ca}^{2+}$  showed that elevation of the intracellular free  $\text{Ca}^{2+}$  concentration directly augmented  $\text{Ca}^{2+}$  channel currents<sup>50</sup> previously reported to provide the bulk of the activator  $\text{Ca}^{2+}$  required for excitation-contraction coupling in frog myocardial fibers.<sup>1</sup> The authors suggested that an increase in the cytosolic  $\text{Ca}^{2+}$  might underlie the  $\text{Ca}^{2+}$  influx-dependent<sup>51,52</sup> potentiation of  $\text{Ca}^{2+}$  currents observed in frog heart cells in response to repetitive stimulation<sup>52</sup> and associated with an increase in contractility of the frog atrium.<sup>52,53</sup> Similarly, it has been reported that low concentrations of digitalis also potentiate cardiac  $\text{Ca}^{2+}$  currents in a  $\text{Ca}^{2+}$ -dependent manner.<sup>54</sup> Together, these findings are consistent with the idea that an increase in intracellular  $\text{Ca}^{2+}$  could represent a critical step in the amplification of cardiac contractility.

It presently is unknown whether frog SR releases  $\text{Ca}^{2+}$  in response to inotropic agents. However, considering how close calsequestrin-containing corbular SR is to the sarcolemma, corbular SR would be in a strategically favorable position for providing a  $\text{Ca}^{2+}$  signal aimed at potentiating the sarcolemmal  $\text{Ca}^{2+}$  channel currents in frog myocardium. Further studies clearly are required to determine whether this is the case.

#### Acknowledgments

We are grateful to Dr. L. Smillie and M. Carpenter (Department of Biochemistry, University of Alberta) for carrying out the  $\text{NH}_2$ -terminal amino acid sequence analysis of frog cardiac calsequestrin.

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KEY WORDS • frog heart • calsequestrin • immunofluorescence • immunoelectron microscopy • NH<sub>2</sub>-terminal sequence