Evidence for the Presence of Calsequestrin in Two Structurally Different Regions of Myocardial Sarcoplasmic Reticulum

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ABSTRACT Localization of calsequestrin in chicken ventricular muscle cells was determined by indirect immunofluorescence and immuno-Protein A–colloidal gold labeling of cryostat and ultracryotomy sections, respectively. Calsequestrin was localized in the lumen of peripheral junctional sarcoplasmic reticulum, as well as in the lumen of membrane-bound structures present in the central region of the I-band, while being absent from the lumen of the sarcoplasmic reticulum in the A-band region of the cardiac muscle cells. Since chicken ventricular muscle cells lack transverse tubules, the presence of calsequestrin in membrane bound structures in the central region of the I-band suggests that these cells contain nonjunctional regions of sarcoplasmic reticulum that are involved in Ca^{2+} storage and possibly Ca^{2+} release. It is likely that the calsequestrin containing structures present throughout the I-band region of the muscle cells correspond to specialized regions of the free sarcoplasmic reticulum in the I-band called corbular sarcoplasmic reticulum. It will be of interest to determine whether Ca^{2+} storage and possibly Ca^{2+} release from junctional and nonjunctional regions of the sarcoplasmic reticulum in chicken ventricular muscle cells are regulated by the same or different physiological signals.

It is generally agreed that the sarcoplasmic reticulum plays an important role in the regulation of the cytoplasmic Ca^{2+} concentration and thereby the state of contraction and relaxation in mammalian and avian cardiac muscle (1–6). Determination of the subcellular distribution of calcium by autoradiography (7) and electron microprobe x-ray analysis (8, 9) have clearly demonstrated that the lumen of the sarcoplasmic reticulum stores Ca^{2+} during relaxation and then releases Ca^{2+} following the depolarization of the transverse tubular membrane to induce contraction in frog skeletal muscle. Biochemical studies (10, 11) and immunocytochemical studies (12–14) have suggested that most of the sarcoplasmic reticulum protein calsequestrin is localized in the lumen of the terminal cisternae in rabbit (10, 11, 14) and rat (12, 13) skeletal muscle. These results strongly support the view that the function of the calcium binding protein calsequestrin in the lumen of the junctional sarcoplasmic reticulum is to "sequester" calcium during the relaxation of skeletal muscle (15, 16).

Recently we have identified, purified, and characterized calsequestrin from canine ventricular muscle tissue (17). In the same study, indirect immunofluorescence labeling showed that most of the calsequestrin is localized in the I-band region of rat and canine ventricular muscle cells, suggesting that most of the cardiac calsequestrin, like skeletal calsequestrin, is confined to the region of the sarcoplasmic reticulum that is in close apposition to either the sarcolemma or the transverse tubules (i.e., the lumen of the peripheral and interior junctional sarcoplasmic reticulum, respectively). If this is the case, one would expect that calsequestrin in myocardial cells without transverse tubules would be localized exclusively in the lumen of the peripheral junctional sarcoplasmic reticulum and absent from the lumen of the sarcoplasmic reticulum in the interior of the myocardial cells. To determine whether or not this is indeed the case, we localized calsequestrin in chicken ventricular muscle cells that lack transverse tubules (4) by the indirect immunofluorescence and the immuno-Protein A–colloidal gold labeling techniques.

The results presented show that calsequestrin is confined to the lumen of the peripheral junctional sarcoplasmic reticulum as well as the corbular sarcoplasmic reticulum while absent from the lumen of "network" sarcoplasmic reticulum. (For terminology of the sarcoplasmic reticulum in cardiac muscle, see reference 27.)

MATERIALS AND METHODS

Preparation and Characterization of Antisera to Canine Cardiac Calsequestrin: Canine cardiac calsequestrin was purified as described by Campbell et al. (17) and then subjected to preparative gel electro-

phoresis on Laemmli slab gels (7.5% acrylamide, 1.5 mm thick) to remove any contaminating proteins. After a brief staining with Coomassie Blue, the 55,000-dalton band of cardiac calsequestrin was excised and then extracted from the gel with 0.1% SDS and 10 mM sodium bicarbonate. Antisera were raised in rabbits by a series of intramuscular injections with 0.5 mg of electrophoretically purified cardiac calsequestrin in Freund's complete adjuvant initially and then in Freund's incomplete adjuvant. The presence of the antibodies to calsequestrin was verified by indirect immunostaining of Western blots (18) of canine sarcoplasmic reticulum separated by SDS PAGE gel electrophoresis (19) before blotting. Affinity-purified antibodies to cardiac calsequestrin were prepared by affinity chromatography of the rabbit antiserum to canine cardiac calsequestrin as previously described (20, 21). The cross-reactivity and specificity of the affinity-purified antibodies used in the present study towards calsequestrin from chicken ventricular muscle was demonstrated by the indirect immunostaining of Western blots of purified chicken cardiac calsequestrin and extracts from chicken ventricular muscle with a goat anti-rabbit γ-globulin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) as the secondary reagent according to the procedure described by Towbin et al. (18). The details of this procedure were performed as previously described (22), except that affinity-purified antibodies to calsequestrin from canine ventricular myocardium (1 μg/ml in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.2, and 3% bovine serum albumin) were used as the primary antibody reagent.

Dissection, Fixation, and Sectioning of Ventricular Muscle: Adult hens were sacrificed with an overdose of Halothane and 10 × 2 × 2 mm^3 bundles of myocardial fibers were quickly dissected from the left ventricular wall, tied to cotton swab sticks, and fixed first in 2% paraformaldehyde and then in a mixture of 2% paraformaldehyde and 0.3% glutaraldehyde as previously described (12). Finally the bundles of fixed ventricular muscle tissue were washed, infused with sucrose, and 4-μm cryostat and 80-nm ultracryotomy (13) sections cut as previously described.

Indirect Immunofluorescence Labeling: Immunofluorescent labeling of cryostat sections of fixed chicken ventricular muscle was carried out as previously described (12). Affinity-purified antibodies to canine cardiac calsequestrin 5 μg/ml in phosphate-buffered saline were used as the primary antibody reagent. Fluorescein-labeled globulin fraction of goat anti-rabbit serum (diluted 1/20; Miles-Yeda, Rehovot, Israel) was used as the secondary reagent. The cells labeled were examined in a Zeiss photomicroscope provided with an epi-fluorescence attachment and a phase-contrast condenser. The fluorescence pictures were photographed on XP1-400 film (Ilford) and developed at 37°C in XPI-400 Processing Chemicals (Ilford). For adsorption 10 μg of affinity-purified antibodies to cardiac calsequestrin were incubated with either 0 or 20 μg of highly purified canine cardiac calsequestrin as previously described (13). The supernatants obtained by centrifugation were diluted four times and used in the indirect immunofluorescence labeling.

Indirect Immunoelectron Microscopic Labeling: Immunoelectron microscopic labeling of ultracryotomy sections of fixed chicken ventricular muscle fibers with affinity-purified antibodies to canine calsequestrin was carried out as previously described (13, 23), except that a Protein A–colloidal gold conjugate was used as the secondary reagent in the labeling procedure.

Colloidal gold particles with a diameter of ~9 nm were prepared by sonication of gold chloride (Matthey Chemical Ltd., Raystone, England) according to the procedure of Baigent and Muller (24) and immediately conjugated to Protein A (Pharmacia Fine Chemicals, Stockholm, Sweden) as described by Slot and Geuze (25). After immunolabeling, the ultrathin frozen sections were adsorption stained and postembedded as prevously described by the technique developed by Tokuyasu (23, 26). Sections were examined with a Philips 300 electron microscope.

RESULTS

Antibody Specificity

The ability of the affinity-purified antibodies to the electrophoretically purified canine myocardial calsequestrin to bind specifically to calsequestrin from chicken ventricular muscle was demonstrated by the indirect immunoperoxidase staining on Western blots as shown in Fig. 1. The affinity-purified antibodies were bound to a single band at 55,000 dalton when tested against an extract from chicken ventricular muscle. The electrophoretic mobility of the 55,000-dalton band corresponds to that of calsequestrin purified from either canine or chicken ventricular myocardium. This finding strongly suggests that the antibodies to canine myocardial calsequestrin bind specifically to calsequestrin in the chicken myocardium.

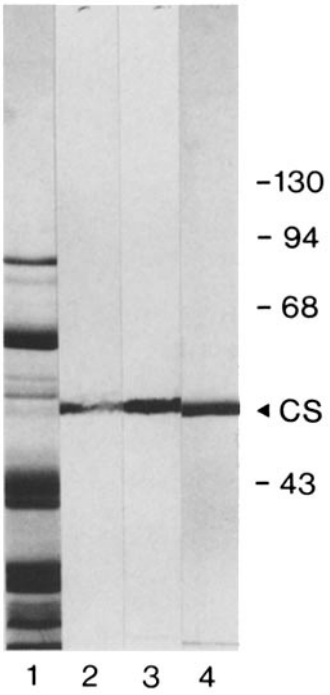


FIGURE 1 Specificity of the affinity-purified antibodies to canine cardiac calsequestrin towards calsequestrin in an extract from chicken ventricular muscle. Lanes *1* and *4*: Coomassie Blue staining pattern of proteins separated on a 7.5% polyacrylamide gel (19). Lanes *2* and *3*: Western blots of muscle proteins labeled with affinity-purified antibodies to canine cardiac calsequestrin by the indirect horseradish peroxidase immunoblotting (18). Lanes *1* and *2*: 20 μg of an ammonium sulfate extract from chicken ventricular muscle. Lanes *3* and *4*: 2 μg of calsequestrin purified from chicken ventricular muscle. *Cs*, calsequestrin. Numbers on the right side of the figure represent molecular weights (× 10^{-3}) of molecular weight standards.

Localization of Calsequestrin by Immunofluorescence

The fluorescent staining pattern observed after labeling transverse (Fig. 2 *c*) and longitudinal (Fig. 2 *f*) crysotat sections of fixed chicken ventricular muscle fibers with antibodies to calsequestrin by the indirect immunofluorescent labeling procedure was compared with a phase-contrast view of the same field as shown in Fig. 2, *d* and *g*, respectively. Specific fluorescent staining in transverse sections was present throughout the cytoplasmic region of the myofibers and consisted of small punctate foci (Fig. 2, *a* and *c*). Although the fluorescent foci were present at the cell periphery they did not appear to be more pronounced in this region than in the interior regions of the cytoplasm (Fig. 2, *a* and *c*). In longitudinal sections (Fig. 2, *e* and *f*), specific fluorescent staining was present in the I-band region of both the interior and the peripheral regions of the myofibers while no regular fluorescent staining was present in the A-band region. The labeling in the I-band region appeared as a row of small foci aligned perpendicular to the long axis of the myofibers (Fig. 2*f*).

To demonstrate the specificity of the staining patterns observed in adult chicken ventricular muscle, we adsorbed antibodies to canine cardiac calsequestrin with canine cardiac calsequestrin. The supernatant was then used in the indirect immunofluorescence staining procedure. As shown in Fig. 2 *b*, the specific staining pattern observed in Fig. 2 *a* was greatly diminished.

Localization of Calsequestrin by Immunoelectron Microscopy

To determine more precisely the localization of calsequestrin in chicken ventricular myocardial cells, we labeled longitudinal sections of fixed muscle tissue with antibodies to

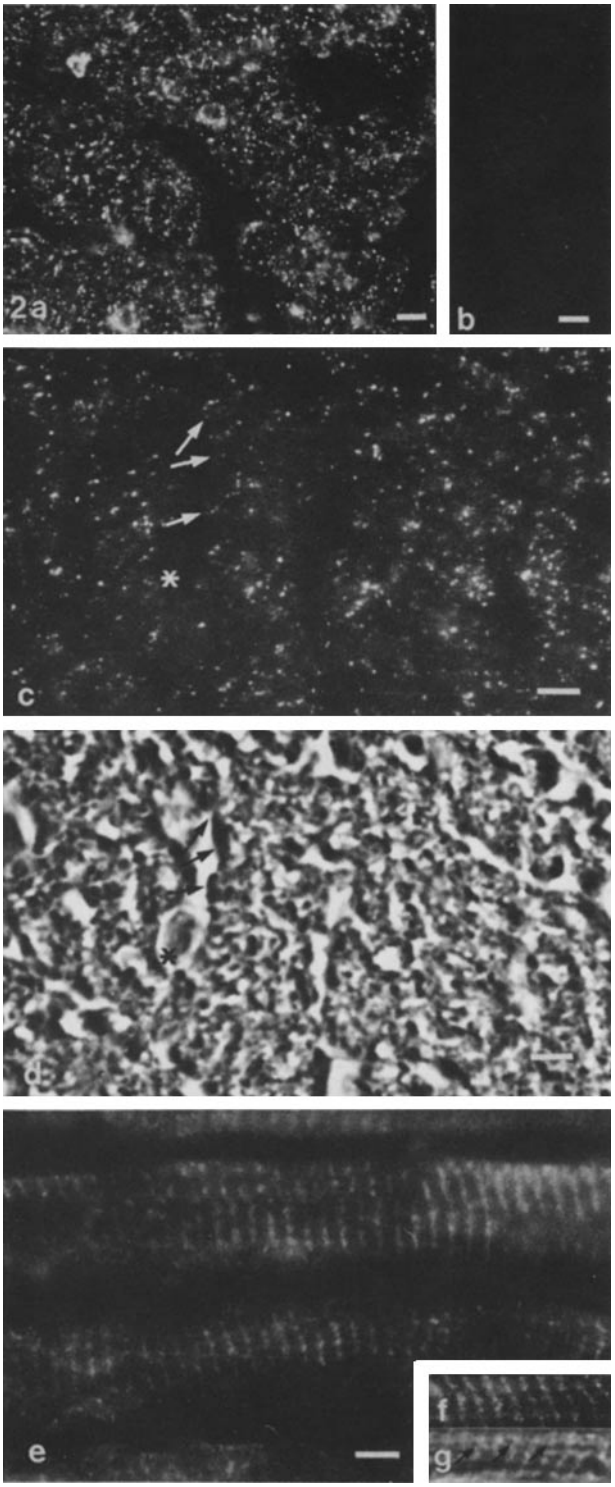


FIGURE 2 Immunofluorescence localization of cardiac calsequestrin in chicken ventricular muscle. Light micrographs of transverse (*a–d*) and longitudinal (*e–g*) sections of bundles of fixed chicken ventricular myocardial cells labeled with cardiac calsequestrin antibodies (*a*, *c*, *e* and *f*) and calsequestrin antibodies adsorbed with canine cardiac calsequestrin (*b*). The fluorescent staining pattern in *c* was compared with the position of the outline of the myocardial cells in *d* (black arrows), a phase-contrast micrograph of the same field. Note that the fluorescent punctate foci in *c* are confined to myocardial cells where they are distributed throughout the cytoplasm. The fluorescent staining pattern in *f* was compared with the position of the A- and I-bands in the same field as seen by phase-contrast microscopy in *g* (*g* is the mirror image of *f*). The fluorescent staining pattern observed in longitudinal sections after labeling with antibodies to cardiac calsequestrin (*e* and *f*) corresponded to the I-band region (black arrows) (only shown for *f*), while no regular staining pattern was observed in the A-band region. *, erythrocyte. Bar, 5 μm. (*a* and *b*) × 765, (*c–g*) × 1,170.

calsequestrin by indirect immuno-Protein A–colloidal gold labeling of ultrathin frozen sections. Examination of the distribution of the gold particles showed that specific calsequestrin labeling was observed over the lumen of vesicular structures present in both the interior (c-SR) and the subsarcolemmal (pj-SR) region of the muscle cell cytoplasm (Fig. 3 and 4), while the sarcolemma (SL in Fig. 3 and Fig. 4, *a–c*), the mitochondria (M in Fig. 4*c*) and the myofibrils were labeled only at background levels. In the interior region of the myocardial cells most of the calsequestrin labeling was observed in vesicular structures localized in the interfibrillar spaces in the I-band region (c-SR in Fig. 4, *a–d*), while the vesicular structures in the A-band region (n-SR in Fig. 4, *a* and *d*) were labeled only at background level. In the subsarcolemmal region of the myocardial cells, calsequestrin labeling was observed in the lumen of some (pj-SR in Fig. 4, *a–c*) of the vesicular structures.

DISCUSSION

Ultrastructural studies of chicken ventricular myocardial cells have shown that the well developed sarcoplasmic reticulum is composed of at least three structurally distinct regions (4). The major region of the sarcoplasmic reticulum that surrounds the myofibrils is composed of an anastomosing network of sarcotubules with an electron lucent lumen and have recently been named "network" sarcoplasmic reticulum by Forbes and Sperelakis (27). The two other regions are extensions of the network sarcoplasmic reticulum and are called the peripheral junctional sarcoplasmic reticulum and the corbular sarcoplasmic reticulum. The peripheral junctional sarcoplasmic reticulum has granular material in its lumen and is closely apposed to the sarcolemma to which it is connected by junctional processes called "feet" (28). Corbular sarcoplasmic reticulum, bulbous and cisternal expansions on the network sarcoplasmic reticulum, also has granular material in its lumen and junctional processes extending from its cytoplasmic surface.

The results presented in this study show that calsequestrin in adult chicken ventricular muscle in situ is localized in the lumen of some, but not all vesicular structures present at both peripheral and interior sites of the muscle cytoplasm. Although junctional processes (feet) between the sarcolemma and the calsequestrin-containing vesicles in the subsarcolemma region of the cytoplasm are not visualized in the present study, it is very likely that these calsequestrin-containing vesicles correspond to the peripheral junctional sarcoplasmic reticulum.

Since calsequestrin localized in the interior regions of the cytoplasm is mostly confined to the lumen of vesicular structures in the interfibrillar spaces in the I-band region but absent from vesicular structures in the A-band region, it is likely that the calsequestrin-containing vesicles present in the I-band region correspond to the specialized extensions on the free sarcoplasmic reticulum called corbular sarcoplasmic reticulum (27).

This interpretation is supported by previous studies of the

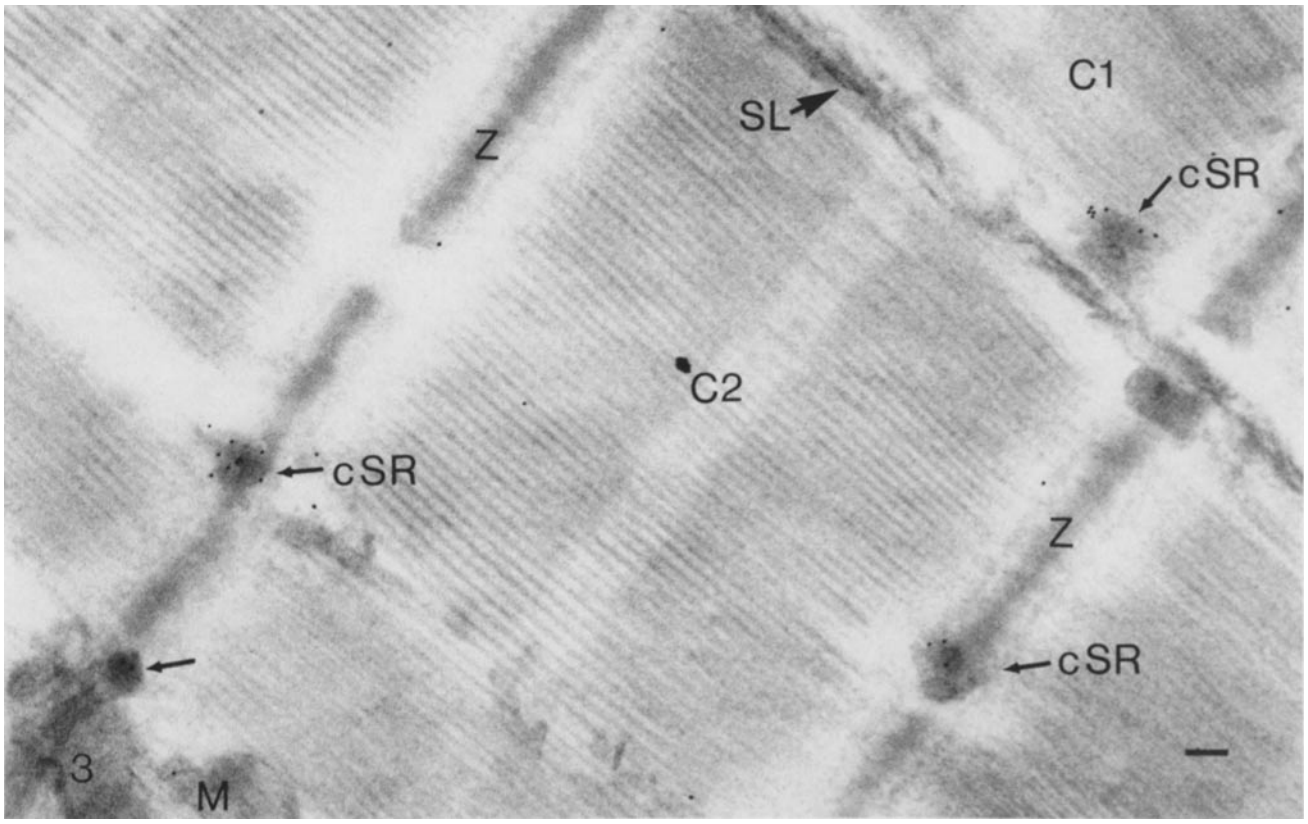


FIGURE 3 Electron micrograph of a longitudinal section of portions of two chicken ventricular muscle cells (*C1* and *C2*) labeled with antibodies to cardiac calsequestrin. Most of the colloidal gold particles were found over spherical structures (*c-SR*, small black arrow) in both peripheral and interior regions of the myocardial cells. In the interior regions of the cells most of these structures were localized in the I-band region, while the rest of the cell was only labeled at background level. *Z*, Z-line; *SL*, sarcolemma; *M*, mitochondrion. Bar, 0.1 μm. × 54,000.

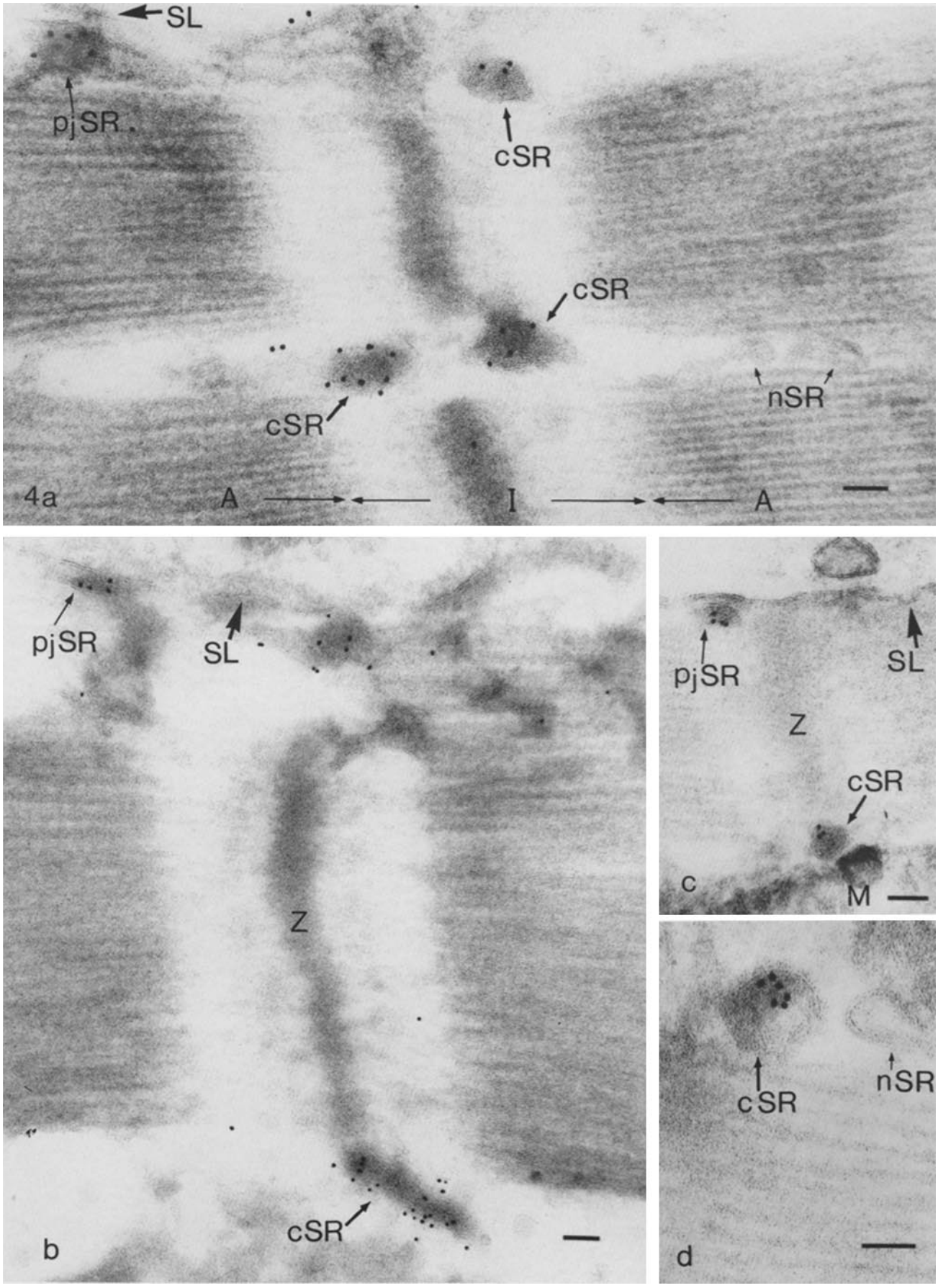
distribution of Ca^{2+} in the sarcoplasmic reticulum of pigeon ventricular muscle by pyroantimonate precipitation (29), which suggested that both the peripheral junctional and the corbular sarcoplasmic reticulum in pigeon myocardial cells might be involved in the regulation of cytoplasmic Ca^{2+} during the contraction-relaxation cycle. However, the localization of Ca^{2+} by this technique might be misleading due to diffusion of Ca^{2+} during the precipitation procedure.

In a recent study we observed by immunofluorescence labeling that calsequestrin-containing structures were present in both interior and peripheral regions of sheep Purkinje fibers, which, like chicken myocardial cells, lack transverse tubules and thus interior couplings (Jorgensen, A. O., A. C.-Y. Shen, A. McLeod, K. P. Campbell, and G. H. Denney, Jr., manuscript in preparation). Furthermore, immunoelectron microscopical localization of calsequestrin in adult rat ventricular cells showed that calsequestrin was present in the lumen of interior and peripheral junctional sarcoplasmic reticulum as well as the lumen of corbular sarcoplasmic reticulum (30) previously shown by ultrastructural studies to be present in the central portion of the I-band region of rat myocardial cells (31). These results support the idea that Ca^{2+} storage and release from both the junctional and the corbular sarcoplasmic reticulum is common to all mammalian and avian myocardial cells.

The obvious structural difference between these two regions of the sarcoplasmic reticulum is that the peripheral junctional sarcoplasmic reticulum is in contact with the sarcolemma, while the corbular sarcoplasmic reticulum is not close to the sarcolemma. This raises the possibility that the Ca^{2+} storage and release at these two different sites are triggered by different stimuli as first noted by Sommer and Waugh (32). Thus it is possible that Ca^{2+} release from the junctional sarcoplasmic reticulum is directly triggered by depolarization of the sarcolemma while Ca^{2+} release from the corbular sarcoplasmic reticulum might be triggered via a diffusible agent.

Studies by Fabiato showed that Ca^{2+} can induce Ca^{2+} release from the sarcoplasmic reticulum in skinned pigeon myocardial and canine Purkinje fibers, both of which lack transverse tubules. Since it is likely that removal of the myocardial sarcolemma resulted in damage to the peripheral junctional sarcoplasmic reticulum, Fabiato concluded that neither the

FIGURE 4 Electron micrographs of longitudinal sections of chicken ventricular muscle cells labeled with antibodies to calsequestrin. Most of the colloidal gold labeling were present over some of the membrane bound structures in the subsarcolemmal region (*pj-SR*) (*a–c*) as well as over membrane bound structures in the I-band region (*c-SR*) (*a–d*) while membrane bound vesicles in the A-band region (*n-SR*) (*a* and *d*), the sarcolemma (*SL*) (*a–c*) and the mitochondrion (*M*) (*c*) were only labeled at the level of the background. *Z*, Z-line. Bar, 0.1 μm. (*a*) × 82,700; (*b*) × 70,000; (*c*) × 75,000; and (*d*) × 102,800.



sarcolemma nor the transverse tubules appear to be essential for Ca^{2+}-induced Ca^{2+} release in these fibers. Thus it is possible that the calsequestrin-containing vesicles in the central region of the I-band in chicken myocardial cells represent the interior Ca^{2+} storage sites from which a diffusible factor Ca^{2+} can induce Ca^{2+} release in skinned as well as intact myocardial fibers on a beat to beat basis.

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