

# The Ca<sup>2+</sup>-release Channel/Ryanodine Receptor Is Localized in Junctional and Corbular Sarcoplasmic Reticulum in Cardiac Muscle

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**Abstract.** The subcellular distribution of the Ca<sup>2+</sup>-release channel/ryanodine receptor in adult rat papillary myofibers has been determined by immunofluorescence and immunoelectron microscopical studies using affinity purified antibodies against the ryanodine receptor. The receptor is confined to the sarcoplasmic reticulum (SR) where it is localized to interior and peripheral junctional SR and the corbular SR, but it is absent from the network SR where the SR-Ca<sup>2+</sup>-ATPase and phospholamban are densely distributed. Immunofluorescence labeling of sheep Purkinje fibers show that the ryanodine receptor is confined to discrete foci while the SR-Ca<sup>2+</sup>-ATPase is distributed in a continuous network-like structure present at the periphery as well as throughout interior regions of these myofibers. Because Purkinje fibers lack T-tubules, these results indicate that the ryanodine receptor is localized not only

to the peripheral junctional SR but also to corbular SR densely distributed in interfibrillar spaces of the I-band regions. We have previously identified both corbular SR and junctional SR in cardiac muscle as potential Ca<sup>2+</sup>-storage/Ca<sup>2+</sup>-release sites by demonstrating that the Ca<sup>2+</sup> binding protein calsequestrin and calcium are very densely distributed in these two specialized domains of cardiac SR in situ. The results presented here provide strong evidence in support of the hypothesis that corbular SR is indeed a site of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release via the ryanodine receptor during excitation contraction coupling in cardiac muscle. Furthermore, these results indicate that the function of the cardiac Ca<sup>2+</sup>-release channel/ryanodine receptor is not confined to junctional complexes between SR and the sarcolemma.

**W**HILE depolarization of the sarcolemma and T-tubules in skeletal muscle is sufficient to elicit the required Ca<sup>2+</sup>-release from the sarcoplasmic reticulum (SR)<sup>1</sup> for muscle contraction (Huxley, 1971; Schneider and Chandler, 1973), depolarization of cardiac sarcolemma and T-tubules does not elicit a Ca<sup>2+</sup>-release from cardiac SR unless extracellular Ca<sup>2+</sup> enters the cytosol, mainly via the slow voltage dependent Ca<sup>2+</sup> channel (Reuter, 1984; Tsien, 1983). This influx of Ca<sup>2+</sup> directly or indirectly induces a release of Ca<sup>2+</sup> from the SR as proposed in the Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release hypothesis (Fabiato, 1983, 1985).

Previous ultrastructural studies have demonstrated that SR in mammalian cardiac myofibers contains at least three distinct but continuous regions, namely the network SR, the interior, and peripheral junctional SR, and the corbular SR (Sommer and Johnson, 1979; Forbes and Sperelakis, 1983; Segretain et al., 1981). The network SR consists of 25–60 nm diam sarcotubules organized in an anastomosing network that surrounds the myofibrils fairly uniformly along the entire length of the sarcomere. The junctional and corbular SR

are structurally specialized domains extending from the network SR and contain electron-dense material in their lumina. They are most densely distributed in the interfibrillar spaces neighboring the central region of the I-band. The prominent structural difference between these two regions of cardiac SR is that junctional SR is physically connected to either T-tubules or to sarcolemma via 'feet' structures, whereas corbular SR is not. However, it has been reported that electron dense structures similar to 'feet' structures project from the surface of corbular SR into the cytoplasm (Sommer and Johnson, 1979). Immunoelectron microscopical studies demonstrated that the SR-Ca<sup>2+</sup>-ATPase (Jorgensen et al., 1982) and its regulator phospholamban (Jorgensen and Jones, 1987) are uniformly distributed in the network SR while calsequestrin is present in the lumen of junctional SR and corbular SR (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985, 1988). Electron microprobe analysis studies showed that the lumen of junctional SR (Jorgensen et al., 1988; Wheeler-Clark and Tormey, 1987) and corbular SR store calcium (Jorgensen et al., 1988).

The studies summarized above support the idea that Ca<sup>2+</sup> is accumulated into the lumen of the SR across the entire surface of the network SR via the Ca<sup>2+</sup>-ATPase. Ca<sup>2+</sup> subse-

1. *Abbreviations used in this paper:* SR, sarcoplasmic reticulum; WGA, wheat germ agglutinin.

quently diffuses to the lumen of junctional and corbular SR where it is sequestered by calsequestrin and stored until the SR receives the next signal to release  $\text{Ca}^{2+}$ . The question then arises as to whether both junctional SR and corbular SR release  $\text{Ca}^{2+}$ , and if so, is  $\text{Ca}^{2+}$  released via the ryanodine receptor or does one of these domains of SR contain a different  $\text{Ca}^{2+}$  release channel such as the  $\text{IP}_3$  receptor (Ferris and Snyder, 1992)?

The clear demonstration that the skeletal ryanodine receptor corresponds to feet structures confined to the junctional face of the terminal cisternae of skeletal SR (Anderson et al., 1989; Block et al., 1988; Smith et al., 1988; Inui et al., 1987; Kawamoto et al., 1986) supports the proposal that the cardiac ryanodine receptor is also associated with the junctional SR (Inui et al., 1988). On the other hand, studies by Fabiato (Fabiato, 1985) have shown that quick elevation of cytosolic  $\text{Ca}^{2+}$  can induce  $\text{Ca}^{2+}$  release from the SR of mechanically skinned canine Purkinje fibers. Because skinned Purkinje fibers presumably lack junctional SR, these results are consistent with the idea that a quick increase in cytosolic  $\text{Ca}^{2+}$  triggers  $\text{Ca}^{2+}$  release from corbular SR. Nonetheless, they do not exclude the possibility that  $\text{Ca}^{2+}$  also induces  $\text{Ca}^{2+}$  release from junctional SR in intact cardiac myofibers.

Considering the striking similarities of the physical characteristics of the tetrameric  $\text{IP}_3$  receptor (Maeda et al., 1991) and the tetrameric ryanodine receptor (Saito et al., 1988), it is reasonable to propose that feetlike structures associated with either junctional SR or corbular SR may represent  $\text{IP}_3$  receptors. In support of this possibility, several pharmacological studies have shown that  $\text{IP}_3$  can trigger elevations of cytosolic free  $\text{Ca}^{2+}$  and induce contraction in skinned cardiac myofibers (Fabiato, 1990; Kentish et al., 1990; Lochner and Bester, 1989; Nosek et al., 1986; Vites and Pappano, 1990; Vites and Pappano, 1992). Furthermore, recent biochemical studies identified and characterized high and low affinity  $\text{IP}_3$  binding sites in isolated canine cardiac SR, where they appear to be most prominent in the subpopulation of cardiac SR proposed to correspond to junctional SR (Kijima and Fleischer, 1992).

To determine directly whether junctional SR and/or corbular SR contain the ryanodine receptor, its subcellular distribution in rat papillary myofibers and sheep Purkinje fibers was determined by immunofluorescence and immunocolloidal gold labeling. The results presented show that the  $\text{Ca}^{2+}$  release channel/ryanodine receptor is localized to both the junctional SR and corbular SR but is apparently absent from the network SR in rat papillary myofibers and sheep Purkinje fibers. The presence of the ryanodine receptor in corbular SR well removed from the sarcolemma indicates that corbular SR represents a site for  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release required for excitation contraction coupling in cardiac muscle.

## Materials and Methods

### Preparation of Microsomal Membranes

Cardiac microsomal membranes were prepared from rat ventricular muscle as previously described for skeletal muscle membranes (Ohlendieck et al., 1991). Protein concentrations were determined according to the procedure of Lowry (Lowry et al., 1951) as modified by Peterson (Peterson, 1977) using BSA as a standard.

### Preparation of Affinity Purified Antibodies to the Ryanodine Receptor

Rabbit antiserum against a COOH-terminal peptide of the skeletal muscle ryanodine receptor (PAGDCFRKQYEDQLS;) (Takeshima et al., 1989) was prepared as described previously (McPherson et al., 1991). This COOH-terminal peptide sequence differs by only a single amino acid from that of the cardiac ryanodine receptor (Otsu et al., 1990). For affinity purification, the COOH-terminal peptide (Robey and Fields, 1989), conjugated to BSA (Parker and Hodges, 1984), was coupled to CNBr-Sepharose according to the manufacturer's instructions. Rabbit serum was diluted 1:4 in TBS (20 mM Tris-HCl, pH 7.4, 200 mM NaCl), and incubated for 16 h at 4°C with 1 ml of the peptide conjugated Sepharose. The Sepharose was loaded on a column, washed 4 × 8 ml with 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, and washed 2 × 8 ml with Tris-HCl containing 100 mM NaCl. Antibody was eluted with 4 M  $\text{MgCl}_2$ , and the eluted fractions were analyzed for protein by reading OD at 280 nm. Peak fractions were pooled, dialyzed for 12 h against PBS, and then dialyzed for 12 h against PBS containing 20% sucrose, 1 mM EGTA, and .01% Na-azide.

### Immunoblotting

Cardiac microsomal proteins were separated on 3–12% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose paper (Towbin et al., 1979), and immunoblotted with ryanodine receptor antibodies followed by HRP-coupled secondary antibodies as previously described (McPherson and Campbell, 1990).

### Immunofluorescence Labeling of Papillary Muscle Cryosections

Small bundles of papillary muscle tissue were dissected from anaesthetized rats and sheep and quickly cryofixed in liquid nitrogen cooled isopentane. Longitudinal and transverse cryostat sections (6–8  $\mu\text{m}$  thick) were cut as previously described (Jorgensen et al., 1982; Jorgensen and Jones, 1987).

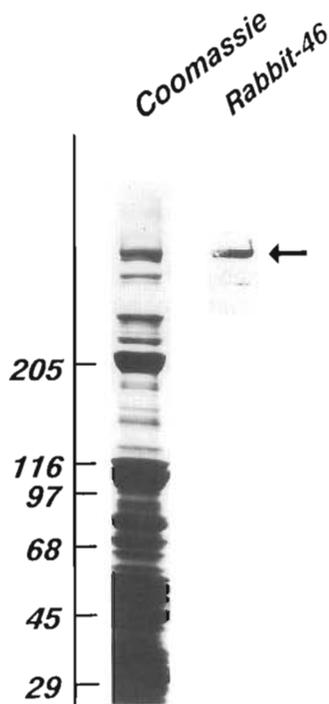
**Single Labeling.** Indirect immunofluorescence labeling of cryostat sections of unfixed rat and sheep papillary muscle was carried out as previously described (Jorgensen and Jones, 1987). Briefly, affinity purified rabbit antibodies to a COOH-terminal peptide of the ryanodine receptor (25  $\mu\text{g}/\text{ml}$ ) were used as the primary reagent. Affinity purified  $\text{F}(\text{ab})_2$  fragments of donkey anti-rabbit gamma globulin-conjugated to fluorescein (20  $\mu\text{g}/\text{ml}$ ; Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) were used as the secondary reagent.

**Double labeling.** Some cryostat sections containing bundles of sheep Purkinje fibers were double labeled with antibodies to the ryanodine receptor and wheat germ agglutinin (WGA) as previously described (McLeod et al., 1991). Briefly, the double labeling procedure composed of the single labeling procedure for the ryanodine receptor followed first by labeling with WGA conjugated to biotin (5  $\mu\text{g}/\text{ml}$ ; Vector Laboratories, Burlingame, CA) and next by streptavidin conjugated to Texas Red (10  $\mu\text{g}/\text{ml}$ ; Vector Laboratories, Burlingame, CA).

Cryosections of sheep papillary muscle were double labeled with antibodies to the ryanodine receptor and to the dog cardiac SR  $\text{Ca}^{2+}$ -ATPase. Briefly, the double labeling procedure was composed of the single labeling procedure for the ryanodine receptor followed first by labeling with Mab IID8 to canine cardiac SR  $\text{Ca}^{2+}$ -ATPase (Jorgensen et al., 1988) and next by affinity purified  $\text{F}(\text{ab})_2$  fragments of goat antimouse  $\text{IgG}_1$  conjugated to Texas Red (20  $\mu\text{g}/\text{ml}$ ; Sera Laboratories, Copthorne, England).

**Controls.** For adsorption, 25  $\mu\text{g}/\text{ml}$  of affinity purified antibodies to the COOH-terminal peptide of the ryanodine receptor were incubated with 0 and 5  $\mu\text{g}/\text{ml}$  of the COOH-terminal peptide, conjugated to rabbit serum albumin (40:1) (Robey and Fields, 1989) as previously described (Jorgensen and Jones, 1987). Thus, the molar ratio of peptide/antibody was ~4 for the adsorption. The supernatants obtained by centrifugation were used as the primary reagent in the indirect immunofluorescence and immunoelectron microscopical labeling procedure.

**Imaging.** Conventional fluorescence microscopy was carried out with a photomicroscope (Carl Zeiss, Inc. Thornwood, NY) provided with an epifluorescence attachment and a phase-contrast condenser. Confocal microscopy was carried out with a photomicroscope (Nikon, Inc., Garden City, NY) provided with a confocal fluorescence imaging system (Laser-sharp MRC Bio-Rad Laboratories Ltd., Richmond, CA) using a krypton argon laser for illumination (White et al., 1987).



**Figure 1.** Coomassie blue and immunoblot staining of rat heart microsomes. Rat heart microsomes were prepared as described in Materials and Methods and separated on SDS-PAGE (200  $\mu$ g/lane) followed by either Coomassie blue staining or immunoblotting with affinity purified antibodies to a COOH-terminal peptide of rabbit skeletal ryanodine receptor (rabbit 46). The arrow indicates the electrophoretic mobility of the rabbit cardiac ryanodine receptor. The migratory position of the molecular weight standards are indicated on the left.

### Immuno-Electron Microscopical Labeling of Thin Sections of Papillary Muscle

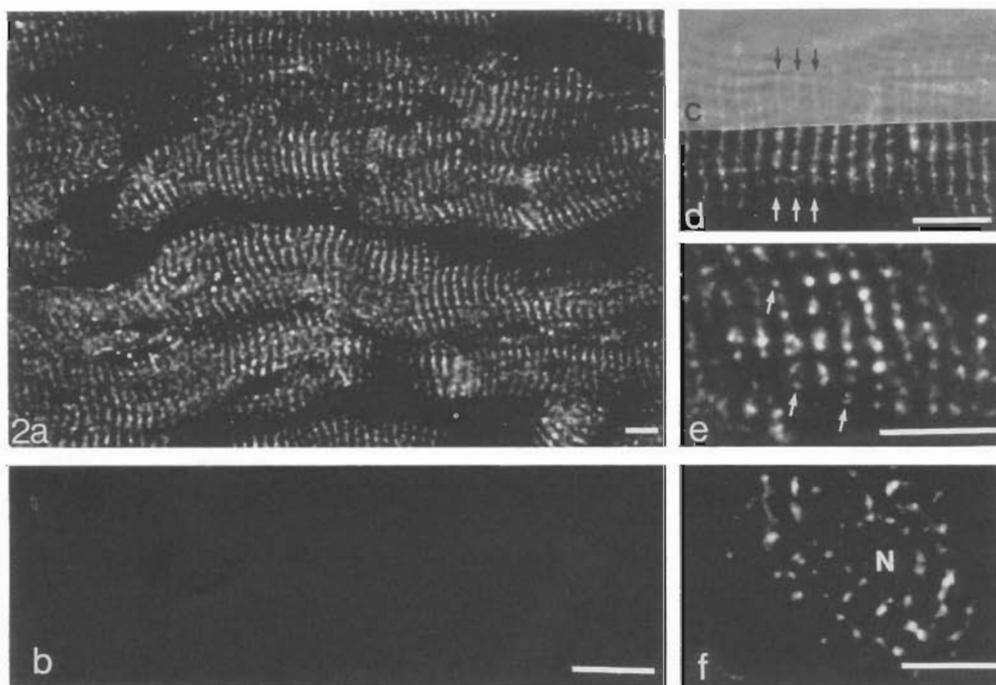
Dissection, specimen preparation, sectioning, and immunocolloidal gold labeling of rat papillary muscle tissue was carried out as previously described (Jorgensen and McGuffee, 1987; Phillips and Boyne, 1984; McGuffee et al., 1981; Chiovetti et al., 1985; Chiovetti et al., 1987) except that cryofixed and freeze-dried tissue to be immunolabeled with antibodies

to the ryanodine receptor was low temperature embedded in LR white according to the procedure of Little and McGuffee (1988), see below.

Briefly, hearts from adult female rats (125–150 g) were quickly dissected and immersed in Krebs-Henseleit buffer (145 mM NaCl, 2.6 mM KCl, 5.9 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , and 10 mM glucose, saturated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ). Whole papillary muscles were dissected and cryofixed using the "Gentleman Jim" quick freezing device (Ted Pella, Inc., Tustin, CA) as described by Phillips and Boyne (Phillips and Boyne, 1984). Subsequently, the cryofixed tissue was freeze-dried in a glass cryosuction pump (McGuffee et al., 1981; Chiovetti et al., 1985). The tissue to be immunolabeled for calsequestrin was exposed to osmium tetroxide vapor and embedded in Spurr as previously described by Jorgensen and McGuffee (1987) while the tissue to be used for immunolabeling of the ryanodine receptor was embedded in LR white, using benzoin-methyl ether as the initiator (McGuffee et al., 1989). Details of the embedding procedure for LR white was carried out according to that described for Lowicryl K4M by Chiovetti et al. (1985, 1987) and modified by Jorgensen and McGuffee (1987). Thin sections (60–80 nm) were collected on nickel grids.

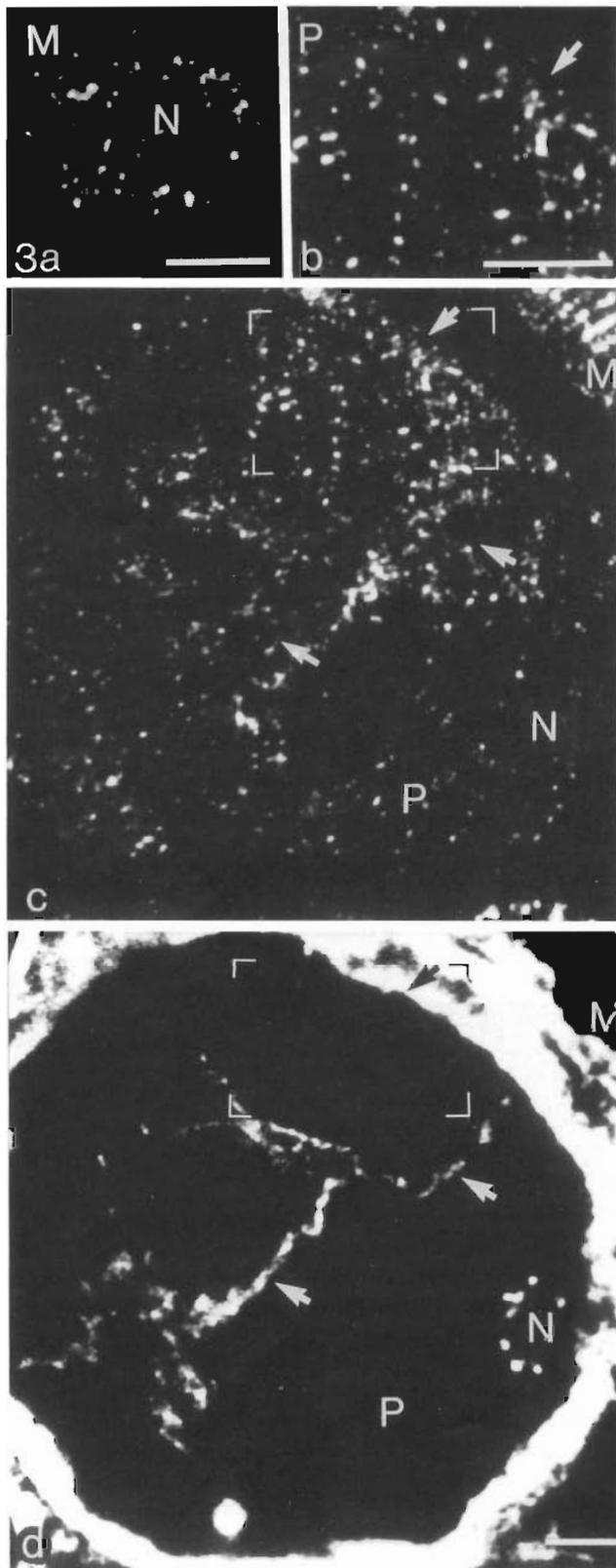
Immunocolloidal gold labeling for the cardiac ryanodine receptor and the cardiac calsequestrin was respectively carried out on thin sections of LR white embedded tissue as previously described for Lowicryl K4M embedded tissue and on thin sections of Spurr embedded tissue (Jorgensen et al., 1988; Jorgensen and McGuffee, 1987). Briefly, affinity purified antibodies to either the COOH-terminal peptide of the ryanodine receptor (25  $\mu$ g/ml) or to canine cardiac calsequestrin (Jorgensen et al., 1985, 1988; Jorgensen and McGuffee, 1987) were used as the primary reagent. An affinity purified goat anti-rabbit gamma-globulin colloidal gold ( $\sim 5$  nm, ryanodine receptor; 10 nm, calsequestrin) conjugate was used at 0.5 mg/ml in PBS containing 3% BSA (Janssen Pharmaceutica; Beerse, Belgium) as the secondary reagent.

Immunocolloidal gold labeling for phospholamban was carried out on thin sections of LR white embedded tissue by a triple-layered procedure as previously described (Jorgensen et al., 1990). Briefly, Mab 2D12 to phospholamban (Sham et al., 1991) was used at 35  $\mu$ g/ml as the primary reagent. Then, affinity purified rabbit anti-mouse ( $\text{F}_c$  fragment) gamma-globulin (Jackson ImmunoResearch Laboratories Inc.) was used at 25  $\mu$ g/ml as the secondary reagent. Finally, affinity purified goat anti-rabbit gamma-globulin colloidal gold ( $\sim 5$  nm) conjugate was used at 0.5 mg/ml in PBS containing 3% BSA (Janssen Pharmaceutica) as the tertiary reagent.



**Figure 2.** Localization of the  $\text{Ca}^{2+}$ -release channel/ryanodine receptor in cryosections of adult rat papillary muscle by immunofluorescence labeling. Longitudinal (a–e) and transverse (f) cryosections (4–8  $\mu$ m) were immunofluorescently labeled with affinity purified antibodies to the COOH-terminal peptide of rabbit ryanodine receptor before (a, d, e, and f) and after (b) adsorption with the COOH-terminal peptide conjugated to BSA. Sections were imaged by either a confocal (a, b, e, and f) or a conventional fluorescence microscope (d). Comparison of the immunofluorescence staining pattern in a longitudinal section (d) with the position of the A- and I-bands in the same field (mirror image) imaged by phase-contrast microscopy (c) showed

that transversely oriented rows of discrete fluorescent foci (d and e; white arrows) were located at the center of the I-band region (c; dark arrows) where most of the junctional SR and corbular SR are localized in rat papillary myofibers. The intensity of labeling observed in (a) was greatly reduced when the ryanodine receptor antibodies were preadsorbed with the COOH-terminal peptide conjugated to BSA (b). In transverse sections, specific labeling was distributed in discrete fluorescent foci present throughout the cytoplasm. N, nucleus; bar, 5  $\mu$ m.



**Figure 3.** Immunofluorescence localization of the  $\text{Ca}^{2+}$  release channel/ryanodine receptor in cryosections of sheep papillary and Purkinje muscle fibers. Cryosections ( $4\text{--}8\ \mu\text{m}$ ) containing papillary myofibers (*a, c*; *M*) and transversely oriented Purkinje fibers (*b* and *c*; *P*) were labeled with either ryanodine receptor antibodies (*a-c*) or with wheat germ agglutinin (*WGA*; *d*) and imaged by scanning laser confocal microscopy. Comparison between the distribution of

After immunolabeling, the sections were positively stained using first 2% osmium tetroxide for 20 min followed by 10 min in saturated uranyl acetate and finally stained for 10 min in lead citrate. The sections were examined with a Hitachi 7000 transmission electron microscope.

## Results

### Antibody Specificity

The specificity of the site specific rabbit antibodies to the COOH terminus of the rabbit skeletal ryanodine receptor towards the rabbit cardiac ryanodine receptor was examined. The cardiac and skeletal ryanodine receptors although products of distinct genes (Otsu et al., 1990; MacKenzie et al., 1990) differ by only one amino acid over the region of the COOH-terminal peptide used to produce the site specific antiserum. Immunoblot analysis of SDS-PAGE separated microsomal proteins from rat heart demonstrates that the antibodies bind to a single band (Fig. 1, *arrow*) present in rat cardiac microsomes with an apparent  $M_r$  of 500,000, corresponding to the  $M_r$  of the rabbit cardiac ryanodine receptor (Otsu et al., 1990). The antibody also reacts with highly purified ryanodine receptor from rabbit skeletal muscle, cardiac muscle, and brain but does not react with the purified  $\text{IP}_3$  receptor from rabbit brain (McPherson and Campbell, submitted for publication).

### Immunofluorescence Labeling

Confocal (Fig. 2 *a, e*, and *f*) and conventional (Fig. 2 *d*) imaging of cryosections of rat papillary muscle labeled with affinity purified antibodies to the COOH terminus of the ryanodine receptor showed strong and specific labeling of muscle fibers. By contrast, vascular smooth muscle fibers, fibroblasts and endothelial cells also present in these sections were only labeled at the level of the background (results not shown).

**Longitudinal Sections.** In longitudinal sections of rat papillary myofibers, specific labeling was confined to narrow parallel strands oriented transversely to the longitudinal axis (Fig. 2 *a, d*, and *e*). These fluorescent strands were frequently resolved into discrete foci (Fig. 2, *d* and *e*, *white arrows*). Comparison between the immunofluorescence staining pattern (Fig. 2 *d*, *white arrows*) and the position of the A- and I-bands as determined by phase-contrast imaging of the same field (Fig. 2 *c*, *dark arrows*) showed that the intensely labeled discrete foci were generally confined to the center of the I-band region where most of the internal junctional and corbular SR are localized in mammalian cardiac myofibers. Absence of specific labeling in the A-band region (Fig. 2, *d* and *e*) suggested that the ryanodine receptor is absent from the network SR where cardiac  $\text{SR-Ca}^{2+}\text{-ATPase}$

the staining pattern for the ryanodine receptor (*c*) and the WGA-labeled cell periphery (*d*; *arrows*) in Purkinje fibers, showed that specific labeling for the ryanodine receptor was localized to discrete foci densely distributed throughout the interior as well as peripheral regions of the cytoplasm. The framed area of a portion of a Purkinje fiber shown in (*c*) is imaged at a higher magnification in (*b*). Arrows point to the cell periphery of Purkinje fibers (*b, c*, and *d*). Specific immunofluorescence labeling for the ryanodine receptor in a transversely oriented papillary myofiber was also distributed in discrete foci present throughout the cytoplasm (*a*). *N*, nucleus; bar,  $10\ \mu\text{m}$ .

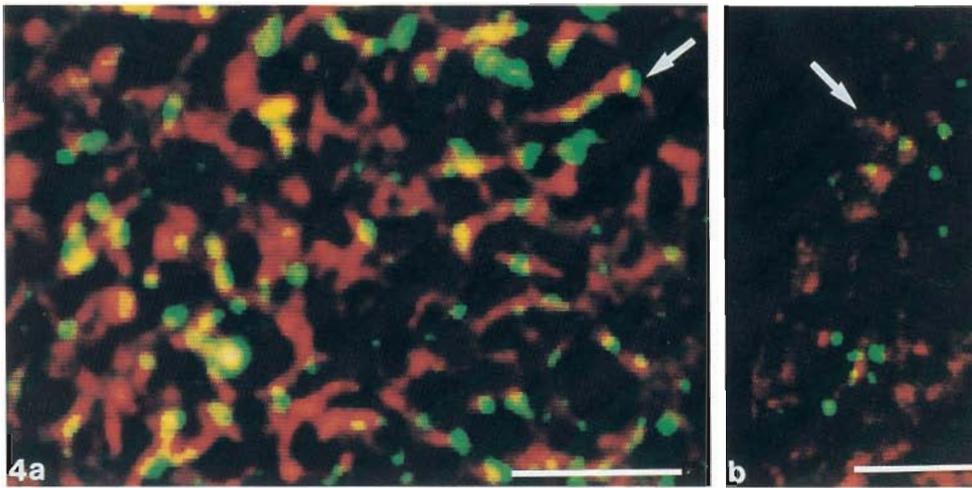


Figure 4. Merged confocal images of sheep papillary and Purkinje fibers double labeled for  $\text{Ca}^{2+}$  release channel/ryanodine receptor and the cardiac SR- $\text{Ca}^{2+}$ -ATPase. Confocal images of transverse cryosections of sheep papillary myofibers (a) and Purkinje fibers (b) double immunofluorescently labeled for  $\text{Ca}^{2+}$ -release channel/ryanodine receptor and the cardiac SR- $\text{Ca}^{2+}$ -ATPase were merged. Comparison of the distribution of the  $\text{Ca}^{2+}$ -release channel/ryanodine receptor (green and yellow) and the cardiac SR- $\text{Ca}^{2+}$ -ATPase (red and yellow)

showed that specific labeling for the  $\text{Ca}^{2+}$ -release channel/ryanodine receptor is confined to discrete foci over the SR- $\text{Ca}^{2+}$ -ATPase labeled networklike structure in papillary myofibers (a) and Purkinje fibers (b). Note that yellow represents regions of the myofibers where the distribution of labeling for the SR- $\text{Ca}^{2+}$ -ATPase (red) overlaps with that of the ryanodine receptor (green). The arrows point to the cell periphery. Bar, 5  $\mu\text{m}$ .

(Jorgensen et al., 1982) has previously been shown to be uniformly distributed by immunofluorescence and immunoelectron microscopical labeling. In transverse sections, specific labeling for the ryanodine receptor was distributed in discrete foci present throughout the cytoplasm of the myofiber (Fig. 2f). Labeling of the ryanodine receptor as seen in Fig. 2a was greatly reduced when the affinity purified antibodies were preadsorbed with the COOH-terminus peptide conjugated to BSA before immunofluorescence labeling (Fig. 2b).

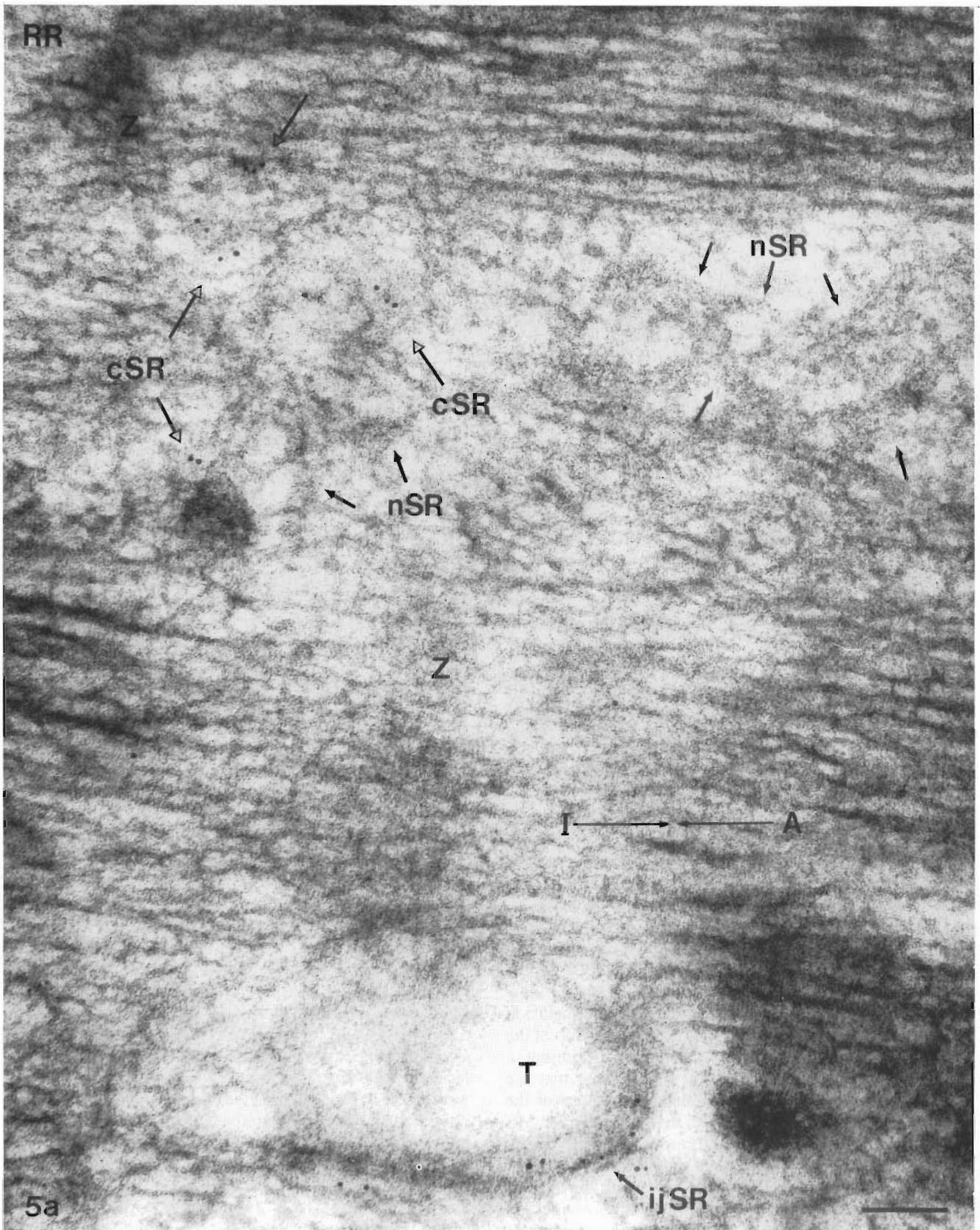
Because both the corbular and junctional SR are present in the interfibrillar spaces at the center of the I band regions in rat papillary myofibers the results presented above do not enable one to conclude whether the labeling for the cardiac ryanodine receptor seen in the cytoplasm represents junctional SR and/or corbular SR. Confocal imaging of transverse sections of sheep papillary muscle immunofluorescently labeled for the ryanodine receptor showed that specific labeling is present in both Purkinje fibers (P; Fig. 3, b and c) and papillary myofibers (M; Fig. 3, a and c), where it is distributed in discrete foci present in both interior and peripheral regions. The position of the cell periphery of Purkinje fibers shown in Fig. 3c was visualized by double labeling the same section with WGA (Fig. 3d). As Purkinje fibers lack T-tubules, these results support the conclusion that the discrete fluorescent foci in the interior regions of the Purkinje fibers indeed correspond to corbular SR present in this region of the fiber. To further demonstrate that the ryanodine receptor is confined to discrete domains of the SR, its distribution was compared to that of the cardiac SR- $\text{Ca}^{2+}$ -ATPase by double immunofluorescently labeled confocal imaging of transverse sections of sheep papillary muscle. The results clearly show that specific labeling for the SR- $\text{Ca}^{2+}$ -ATPase in agreement with previous studies (Jorgensen et al., 1982) is distributed in a continuous networklike structure in both papillary myofibers (Fig. 4a, red and yellow) and Purkinje fibers (Fig. 4, b, red and yellow). By contrast, specific labeling for the ryanodine receptor was confined to discrete foci distributed along the SR- $\text{Ca}^{2+}$ -ATPase labeled networklike structure in both papillary myofibers (Fig. 4, a,

green and yellow) and Purkinje fibers (Fig. 4, b, green and yellow). These results strongly support the conclusion that the ryanodine receptor is confined to specialized domains within the SR in both papillary myofibers and Purkinje fibers. Because junctional SR is absent from interior regions of Purkinje fibers it is likely these specialized regions correspond to corbular SR present in interior regions of these fibers.

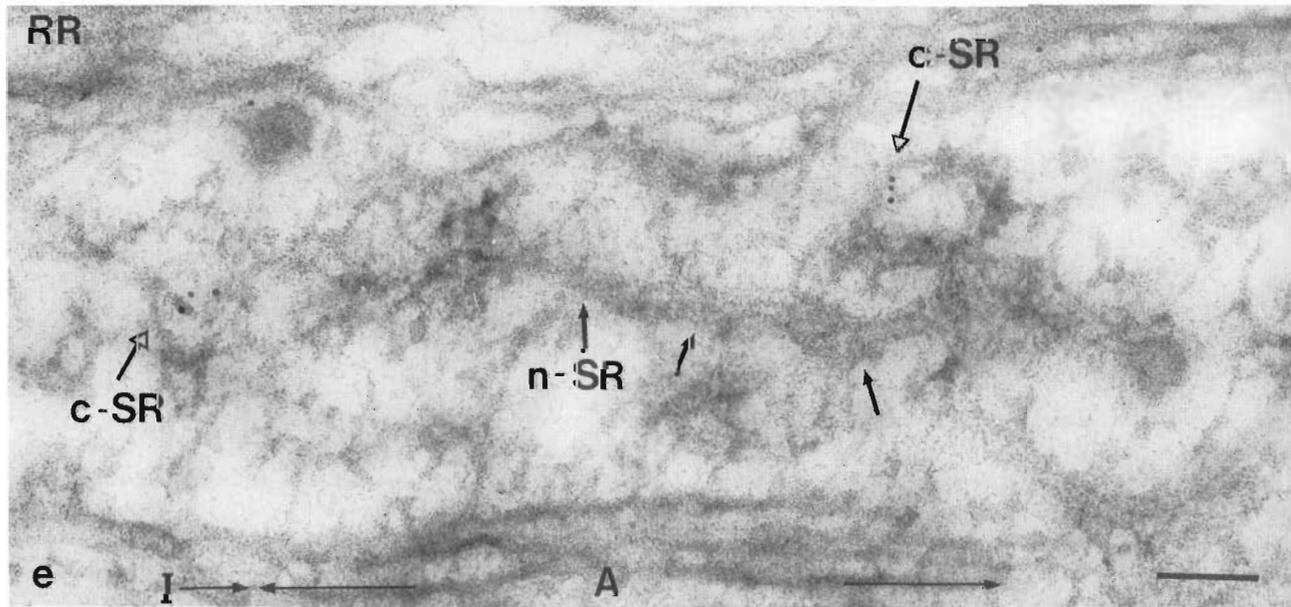
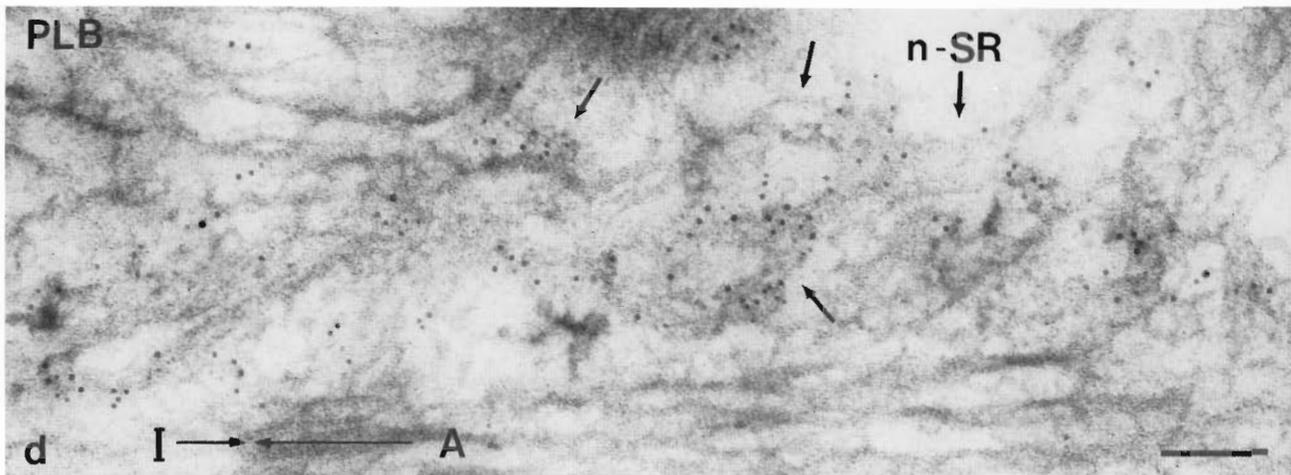
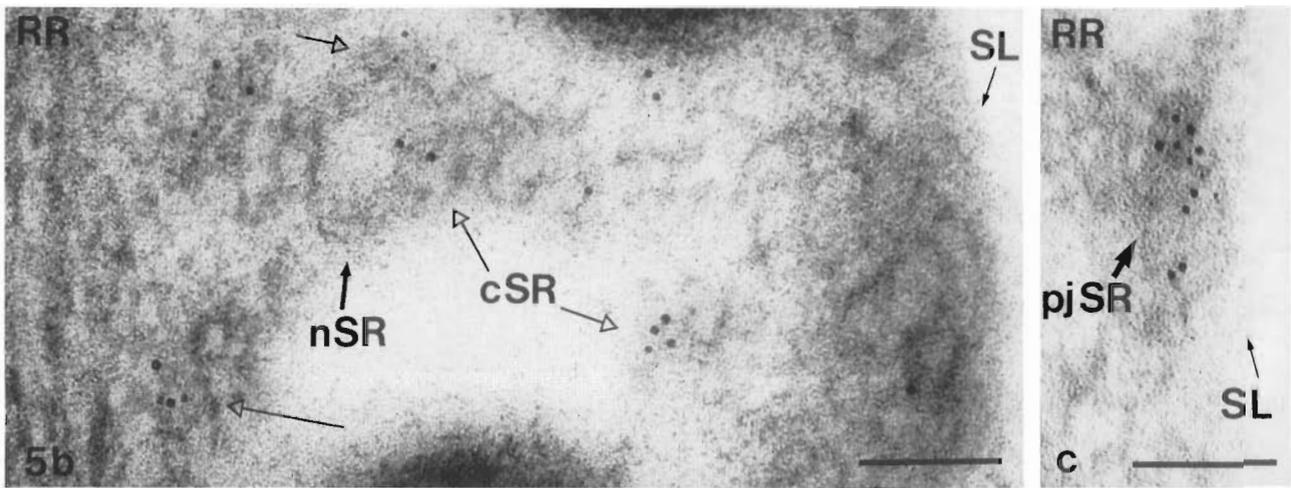
**Immunocolloidal Gold Localization of the Ryanodine Receptor.** Examination of immunolabeled thin sections of rat papillary muscle showed that most of the colloidal gold particles were confined to I-band regions but were not detected in A-band regions of the myofibers (Fig. 5a). Examination of specific regions showed that a majority of these particles were closely associated with discrete regions of the SR in both interior (*ijSR* and *cSR*, Fig. 5a) and subsarcolemmal regions (*cSR*, Fig. 5b and *pjSR*, Fig. 5c) of the myofibers.

Subsarcolemmal regions of myofibers at higher magnification showed that clusters of colloidal-gold particles were frequently present at variable distances from free surface SL (*cSR* and *open arrows*, Fig. 5b). When simultaneous visualization of SR and colloidal gold particles was obtained, clusters of colloidal gold particles were frequently observed to be closely associated with unlabeled network SR (*n-SR*, Fig. 5b). Occasionally, colloidal gold particles were also associated with tubular profiles of SR closely apposed to free surface SL (*pjSR*, Fig. 5c). Although electron dense structures were not visualized between the tubular profile of SR and the closely apposed SL, we tentatively conclude that these structures correspond to peripheral junctional SR.

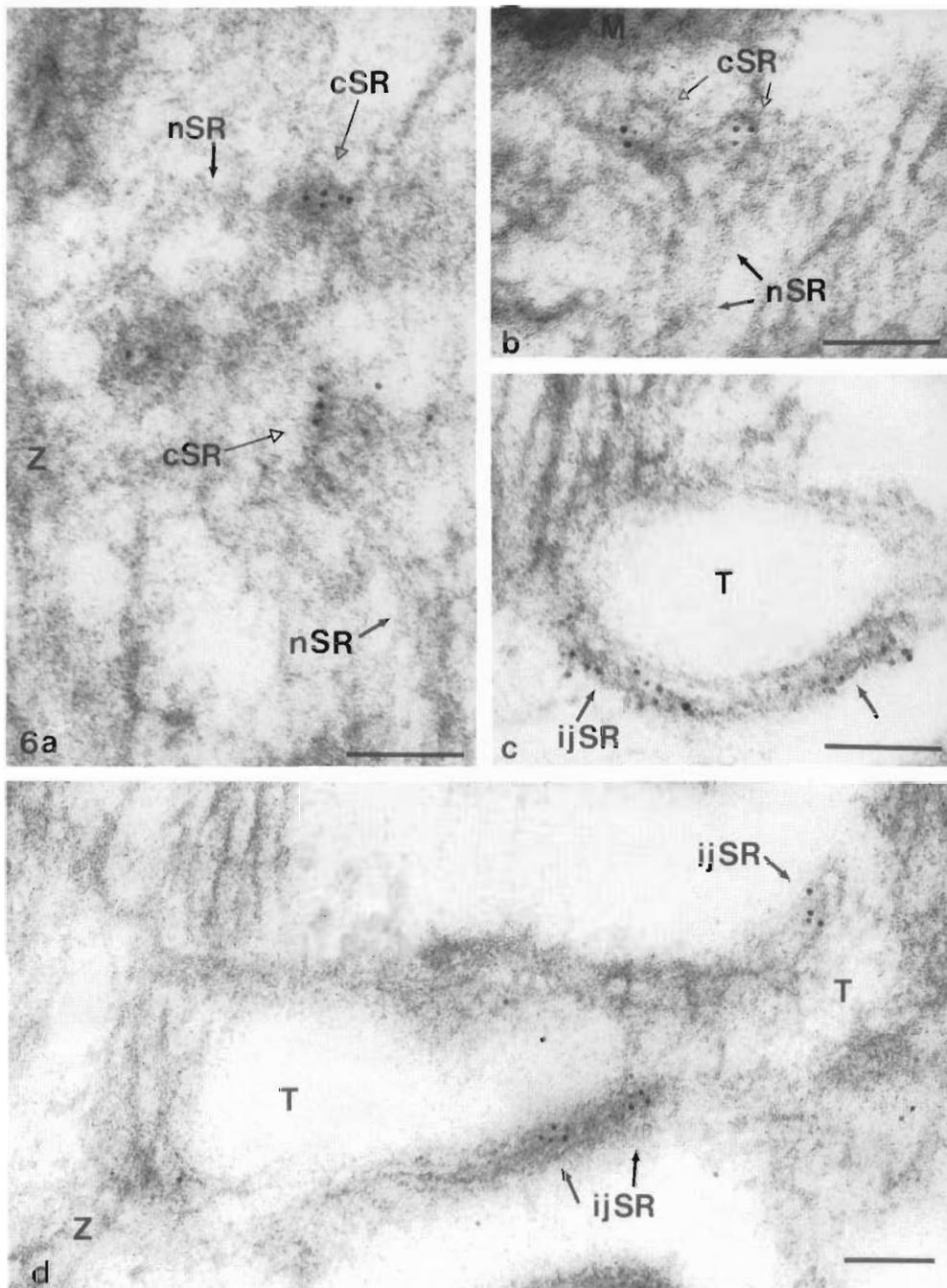
In interior regions of the myofibers, colloidal gold particles were associated with junctional SR closely apposed to T-tubules (*ijSR*, Fig. 5a). Furthermore, in the same region of the myofibers, small clusters of colloidal gold particles were frequently present in discrete foci and over the lumen of vesicular structures (*cSR*, Fig. 5a, *open arrows*) generally confined to the interfibrillar spaces at the level of the I-band region and closely associated with network SR. By contrast network SR present in I-band regions (*nSR*, Fig. 5a) and



**Figure 5.** Immunocolloidal gold localization of the  $\text{Ca}^{2+}$  release channel/ryanodine receptor (RR) and phospholamban (PLB) in rat papillary myofibers. Electronmicrographs of longitudinal thin sections (60–80 nm) of cryofixed, freeze-dried and low temperature LR White embedded rat papillary muscle. Immunocolloidal gold labeling for the ryanodine receptor (RR) (a, b, c, and e) was present over interior junctional SR (ijSR; a), over peripheral junctional SR (pjSR; c) and over corbular SR (cSR, open arrows; a, b, and e) localized at the level of the I-band region and closely associated with unlabeled network SR (nSR). Network SR (nSR and closed arrows; a, b, and e) in both



A- and I-band regions as well as myofibers were labeled only at the level of the background ( $<1$  colloidal gold particle per  $\mu\text{m}^2$ ). Membranes of the network SR were well visualized in *d* and *e* (*nSR* and *closed arrows*). Note that intense immunocolloidal gold labeling for phospholamban (*PLB*) (*d*) was fairly uniformly distributed over the network SR (*nSR* and *closed arrows*, *d*) while clusters of colloidal-gold immunolabeling for the ryanodine receptor (*RR*) was only detected over corbular SR (*cSR*, *e*) A, A-band; I, I-band; T, T-tubule; Z, Z-line; SL, sarcolemma. Bar,  $0.1 \mu\text{m}$ .



**Figure 6.** Immunocolloidal gold localization of the  $\text{Ca}^{2+}$  release channel/ryanodine receptor in rat papillary myofibers. Electronmicrographs of longitudinal thin sections (60–80 nm) of cryofixed, freeze-dried and low temperature LR white embedded rat papillary muscle showing high magnification images from interior regions of cardiac myofibers where T-tubules (T) and structurally distinct but continuous regions of SR are well visualized. Immunocolloidal gold particles labeling the ryanodine receptor were generally confined to the I-band region of the fiber where they were clustered over corbular SR (cSR, a and b), continuous with unlabeled network SR (nSR, a and b), and densely distributed over junctional SR (ijSR, c and d). Z, Z-line; M, mitochondria; Bar, 0.1  $\mu\text{m}$ .

A-band regions (nSR, Fig. 5 a) were only labeled at the level of the background.

When simultaneous visualization of SR and colloidal gold particles was obtained, it was clearly demonstrated that colloidal-gold particles were observed over vesicular structures (cSR, Figs. 5 e, 6, a and b) continuous with network SR (nSR, Figs. 5 e, 6, a and b) while the network SR itself was labeled only at the level of the background (i.e.,  $<1$  colloidal-gold particle/ $\mu\text{m}^2$ ). By contrast, labeling for phospholamban previously shown to be a marker of network SR (Jorgensen and Jones, 1987) was demonstrated to be uniformly distributed throughout the network SR in both the A band and I band region of the myofibers (Fig. 5 d). Furthermore, it was clearly demonstrated that colloidal gold particles were associated with junctional SR (ijSR, Fig. 6, c and

d). By contrast, T-tubular membranes (Fig. 6, c and d), sarcolemma (SL, Fig. 5, b and c), mitochondria (not shown) and myofibrils were labeled only at the level of the background ( $<1$  colloidal gold particle/ $\mu\text{m}^2$ ). The specificity of the immunocolloidal gold labeling was demonstrated by labeling thin sections with affinity purified antibodies to the ryanodine receptor that had been preadsorbed with COOH-terminal peptide to the skeletal ryanodine receptor before immunolabeling. Specific labeling of the distinct structural regions of the SR shown in Fig. 5 a was greatly diminished and less than 2 colloidal gold particles/ $\mu\text{m}^2$  (i.e., less than twofold that of the density of background labeling).

**Immunocolloidal Gold Localization of Calsequestrin in Spurr Embedded Tissue.** The immuno-electronmicroscopical studies presented in Fig. 7 and elsewhere (Jorgensen and

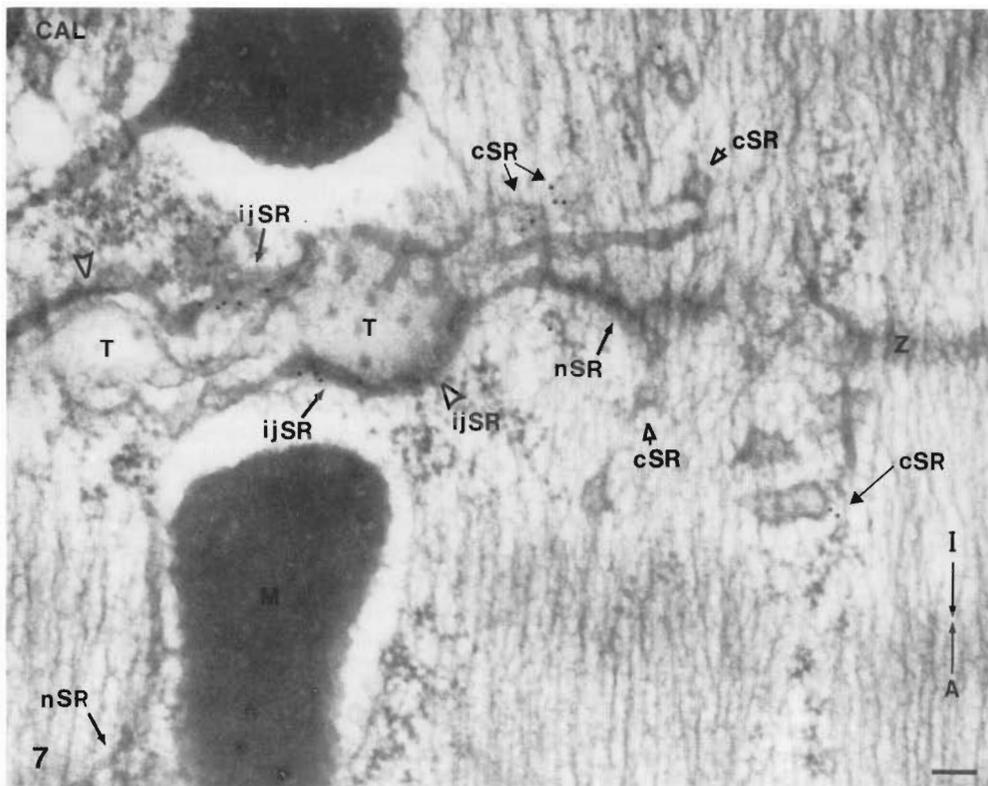


Figure 7. Electronmicrograph showing the distribution of immunocolloidal gold labeling for calsequestrin (CAL) in rat papillary myofiber embedded in Spurr. Most of the colloidal gold particles were present over the lumen of interior junctional (ijSR) and corbular SR (cSR) observed in the central regions of the I-band region. Well visualized but unlabeled ijSR and cSR were represented by open arrowheads and open arrows, respectively. Network SR (nSR) was not labeled; Z, Z-line; A, A-band; I, I-band; M, mitochondria; T, T-tubule. Bar, 0.1  $\mu$ m.

Campbell, 1984; Jorgensen et al., 1984, 1985, and 1988; Jorgensen and McGuffee, 1987) show that in interior regions of rat cardiac myofibers labeling for calsequestrin is distributed to corbular SR (cSR, Fig. 7) and interior junctional SR (ijSR, Fig. 7) both of which are continuous with unlabeled network SR (nSR, Fig. 7) and mainly located in the central portion of the I-band region. Similarly, in subsarcolemmal regions of rat cardiac myofibers calsequestrin has been localized to corbular SR and peripheral junctional SR (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985, and 1988; Jorgensen and McGuffee, 1987). These calsequestrin containing structures are continuous with nSR. It is noteworthy that corbular SR and junctional SR are frequently in close vicinity to one another (Fig. 7). It is observed that some (ijSR, arrows) but not all of the junctional SR (ijSR, open arrowheads) and similarly some (cSR, arrows) but not all of the corbular SR (cSR, open arrows) visualized as a result of the vapor osmication of the cryofixed and freeze-dried tissue before embedment in Spurr were labeled for calsequestrin. We suggest that unlabeled junctional and corbular SR are either not exposed to the surface of the section or that the cytoplasmic face but not the luminal face of these structures where calsequestrin is localized are exposed to the surface. In summary, it appears that the  $\text{Ca}^{2+}$ -release channel/ryanodine receptor and calsequestrin codistribute in corbular SR and junctional SR at both interior and subsarcolemmal regions of rat papillary myofibers but are absent from the network SR.

## Discussion

The results of the immunolocalization studies of rat papillary myofibers presented here show that the  $\text{Ca}^{2+}$ -release chan-

nel/ryanodine receptor, like calsequestrin, is distributed over junctional and corbular SR present in both interior and subsarcolemmal regions of the same myofiber, but is absent from the network SR where the SR  $\text{Ca}^{2+}$ -ATPase (Jorgensen et al., 1982) and phospholamban (Jorgensen and Jones, 1987) is densely distributed. Furthermore, the observation that specific immunofluorescence labeling for the ryanodine receptor, like that for calsequestrin is confined to discrete foci closely associated with the SR- $\text{Ca}^{2+}$ -ATPase labeled network present throughout the cytoplasm of sheep Purkinje fibers, which lack T-tubules, strongly support the conclusion that the discrete foci represent a specialized domain of the SR and may correspond to corbular SR.

On the basis of the results presented here and previous results on the subcellular distribution of calsequestrin (Jorgensen et al., 1985, 1988; Jorgensen and McGuffee, 1987) and calcium (Jorgensen et al., 1988), we conclude that the ryanodine receptor codistributes with calsequestrin and calcium in relaxed rat papillary myofibers. These results show that not only junctional SR but also corbular SR are sources of  $\text{Ca}^{2+}$  release into the myofibrillar space of cardiac myofibers via the  $\text{Ca}^{2+}$ -release/ryanodine receptor during excitation-contraction coupling.

The relative amount of corbular and junctional SR have only been determined in finch ventricles and mouse atria which lack T-tubules. It was reported that corbular/extended junctional SR and junctional SR constitute  $\sim 80$  and  $20\%$ , respectively, of the SR containing electron dense material in the lumina (Bossen et al., 1978, 1983). Evaluation of the relative amount of calsequestrin containing SR corresponding to corbular SR and junctional SR suggested that they constitute  $\sim 40$  and  $60\%$ , respectively, in rat papillary myofibers (Jorgensen et al., 1985). These estimates indicate that corbular SR like junctional SR represents a considerable propor-

tion of the total  $\text{Ca}^{2+}$  storage and release sites in cardiac myofibers.

The functional significance of the presence of two structurally distinct  $\text{Ca}^{2+}$ -release sites, both of which presumably release  $\text{Ca}^{2+}$  via the ryanodine receptor is presently unknown. The structural analogy of cardiac junctional SR and terminal cisternae of skeletal SR has favored junctional SR as the site of  $\text{Ca}^{2+}$  release relevant to excitation-contraction coupling in cardiac muscle (Fleischer and Inui, 1989; Wier, 1992). The possibility that corbular/extended junctional SR also plays a significant role in this process was originally proposed by Sommer (Jewett et al., 1973; Sommer and Waugh, 1976) on the basis of ultrastructural studies in avian cardiac myofibers. Its proposed potential role has been extended to include mammalian myocardial myofibers on the basis of more recent ultrastructural (Segretain et al., 1981), immunocytochemical (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985, 1988; Jorgensen and McGuffee, 1987) and electronmicroprobe analytical studies (Jorgensen et al., 1988). Regarding the specific role of corbular SR, it is noteworthy that it has not been observed in skeletal muscle. Thus, the presence of corbular SR at variable distances from junctional SR and from the SL may be relevant to features of excitation-contraction coupling in cardiac myofibers distinct from those in skeletal muscle. We have previously proposed that  $\text{Ca}^{2+}$  release from these two structurally distinct but continuous regions of the SR occurs in response to different stimuli during excitation-contraction coupling (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985). Thus, it was suggested that  $\text{Ca}^{2+}$  release from junctional SR might be triggered by signals depending on the physical contact via feet structures between junctional SR and sarcolemma, while corbular SR which is not in physical contact with the SL might be triggered to release  $\text{Ca}^{2+}$  by a diffusible agent. Considering our finding that the  $\text{Ca}^{2+}$  release channel/ryanodine receptor is present in both corbular SR and junctional SR, is it still reasonable on the basis of present evidence to propose that  $\text{Ca}^{2+}$  release from these two storage sites is triggered by different mechanisms?

Studies in skinned mammalian and avian myofibers have demonstrated that graded  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal SR occurs in cardiac but not skeletal muscle (Fabiato, 1983, 1985). These studies also showed that the size of the  $\text{Ca}^{2+}$  transient is determined by the rate of change of the free cytosolic  $[\text{Ca}^{2+}]$ . As skinned cardiac myofibers lack T-tubules, and thus functional junctional SR, it is highly likely that the corbular SR is the prominent site of  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release in these fibers (Fabiato 1983, 1985). However, these studies do not exclude the possibility that  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release also occurs at the junctional SR. On the basis of more recent studies of isolated rat and guinea pig ventricular myofibers it was concluded that  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release from the SR is a prominent component of the  $\text{Ca}^{2+}$ -transient leading to contraction in these myofibers (Wier, 1990; Nabauer et al., 1989; Cleemann and Morad, 1991; Wier, 1992; Beuckelmann and Wier, 1988). Furthermore, the  $\text{Ca}^{2+}$  release from SR was demonstrated to be triggered directly by the  $\text{Ca}^{2+}$  current entering the cytosol via the voltage gated L-type  $\text{Ca}^{2+}$  channel (Nabauer et al., 1989; Cleemann and Morad, 1991). Although this  $\text{Ca}^{2+}$  entry is triggered by depolarization of the SL and T-tubules,  $\text{Ca}^{2+}$  release from SR and the ensuing contraction does not

occur without the  $\text{Ca}^{2+}$  current, implying that the depolarization dependent mechanism leading to  $\text{Ca}^{2+}$ -release from skeletal SR (Schneider and Chandler, 1973; Bean and Rios, 1989) does not operate in cardiac muscle. Nonetheless, it has been observed that early repolarization of the SL shortens the  $[\text{Ca}^{2+}]$  transient in cardiac myofibers (Beuckelmann and Wier, 1988; Cannell et al., 1987), a finding that does not support the hypothesis of  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release. Thus, the possibility remains that a depolarization-dependent mechanism distinct from that required for excitation-contraction coupling in skeletal muscle may modulate  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release from cardiac SR (Wier, 1992).

These considerations, combined with our findings that the  $\text{Ca}^{2+}$  release channel/ryanodine receptor in cardiac SR is present in both junctional SR and corbular SR, makes it tempting to speculate that a combination of depolarization and  $\text{Ca}^{2+}$  current first induces  $\text{Ca}^{2+}$  release via the ryanodine receptor from junctional SR, which is closer to the SL and the site of  $\text{Ca}^{2+}$  entry via the L-type  $\text{Ca}^{2+}$  channel. This  $\text{Ca}^{2+}$  release then in turn, possibly by a regenerative mechanism, induces further release of  $\text{Ca}^{2+}$  via ryanodine receptors from corbular SR located further away from the SL and junctional SR. While a sufficiently large  $\text{Ca}^{2+}$  current might directly trigger  $\text{Ca}^{2+}$  release from corbular SR, the proposal that the initial  $\text{Ca}^{2+}$  release from junctional SR is an essential step in cardiac E-C coupling are consistent with the idea that  $\text{Ca}^{2+}$ -release from both corbular and junctional SR is triggered by  $\text{Ca}^{2+}$  but modulated only at the junctional SR by a yet unknown depolarization-dependent mechanism. Because the size of the  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release in skinned fibers is graded and determined by the rate of change of the free cytosolic  $\text{Ca}^{2+}$  concentration at the  $\text{Ca}^{2+}$  release site (Fabiato, 1983, 1985), an increase in the  $\text{Ca}^{2+}$  current could provide a steeper  $\text{Ca}^{2+}$  gradient triggering  $\text{Ca}^{2+}$  release from corbular SR located further into the fiber thus resulting in a larger  $\text{Ca}^{2+}$  transient and thereby an increase in contraction as observed in response to various inotropic agents such as epinephrine (Callewaert et al., 1988). To test this hypothesis it will be important in future studies to determine whether the spatial distribution of gradients of cytosolic free  $\text{Ca}^{2+}$  during the earliest stages of excitation-contraction coupling is consistent with this model. Although this is not feasible with presently available techniques (Wier, 1992), it is likely to be feasible with improved techniques in the near future.

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