Immunoelectron Microscopical Localization of Phospholamban in Adult Canine Ventricular Muscle

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Abstract. The subcellular distribution of phospholamban in adult canine ventricular myocardial cells was determined by the indirect immunogold-labeling technique. The results presented suggest that phospholamban, like the Ca^{2+}-ATPase, is uniformly distributed in the network sarcoplasmic reticulum but absent from the junctional portion of the junctional sarcoplasmic reticulum. Unlike the Ca^{2+}-ATPase, but like cardiac calsequestrin, phospholamban also appears to be present in the corbular sarcoplasmic reticulum. Comparison of the relative distribution of phospholamban immunolabeling in the sarcoplasmic reticulum with that of the sarcolemma showed that the density of phospholamban in the network sarcoplasmic reticulum was ~35-fold higher than that of the cytoplasmic side of the sarcolemma, which in turn was found to be three- to fourfold higher than the density of the background labeling. However, a majority of the specific phospholamban labeling within 30 nm of the cytoplasmic side of the sarcolemma was clustered and present over the sarcoplasmic reticulum in the subsarcolemmal region of the myocardial cells, suggesting that phospholamban is confined to the junctional regions between the sarcolemma and the sarcoplasmic reticulum, but absent from the nonjunctional portion of the sarcolemma. Although the resolution of the immunogold-labeling technique used (60 nm) does not permit one to determine whether the specific labeling within 30 nm of the cytoplasmic side of the sarcolemma is associated with the sarcolemma and/or the junctional sarcoplasmic reticulum, it is likely that the low amount of labeling in this region represents phospholamban associated with sarcoplasmic reticulum. These results suggest that phospholamban is absent from the sarcolemma and confined to the sarcoplasmic reticulum in cardiac muscle.

THE key regulator of the contraction relaxation cycle of cardiac muscle cells is Ca^{2+}. It is generally accepted that the sarcoplasmic reticulum together with the sarcolemma regulates the Ca^{2+} fluxes in the myofibril (2, 9, 19, 32, 35). The ability of beta-adrenergic agonists to enhance the contractility and rate of relaxation in cardiac muscle cells is believed to be mediated by transient changes in the Ca^{2+} transport properties of the sarcoplasmic reticulum and/or the sarcolemma (9, 30–32).

Phospholamban is the main membrane protein of cardiac sarcoplasmic reticulum to become phosphorylated in response to perfusion of hearts with beta-adrenergic agonists (20). Since beta-adrenergic agonists cause an increase in intracellular cAMP concentration in myocardium, which in turn is associated with cAMP-activated phosphorylation of phospholamban and stimulation of Ca^{2+}-transport by sarcoplasmic reticulum (17, 20, 30, 31), it has been proposed that the increase in the rate of relaxation of cardiac muscle produced by beta-adrenergic agonists is at least in part mediated by the cAMP-activated phosphorylation of phospholamban (20, 30, 31). Assuming that the sole function of phospholamban is to enhance Ca^{2+} uptake by the Ca^{2+}-ATPase of sarcoplasmic reticulum, one would predict that phospholamban and the Ca^{2+}-ATPase should colocalize in this membrane system. However, on the basis of in vitro (26, 27) and in situ (7, 8) studies on the phosphorylation of sarcolemmal proteins, it has been reported that a phospholamban-like protein called calciductin is also present in isolated cardiac sarcolemmal vesicles. Furthermore, it has been suggested that beta-adrenergic–stimulated phosphorylation of the sarcolemmal-associated phospholamban enhances the Ca^{2+} flux into the myocardial cells via the voltage-gated Ca^{2+} channel during the depolarization of the sarcolemma (26, 27). This possibility is, however, disputed by biochemical studies suggesting that the phospholamban-like protein detected in cardiac sarcolemmal vesicles is present as a contaminant (9, 22).

Ultrastructural studies have previously suggested that the sarcoplasmic reticulum of mammalian cardiac myocytes is composed of at least three structurally distinct but continuous regions (1, 4, 28, 29). They are called the network, the junctional, and the corbular sarcoplasmic reticulum. The network sarcoplasmic reticulum consists of an anastomosing network of electron lucent sarcotubules and surrounds the myofibrils. The junctional and corbular sarcoplasmic reticulum both contain electron dense material in their lumens and

have electron dense structures extending from their cytoplasmic surface. The junctional sarcoplasmic reticulum is bridged to the sarcolemma or its derivative, the transverse tubules, via electron dense structures called "feet" (6). The corbular sarcoplasmic reticulum, composed of corbular and cisternal expansions on the network sarcoplasmic reticulum, is mainly localized at the Z-line region in the interior regions of the myocardial cells. It is not closely apposed to either the sarcolemma or the transverse tubules.

It has previously been shown by immunoelectron microscopical labeling that the Ca^{2+}-ATPase is confined to the sarcoplasmic reticulum in cardiac muscle where it is uniformly distributed in the network sarcoplasmic reticulum but apparently absent from the junctional portion of both the interior and the peripheral junctional sarcoplasmic reticulum (15). Freeze fracture studies suggest that it is also absent from the corbular sarcoplasmic reticulum (1). To ascertain whether phospholamban like the Ca^{2+}-ATPase of the sarcoplasmic reticulum (15) is confined to the network sarcoplasmic reticulum or whether phospholamban is also a component of the cardiac sarcolemma, as proposed by several investigators (7, 8, 26, 27), we have determined the subcellular distribution of phospholamban in adult canine ventricular muscle cells by the indirect immunofluorescence and immunogold labeling techniques. The results presented show that phospholamban is densely distributed throughout the sarcoplasmic reticulum but is apparently absent from the sarcolemma, including the transverse tubules and the intercalated discs joining adjacent ventricular cells. Within the sarcoplasmic reticulum, phospholamban appears to be uniformly distributed in the network and the corbular sarcoplasmic reticulum but apparently absent from the junctional portion of the junctional sarcoplasmic reticulum.

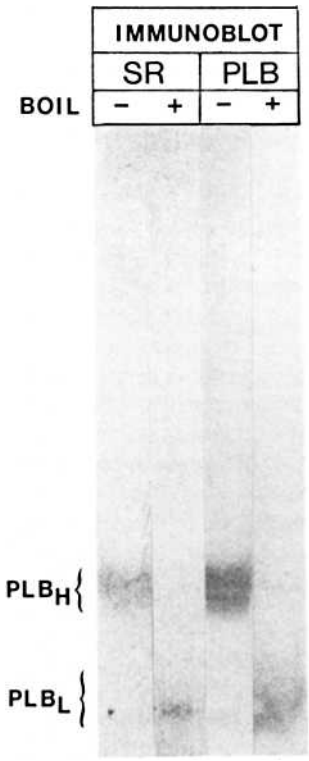

Figure 1. Immunoblot showing specificity of affinity-purified antibodies to canine ventricular phospholamban. Canine ventricular sarcoplasmic reticulum proteins (*SR*) and purified phospholamban (*PLB*) were separated by SDS PAGE and subjected to electrophoresis onto nitrocellulose (24). The nitrocellulose sheet containing the transferred proteins was first incubated with affinity-purified antibodies to canine cardiac phospholamban and then with horseradish peroxidase conjugated to affinity-purified goat anti-rabbit $F(ab)_2$. Plus and minus signs indicate whether or not the samples were boiled in SDS before electrophoresis. The immunoblot shows that the affinity-purified antibodies to canine ventricular phospholamban specifically bind to the high (25 kD; PLB_H) and low (5–6 kD; PLB_L) molecular mass forms of phospholamban.

Materials and Methods

Preparation and Characterization of Affinity-purified Antibodies to Canine Cardiac Phospholamban

Canine cardiac phospholamban was purified according to the procedure of Jones et al. (10). Rabbit antiserum to canine cardiac phospholamban was prepared and characterized as previously described by Jones et al. (10). Affinity-purified rabbit antibodies to cardiac phospholamban were prepared according to the procedure of Fowler and Bennett (5) by adsorption of the rabbit antiserum to phospholamban electrophoretically transferred to nitrocellulose sheets (12). Briefly, highly purified untreated phospholamban (200 μg) was separated from minor contaminants by electrophoresis on 8% slab gels (23). The separated proteins on the slab gels were subsequently transferred electrophoretically to nitrocellulose paper. The position of the high molecular mass form of phospholamban on the nitrocellulose was identified by indirect immunostaining of a narrow strip of the nitrocellulose sheet according to the procedure described by Towbin et al. (34). The portion of the nitrocellulose sheet containing the high molecular mass form of phospholamban was then excised and used as the affinity support for adsorption and elution of purified phospholamban antibodies (12).

The specificity of affinity-purified antibodies to canine cardiac phospholamban was verified by indirect immunostaining of nitrocellulose blots of canine sarcoplasmic reticulum proteins separated by SDS PAGE (34). The details of this procedure were performed as previously described (11, 15), except that affinity purified antibodies to phospholamban from canine ventricular muscle (0.5 μg/ml in 0.15 M NaCl, 10 mM Tris-HCl [pH 7.2], and 3% BSA) were used as the primary reagent. Goat anti-rabbit gamma globulin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was used as the secondary reagent.

Dissection, Fixation, and Sectioning of Canine and Rat Ventricular Muscle

Three adult mongrel dogs and two adult female Wistar rats were used in this study. The dogs were anaesthetized with sodium barbitol and the hearts excised. The rats were killed by cervical dislocation. Bundles of ventricular muscle fibers were quickly dissected and fixed by a two-step procedure as previously described (13). Subsequently, the bundles of fixed cardiac muscle tissue were washed, infused with 0.6 M sucrose, and 4–6-μm thick cryostat (13) and 80–100-nm thick ultracryomicrotomy sections (15, 33) cut as previously described.

Indirect Immunofluorescence Labeling

Immunofluorescence labeling of cryostat sections of fixed canine and rat ventricular muscle tissue was carried out as previously described (13). Affinity-purified antibodies to canine cardiac phospholamban (20 μg/ml) in pH 7.4 PBS were used as the primary reagent. The gamma globulin fraction of goat anti-rabbit serum conjugated to fluorescein (diluted 1:20; Miles-Yeder, Rehevat, Israel) was used as the secondary reagent. For preadsorption, 10 μg of affinity purified antibodies to canine cardiac phospholamban were incubated with either 0 or 7.5 μg highly purified phospholamban (10) as previously described (13). The supernatants obtained by centrifugation were used in the indirect immunofluorescence-labeling assay. The immunofluorescence cryostat sections were examined in a Zeiss photomicroscope provided with an epi-fluorescence attachment and a phase contrast condenser. The fluorescence pictures were photographed on TRI X-100 film.

Indirect Immunoelectron Microscopical Labeling

Immunoelectron microscopical labeling of ultracryomicrotomy sections of fixed canine ventricular muscle fibers was carried out as previously described (14, 33). Affinity-purified antibodies to phospholamban (20 μg/ml in PBS) were used as the primary reagent. Affinity purified goat anti-rabbit gamma globulin conjugated to colloidal gold (5–10 nm; Janssen Pharmaceutica, Beerse, Belgium) was used as the secondary reagent in the labeling procedures. After the immunolabeling, the ultrastructural details of the ultracryomicrotomy sections were visualized by first osmicating, dehydrating, and postembedding the immunolabeled frozen sections in LR white acrylic resin (London Resin Co. Ltd., London, United Kingdom) and then subjecting the specimen to positive staining with uranyl acetate and lead citrate as described by Keller et al. (16). In some cases, treatment of the immunolabeled sections with tannic acid (1%) followed the osmication step. This

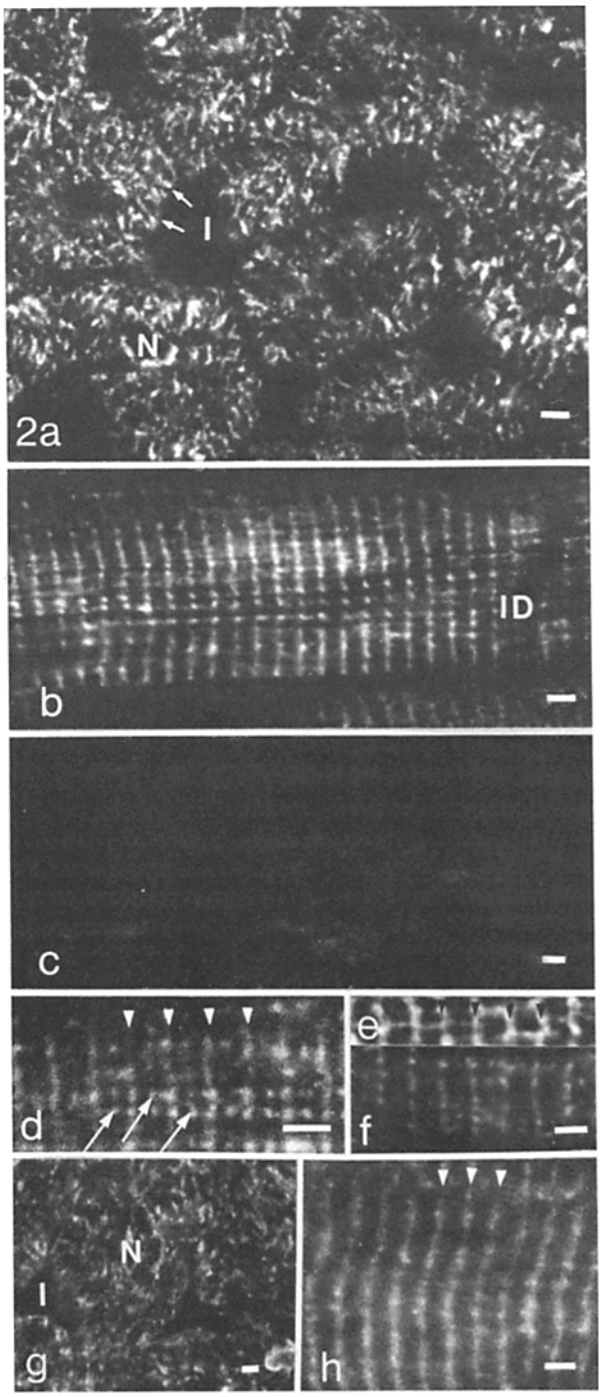

Figure 2. Distribution of phospholamban in adult canine (*a–d* and *f*) and rat (*g* and *h*) ventricular myocardium as observed by indirect immunofluorescence labeling. Fixed adult canine (*a–f*) and unfixed rat (*g* and *h*) ventricular muscle tissues were cryostat-sectioned (5–8 μm) transversely (*a* and *g*) and longitudinally (*b–f* and *h*), and labeled with phospholamban antibodies (*a*, *b*, *d*, *f–h*) and with phospholamban antibodies preadsorbed with highly purified phospholamban (*c*). In transverse sections (*a* and *g*), an irregular polygonal-staining pattern was observed. Comparison between the fluorescent staining pattern in *f* with the phase-contrast image of the same field (*e*, *black arrowheads*) shows that regular fluorescent staining present in the I-band region appears as short rods oriented parallel to the long axis of the myofiber (*d* and *h*, *white arrowheads*). Regular fluorescent staining in the A-band region consists of small bright foci in the central region of the A-band (*d*, *white arrows*). The intensity of staining observed after labeling with phospholamban antibodies (*b*) was greatly reduced when the phospholamban antibodies were preadsorbed with purified phospholamban before labeling (*c*). *I*, Intercellular space; *N*, nucleus; *ID*, intercalated disc. Short white arrows in *a* point to the outline of a myocardial cell. Bars, 2.5 μm.

treatment enhanced the visualization of the electron dense structures (feet) spanning the gap between junctional sarcoplasmic reticulum and sarcolemma.

Quantitation

The relative density of the phospholamban labeling per unit length of sarcoplasmic reticulum membrane was compared with that of sarcolemmal membrane of canine ventricular muscle cells as follows.

Ultracryomicrotomy sections of canine ventricular myocardium obtained from three dogs were labeled with either affinity-purified rabbit antibodies to phospholamban or with normal rabbit gamma globulin by the indirect immunogold-labeling technique described above. Sections containing well-delineated sarcoplasmic reticulum and sarcolemma were identified, and ten regions of myocardial cells containing well-delineated sarcoplasmic reticulum were photographed at random. For each photomicrograph (80,000×) containing well-delineated sarcoplasmic reticulum, three to five micrographs containing sarcolemma were photographed at random along the periphery of myocardial cells in the same region of the section. The length of the sarcoplasmic reticulum membrane and the sarcolemma in each micrograph was measured using a computer-aided digital tracer. The number of colloidal gold particles (Nt) present within 30 nm of either side of the total length (Lt) of the sarcolemma and/or the sarcoplasmic reticulum membranes were counted. The maximal distance between a membrane-associated antigen and a colloidal gold particle conjugated to the secondary antibody reagent is equal to the sum of the maximal diameter of each of the labeling reagents, i.e., 24–28 nm. The relative density (Nd) of the labeling per micrometer sarcoplasmic reticulum was calculated for each micrograph according to the formula Nd = Nt/Lt. The total number of colloidal gold particles present within 30 nm of the total length of the extracellular and cytoplasmic side of the sarcolemma was counted and the density of gold particles per micrometer of the extracellular and intracellular side of the membrane calculated for groups of three to five micrographs as described above.

Results

Antibody Specificity

The specificity of affinity-purified antibodies to canine cardiac phospholamban studies was demonstrated by indirect immunostaining of purified canine cardiac phospholamban and sarcoplasmic reticulum proteins previously separated by SDS PAGE (34) and blotted onto nitrocellulose. As shown in Fig. 1, affinity-purified antibodies to phospholamban were bound to single protein bands of 25,000 and 5,000–6,000 d, which correspond to the high (PLB_H) and low (PLB_L) molecular mass forms of phospholamban in cardiac sarcoplasmic reticulum vesicles. These results demonstrate that the affinity-purified antibodies to cardiac phospholamban are indeed monospecific for phospholamban in canine ventricular muscle.

Immunofluorescence Labeling

To determine the distribution of phospholamban in myocardial tissue, transverse and longitudinal cryostat sections of fixed canine (Fig. 2, *a–f*) and rat ventricular muscle (Fig. 2, *g* and *h*) were labeled with antibodies to phospholamban by the indirect immunofluorescence-staining technique. Examination of these sections showed that all myocardial fibers were strongly and specifically labeled (Fig. 2 *a*). At a particular phospholamban antibody concentration, the labeling

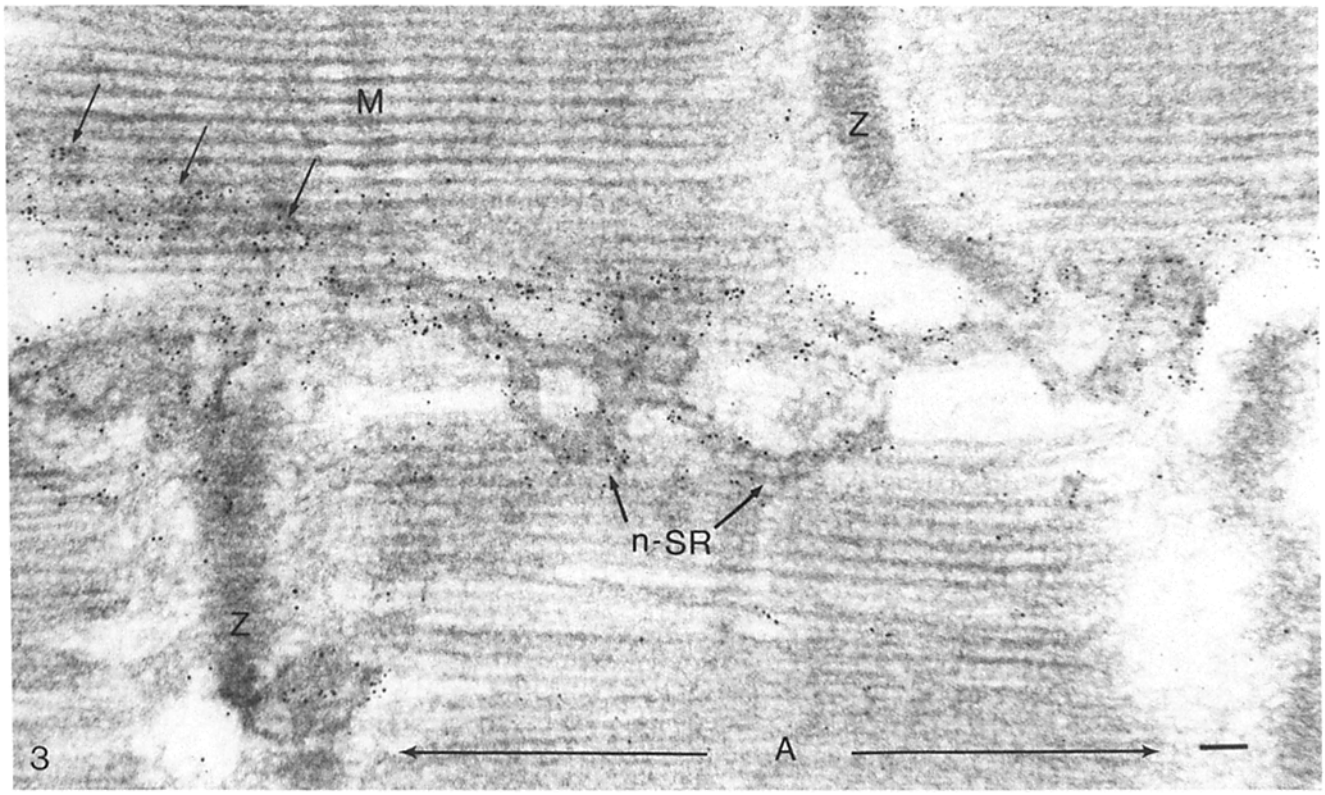

Figure 3. Gold particles are densely and fairly uniformly distributed throughout the network sarcoplasmic reticulum (*n-SR*) in the A-band and the I-band regions of the myocardial cell. Generally, myofibrils are unlabeled by gold particles. However, discrete areas densely labeled with gold particles can occasionally be observed (*thin arrows*). Since myofibrils are generally unlabeled, it is assumed that such discrete areas labeled with colloidal gold represent labeling of SR not visualized by the positive-staining procedure. *Z*, Z-line; *M*, M-line. Bar, 0.1 μm.

was more intense in canine (Fig. 2 *a*) than in rat ventricular myocardial cells (Fig. 2 *g*). By contrast, smooth muscle cells, fibroblasts, and endothelial cells were only labeled at the level of the background (not shown). Arterial smooth muscle cells in cryosections from unfixed myocardial tissue were faintly but positively labeled in the perinuclear region of the cells (not shown). Since this staining intensity was greatly diminished after fixation, it was unfeasible to assess the specificity of this staining pattern by adsorption of phospholamban before the immunofluorescence labeling. However, the presence of phospholamban in microsomes isolated from canine aorta was recently demonstrated by the Western blotting technique (25).

In transverse sections of canine (Fig. 2 *a*) and rat myocardial tissue labeled with antibodies to phospholamban (Fig. 2 *g*), a polygonal staining pattern was observed within the cytosol of each myofiber. The number of sides, as well as the length of the sides, varied from one polygon to the next. The labeling of the cell periphery appeared to be discontinuous (*arrows*, Fig. 2 *a*).

In longitudinal sections of canine (Fig. 2, *b*, *d*, and *f*) and rat (Fig. 2 *h*) myocardial tissue, comparison between the immunofluorescence staining pattern (Fig. 2 *f*) and the position of the A- and I-bands (*black arrowheads*, Fig. 2 *e*) in the same field, as determined by phase contrast microscopy (Fig. 2 *e*), showed that intense regular fluorescence labeling was present throughout the I-band region (*white arrowheads*, Fig. 2, *d* and *h*). Furthermore, a regular staining pattern limited to the center of the A-band region could frequently be observed (*arrows*, Fig. 2 *d*). It should be noted that intercalated discs (*ID*; Fig. 2 *b*) were only labeled at the level of the background (Fig. 2 *c*).

To demonstrate the specificity of the immunofluorescence-staining patterns observed in canine cardiac muscle labeled with antibodies to canine phospholamban (Fig. 2, *a*, *b*, *d*, and *f*), the supernatant from phospholamban-adsorbed antibodies to phospholamban were used in the indirect immunofluorescence-staining procedure. As shown in Fig. 2 *c*, the intensity of the immunofluorescence-staining pattern observed in Fig. 2 *b* was greatly diminished after labeling with the adsorped phospholamban antibodies.

Immunoelectron Microscopical Labeling

The distribution of phospholamban in myocardial cells as determined by immunofluorescence studies corresponded in general to that of the Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum in rat myocardial cells as previously determined by immunoelectron microscopical labeling (15). However, to determine the distribution of phospholamban more precisely, these studies were extended to the ultrastructural level of resolution.

Longitudinal ultracryomicrotomy sections of fixed adult canine myocardial tissue were labeled with antibodies to phospholamban by the indirect immunogold-labeling technique (14, 33). To visualize the ultrastructural detail of the

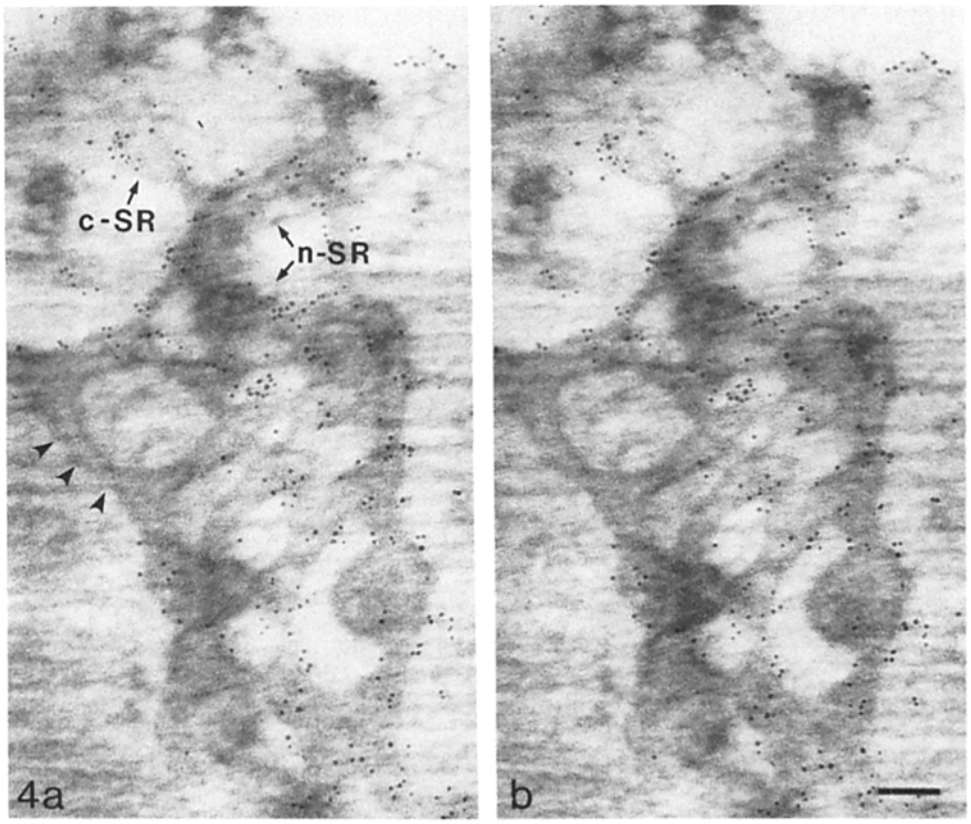

Figure 4. Stereoimaging of phospholamban labeling in the network sarcoplasmic reticulum. The photographs show the same field of a portion of Fig. 3 *b* taken at +6 and −6° angles from the original (0°) position of the goniometer stage. Examination of the stereopair with a steroscopic binocular lens shows that the region of the network sarcoplasmic reticulum that is not labeled with gold particles (*black arrowheads*) is positioned relatively deep into the section, whereas the gold particles are generally confined to the upper portion of the section. Note that the structure labeled *c-SR* is continuous with the network sarcoplasmic reticulum (*n-SR*). *c-SR*, corbular sarcoplasmic reticulum. Bar, 0.1 μm.

cardiac muscle membranes, the immunolabeled ultracryomicrotomy sections were subsequently osmicated, dehydrated, embedded in LR white resin (London Resin Co. Ltd.), and positively labeled according to the procedure of Keller et al. (16). Although this procedure for visualizing ultrastructural detail has greatly improved the delineation of cardiac membranes, it has so far not permitted the visualization of the feet, which in sections of epon-embedded muscle tissue have been shown to bridge the peripheral and the interior junctional sarcoplasmic reticulum membrane with the sarcolemmal and the transverse tubular membranes, respectively (3). The addition of tannic acid during the osmication step has now enabled us to visualize the feet structures described above in immunolabeled ultracryomicrotomy sections (*arrowheads*; Figs. 6, *c* and *d*, and 8).

Examination of the distribution of gold particles in myocardial cells showed that the gold particles were densely distributed over the sarcoplasmic reticulum (*SR*, Figs. 3–8; Table I). By contrast, the sarcolemma (*SL*, Fig. 6; Table I), the intercalated disc (*ID*, Fig. 7), the transverse tubular membrane (*T*, Fig. 8), the mitochondria (*M*, Fig. 8), and the myofibrils were very sparsely labeled at a level similar to that of the background. There were also, however, discrete regions over some myofibrils (*thin arrows*; Fig. 3) and over the periphery of the occasional mitochondrion (not shown) that were densely labeled with gold particles but apparently lacked sarcoplasmic reticulum. Since most mitochondria (Fig. 8) and myofibrils (Figs. 3, 5 *a*, 6, *a–d*, and 8) were only sparsely labeled and since the sarcoplasmic reticulum has been shown to be present in these regions, it seems reasonable to assume that these discrete regions of labeling over myofibrils and mitochondria represent sarcoplasmic reticulum not visualized by the positive staining.

Network Sarcoplasmic Reticulum

Simultaneous visualization of the sarcoplasmic reticulum and the colloidal gold particles showed that the density of colloidal gold particles was quite uniform throughout the network sarcoplasmic reticulum in both the I-band and the A-band (*n-SR*, Fig. 3) regions of the sarcomeres present in the interior as well as the subsarcolemmal regions (*n-SR*, Fig. 6, *b* and *d*) of myocardial cells.

There are also, however, regions of network sarcoplasmic reticulum without colloidal gold particles (*arrowheads*; Fig. 4 *a*). Since ultrastructural features, including the sarcoplasmic reticulum, can be visualized throughout the thickness of the section while the antibodies might only penetrate the upper portion of the section, it is likely that the unlabeled regions of the network sarcoplasmic reticulum were not at the upper region of the section and thus not exposed to the antibody-labeling reagents. To evaluate this possibility some sections were examined by stereo electron microscopy. The results showed that generally most of the colloidal gold particles were present near the top surface of the thin frozen section (Fig. 4, *a* and *b*).

Corbular Sarcoplasmic Reticulum

Vesicular structures localized in the interfibrillar spaces close to the Z-line were frequently observed to be labeled with gold particles (*c-SR*; Figs. 4 and 5). The gold particles were either distributed over the surface of the vesicular struc-

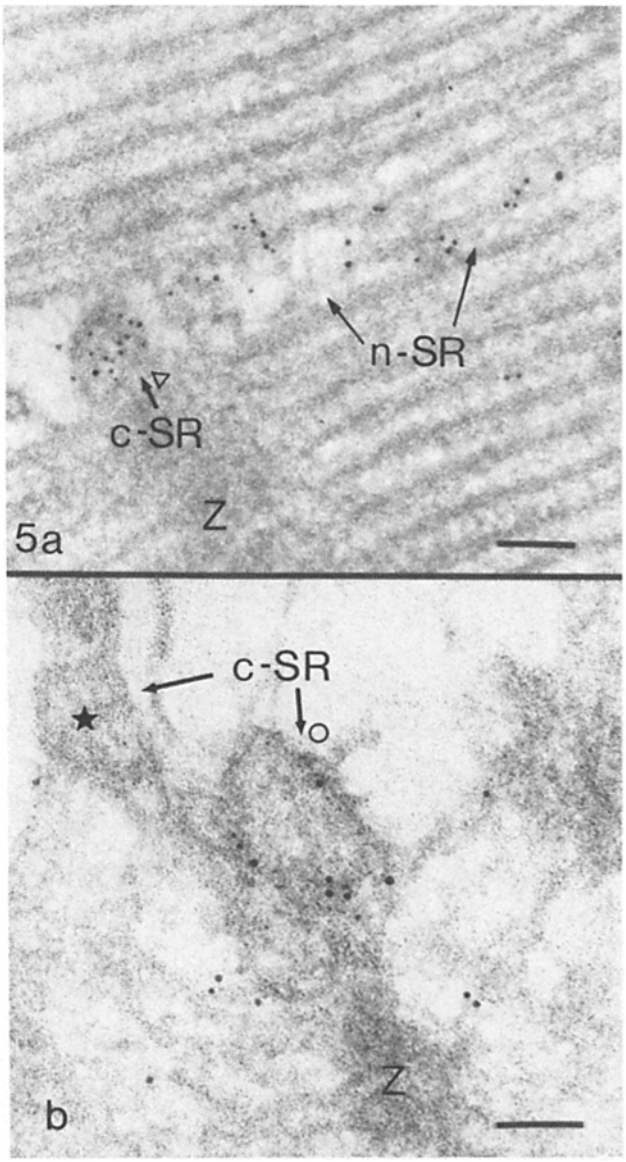

Figure 5. Gold particles are associated with most vesicular structures present in close vicinity to the Z-lines (*c-SR* in *a* and *b*), however some vesicular structures (*asterisk*, *b*) are not labeled. Note that gold particles are either associated with the surface of the vesicular structure (*triangle*, *a*) or associated with the cytoplasmic side of its membrane (*circle*, *b*). Z, Z-line; *n-SR*, network sarcoplasmic reticulum, *c-SR*, corbular sarcoplasmic reticulum. Bar, 0.1 μm.

ture (*triangle*, Fig. 5 *a*) or associated with the cytoplasmic surface of these vesicles (*circle*; Fig. 5 *b*). Stereo imaging of these vesicles suggested that they are continuous with the network sarcoplasmic reticulum (Fig. 4). Some of the vesicular structures were unlabeled by gold particles (*asterisk*, Fig. 5 *b*).

Junctional Sarcoplasmic Reticulum

Examination of the distribution of gold particles in the subsarcolemmal region of the ventricular myocytes showed that the gold particles were fairly densely distributed over the nonjunctional regions of the junctional sarcoplasmic reticulum not closely apposed to either the sarcolemma (*large arrows*; Fig. 6, *c* and *d*) or the transverse tubules (*J-SR*; Fig. 8). By contrast, the density of gold particles over the junctional portion of the junctional sarcoplasmic reticulum closely apposed to either the interior or the peripheral junctional sarcoplasmic reticulum was much lower than that over the nonjunctional portion and similar to that of the background. The apparent absence of gold particles over the junctional portion of the junctional sarcoplasmic reticulum was especially evident when the fixation procedure had resulted in a moderate increase in the distance between the sarcolemma and the junctional portion of the sarcoplasmic reticulum (Fig. 6, *c* and *d*).

The Relative Distribution of Phospholamban in Sarcoplasmic Reticulum and Sarcolemma

From the qualitative examination of the density of gold labeling of the different subcellular organelles, the results presented showed that the network and perhaps also the corbular sarcoplasmic reticulum are densely labeled with antibodies to phospholamban, while the junctional portion of the interior and peripheral junctional sarcoplasmic reticulum and the sarcolemma appeared to be labeled at the level of the background.

Comparison of the relative density of phospholamban labeling in the sarcoplasmic reticulum with that of the cytoplasmic and extracellular sides of the sarcolemma showed that the density of gold particles per micrometer of sarcoplasmic reticulum was ~35 times higher than that of the cytoplasmic side of the sarcolemma, and ~150 times higher than that of the extracellular side of the sarcolemma (Table I). The density of the gold particles associated with the extracellular side of the sarcolemma was found to be similar to that of the background (Table I). Ultrastructural studies of the cardiac sarcoplasmic reticulum have shown that the distance between the sarcolemma and the junctional sarcoplasmic reticulum is between 10–20 nm (3). Examination of the distribution of the gold particles that could theoretically be associated with the cytoplasmic side of the sarcolemma (i.e., located within 30 nm of the sarcolemma) showed that most (~70%) of these particles were clustered and present over positively stained sarcoplasmic reticulum in the subsarcolemmal region of myocardial cells (Fig. 6 *a*).

Discussion

The results presented show that phospholamban is densely and uniformly distributed throughout the network sarcoplasmic reticulum in canine ventricular muscle cells in situ. The vesicular structures containing phospholamban present in the interfibrillar spaces in the vicinity of the Z-line region are tentatively identified as the specialized nonjunctional sarcoplasmic reticulum called the corbular sarcoplasmic reticulum (1, 4, 28). Phospholamban appears to be absent from the junctional portion of the junctional sarcoplasmic reticulum closely apposed to either the sarcolemma or the transverse tubules in cardiac cells. Comparison of these results with our previous immunoelectron microscopical studies of the distribution of the Ca^{2+}-ATPase in rat ventricular muscle (15) suggests that both the Ca^{2+}-ATPase and phospholamban are present in the network sarcoplasmic reticulum but absent from the junctional portion of the sarcoplasmic reticulum.

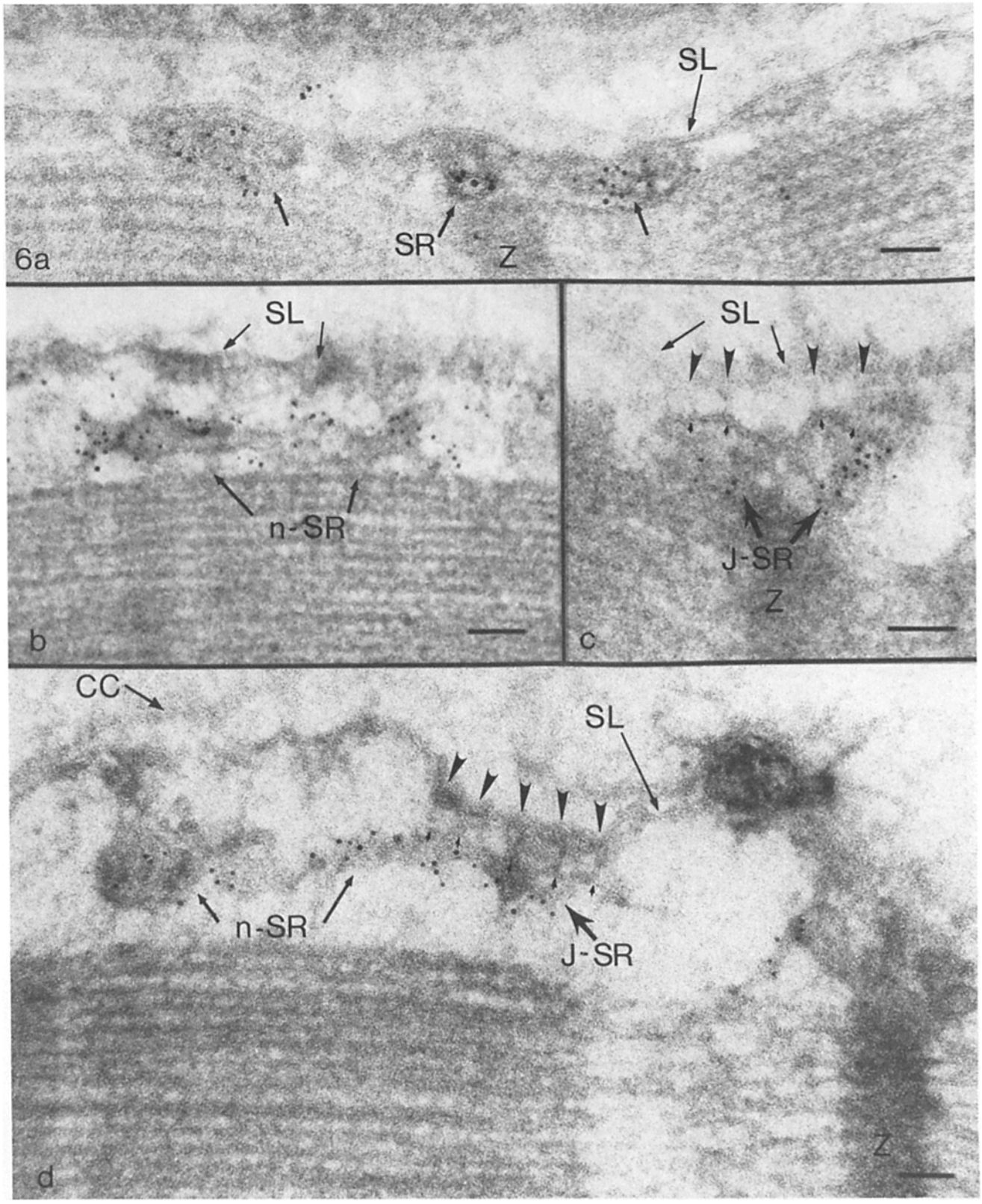

Figure 6. In the subsarcolemmal region along the lateral sarcolemmal membrane of canine ventricular myocardial cells, phospholamban is fairly uniformly distributed over network sarcoplasmic reticulum (*a*, *b*, and *d*, *medium arrows*; *b* and *d*, *n-SR*) and the cytoplasmic phase of the junctional sarcoplasmic reticulum (*J-SR*, *c* and *d*, *thick arrows*) present in this region of the cell. By contrast, the junctional portion of the junctional sarcoplasmic reticulum (*c* and *d*, *small arrows*) is not labeled by gold particles. Note that regularly spaced electron dense material is bridging the sarcolemma (*SL*) and the junctional portion of the junctional sarcoplasmic reticulum (*large arrowheads*, *c* and *d*). Similarly the sarcolemma appears to be labeled only at the level of the background (*a–d*, *thin arrows*). *Z*, Z-line; *CC*, cell coat. Bars, 0.1 μm.

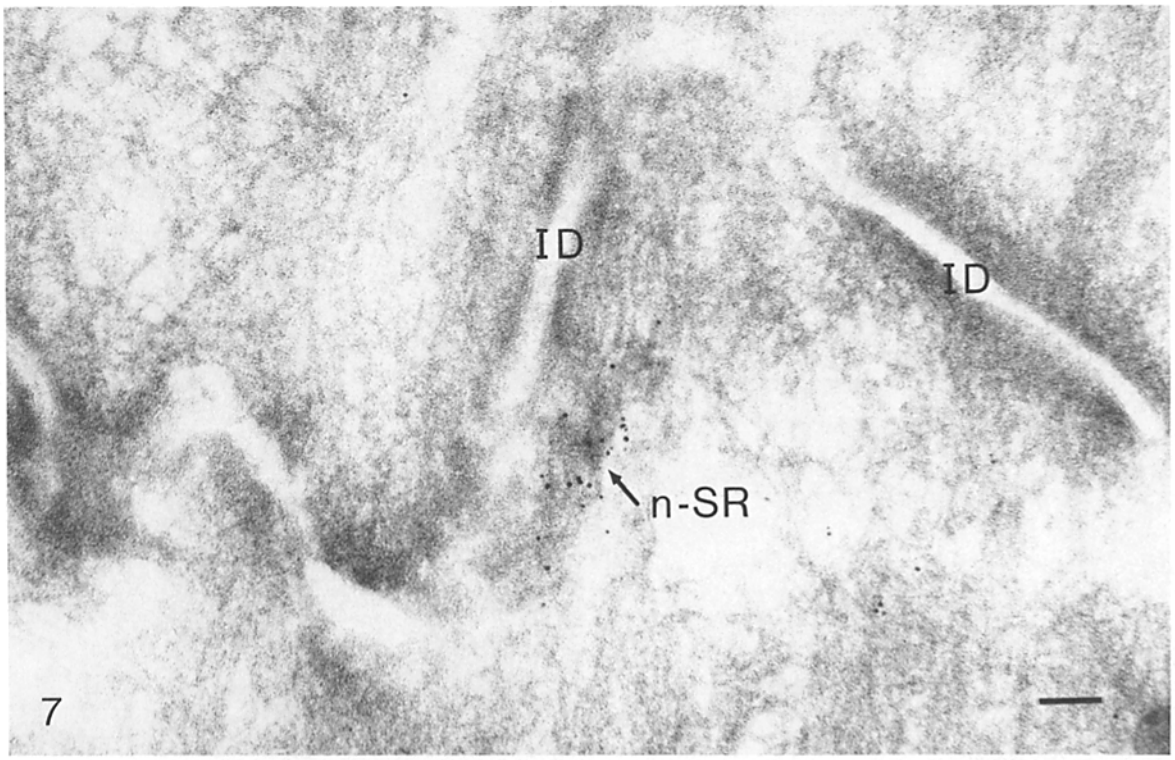

Figure 7. In the subsarcolemmal region along the intercalated discs (*ID*), gold particles are present over the network sarcoplasmic reticulum (*n-SR*) but absent from the sarcolemma. Bar, 0.1 μm.

These results are consistent with the idea first proposed by Kirchberger et al. (17) that phospholamban regulates the Ca^{2+} transport activity of the Ca^{2+}-ATPase localized to the network sarcoplasmic reticulum in cardiac muscle.

Recent immunoelectron microscopical studies (unpublished observations) and freeze fracture studies (1) of cardiac sarcoplasmic reticulum suggest that the Ca^{2+}-ATPase is absent from corbular sarcoplasmic reticulum. This region of the sarcoplasmic reticulum has previously been shown to contain the Ca^{2+}-binding protein calsequestrin and may perhaps function as a Ca^{2+} storage and/or release site in the sarcoplasmic reticulum (11, 14). The localization of phospholamban to the corbular sarcoplasmic reticulum supports the possibility that phospholamban might have an additional function in the corbular sarcoplasmic reticulum distinct from its function as a regulator of the Ca^{2+}-ATPase. Double-labeling experiments with antibodies to the Ca^{2+}-ATPase at the network sarcoplasmic reticulum and phospholamban are in progress to determine definitively whether phospholamban is indeed present in this specialized nonjunctional sarcoplasmic reticulum previously shown to lack the Ca^{2+}-ATPase.

Several investigators studying sarcolemmal protein phosphorylation in intact cellular (7, 8) and isolated membrane fractions (26, 27) have demonstrated the presence of a phospholamban-like protein in membrane fractions enriched in sarcolemmal vesicles. Thus it has been proposed that beta-adrenergic stimulation of phosphorylation of the putative sarcolemmal-associated phospholamban might enhance the inotropic state of myocardial cells (7, 8, 26, 27). However, this interpretation has been disputed on the basis of biochemical studies suggesting that the sarcolemmal-associated phospholamban is not an integral component of the sarcolemma but rather a contaminant originating from the sarcoplasmic reticulum (9, 10, 22). This interpretation is further supported by the recent finding that the phospholamban content of highly purified sarcolemmal vesicles is only 8% of that of purified sarcoplasmic reticulum vesicles when com-

*Table I. Relative Distribution of Phospholamban in the Sarcoplasmic Reticulum and the Sarcolemma of Canine Ventricular Myocytes**

Label	Membrane	Total membrane size	No. gold‡ particles/μm of membrane
		μm	
Phospholamban antibodies	Network sarcoplasmic reticulum	33.3	31.1 ± 7.6
Phospholamban	§Sarcolemma:	197	
	outside		0.2 ± 0.2
	inside		0.9 ± 0.3
Normal rabbit globulin	§Sarcolemma:	50	
	outside		0.2
	inside		0.2

* See Materials and Methods.

‡ The entire length of network sarcoplasmic reticulum or sarcolemma was measured in each micrograph. The number of gold particles/micrometer of membrane was then averaged over the number of micrographs used in the study and a SD calculated.

§ Since the maximum distance between a sarcolemma-associated antigen and the center of the colloidal gold particles conjugated to the secondary antibody is 25 nm, gold particles within 30 nm of the outer or inner surface of the sacrolemma were enumerated.

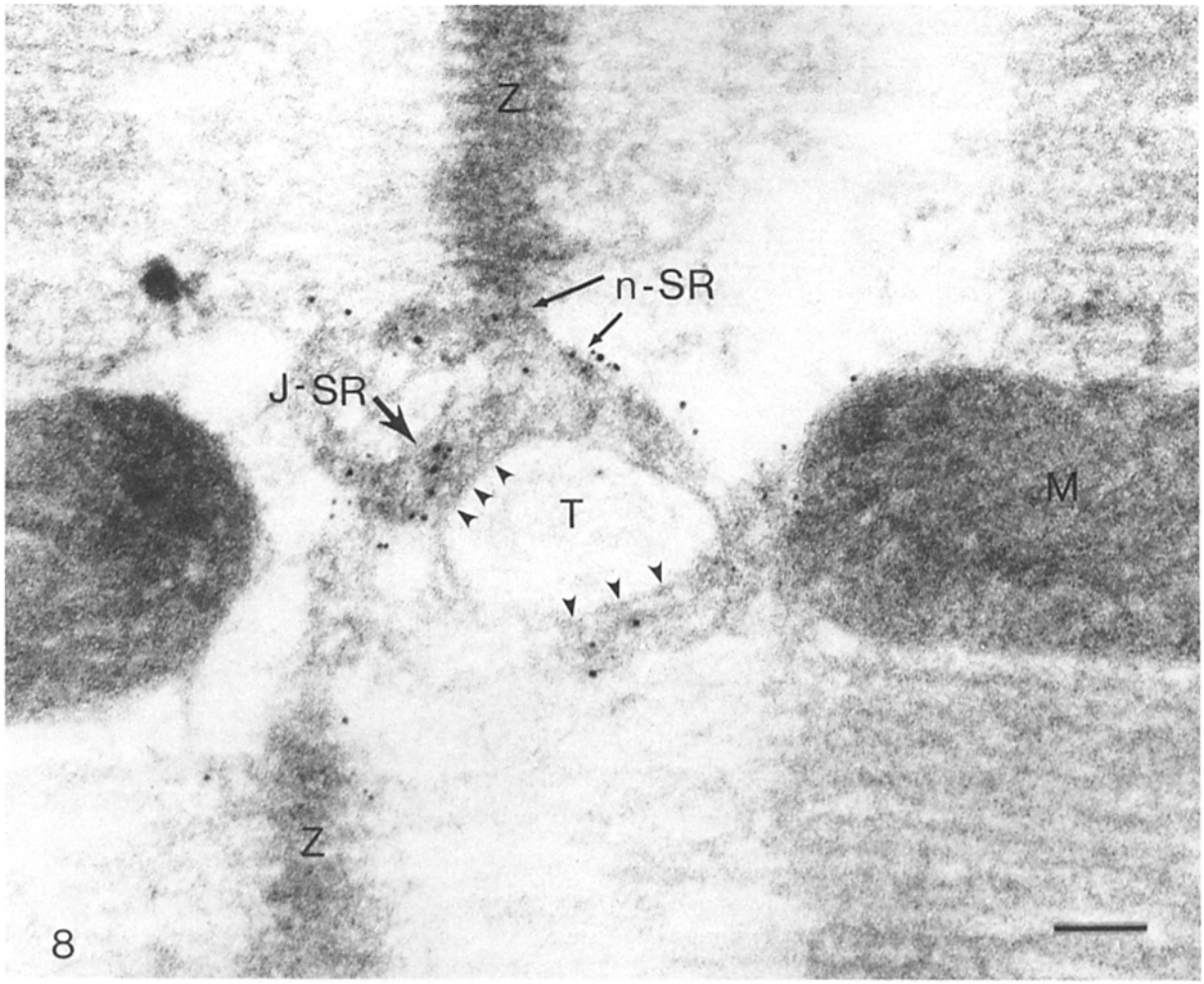

Figure 8. In the region where the junctional sarcoplasmic reticulum (*J–SR*) is closely apposed to transverse tubules (*T*), gold particles are densely distributed over the cytoplasmic phase of the junctional sarcoplasmic reticulum (*black arrows*) and less densely over the junctional portion of the junctional sarcoplasmic reticulum. Gold particles are absent from the T-tubular membrane (*arrowheads*). *M*, Mitochondrion; *Z*, Z-line; *n-SR*, network sarcoplasmic reticulum. Bar, 0.1 μm.

parisons are expressed per milligram of membrane protein isolated (24).

Comparison of the relative density of phospholamban immunolabeling of the sarcoplasmic reticulum with that of the sarcolemma supports the proposal that phospholamban is confined to the sarcoplasmic reticulum in cardiac muscle. Thus, the density of phospholamban in the network sarcoplasmic reticulum was found to be ~35-fold higher than on the cytoplasmic side of the sarcolemma, which was only about three- to fourfold higher than background labeling. The finding that the majority of the phospholamban labeling on the cytoplasmic side of the sarcolemma was clustered and present over the sarcoplasmic reticulum in the subsarcolemmal region of the cell is consistent with the possibility that specific phospholamban labeling in this region of the myocardial cell represents phospholamban associated with the nonjunctional portion of the junctional sarcoplasmic reticulum and that phospholamban is absent from the nonjunctional portion of the sarcolemma. This suggestion is also supported by the observation that phospholamban labeling of well-delineated and perpendicularly cut junctional regions of the sarcolemma and junctional sarcoplasmic reticulum (Fig. 6, *c* and *d*) is densely distributed over the nonjunctional portion of the junctional sarcoplasmic reticulum while absent from the sarcolemma and the junctional portion of the junctional sarcoplasmic reticulum. However, since the maximal distance between an antigen associated with the cytoplasmic side of the sarcolemma and the center of the colloidal gold particle conjugated to the secondary antibody reagent is 30 nm while the distance between the sarcolemma and the junctional sarcoplasmic reticulum is only 10–20 nm, the immunogold-labeling technique used in the present study cannot exclude the possibility that the junctional portion of the sarcolemma closely apposed to junctional sarcoplasmic reticulum is specifically labeled with antibodies to phospholamban. Such labeling, however, is well below the levels of phospholamban detected in partially purified sarcolemmal fractions (7, 8, 26, 27).

In conclusion, the results presented clearly show that the bulk of the phospholamban detected in isolated sarcolemmal vesicles (7, 8, 26, 27) is likely to represent contamination with sarcoplasmic reticulum vesicles. Thus, recent reports by Louis et al. (21) and Lamers et al. (18) suggesting that phospholamban in cardiac muscle is an integral component

of both the sarcoplasmic reticulum and the sarcolemma should be viewed with caution.

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