

Localization of Phospholamban in Smooth Muscle Using Immunogold Electron Microscopy

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Abstract. Phospholamban, the putative regulator of the Ca^{2+} -ATPase in cardiac sarcoplasmic reticulum, was immunolocalized in canine visceral and vascular smooth muscle. Gently disrupted tissues were labeled with an affinity-purified phospholamban polyclonal antibody and indirect immunogold, using preembedding techniques. The sarcoplasmic reticulum of smooth muscle cells was specifically labeled with patches of

immunogold distributed in a nonuniform fashion, while the sarcolemma did not appear to contain any phospholamban. The outer nuclear envelopes were also observed to be heavily labeled with the affinity-purified phospholamban polyclonal antibody. These findings suggest that phospholamban may play a role in the regulation of cytoplasmic and intranuclear calcium levels in smooth muscle cells.

PHOSPHOLAMBAN (PL)¹ is a low molecular mass, integral membrane protein that has been purified from cardiac sarcoplasmic reticulum (SR) and well characterized both biochemically and functionally (5, 7). In vitro phosphorylation of PL by three different kinases, at distinct sites, enhances Ca^{2+} uptake by cardiac microsomes (2, 9, 10, 14, 15, 17, 19, 20). Recently, it has been shown that an endogenous protein phosphatase can reverse the stimulatory effects of kinase activity (12). Furthermore, PL is the major cardiac SR protein phosphorylated in response to β -adrenergic stimulation in vivo (13, 18). These studies suggest that PL is an important regulator of the Ca^{2+} -ATPase in cardiac SR.

A similar regulatory system for the calcium pump may be present in the SR of smooth muscle cells. In a recent study, a PL-like polypeptide with the appropriate molecular mass and exhibiting the characteristic mobility shift in SDS-PAGE was found in porcine gastric smooth muscle (23). This polypeptide was abundant in Western blots of an enriched SR fraction but was only detected at very low levels in an enriched plasma membrane fraction (23). Phosphorylation of this PL-like polypeptide is mediated by endogenous cAMP- and Ca^{2+} /calmodulin-dependent protein kinases (23). cAMP-dependent phosphorylation of smooth muscle microsomes enhances the rates of Ca^{2+} uptake in the presence of oxalate (1, 21) and cAMP can stimulate Ca^{2+} sequestration by the SR in permeabilized arterial muscle cells (25). Thus, smooth muscle cells contain a protein with characteristics similar to those of PL. In this study, we have used immunogold EM to localize the binding sites of an affinity-purified PL antibody in visceral and vascular smooth muscle. Our results indicate that PL is present in the SR and the outer nuclear envelope of smooth muscle cells.

1. *Abbreviations used in this paper:* GAR, goat anti-rabbit antibody; PL, phospholamban; SL, sarcolemma; SR, sarcoplasmic reticulum.

Materials and Methods

Isolation of Cardiac SR and Purification of PL

Canine cardiac SR membranes were isolated as previously described (16). PL was purified from the cardiac SR using a procedure which will be described in detail elsewhere. Briefly, calsequestrin was extracted with Na_2CO_3 at pH 11.4, PL was solubilized in the presence of 0.7 mg deoxycholate per mg protein, and subsequently purified by sulfhydryl group affinity chromatography.

Preparation and Purification of Antibodies

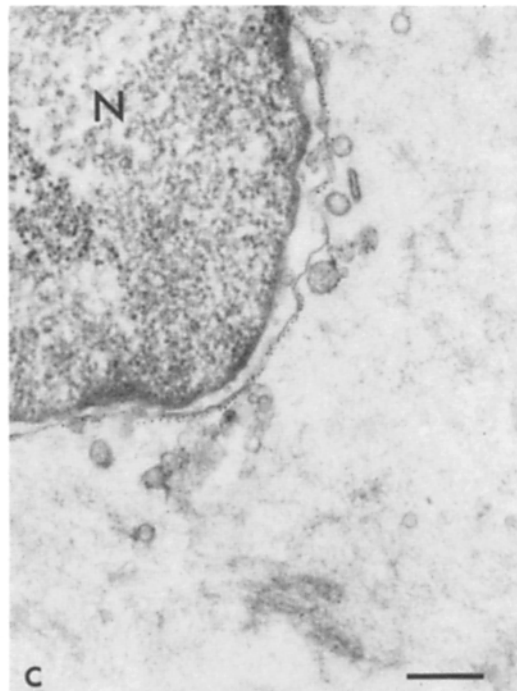
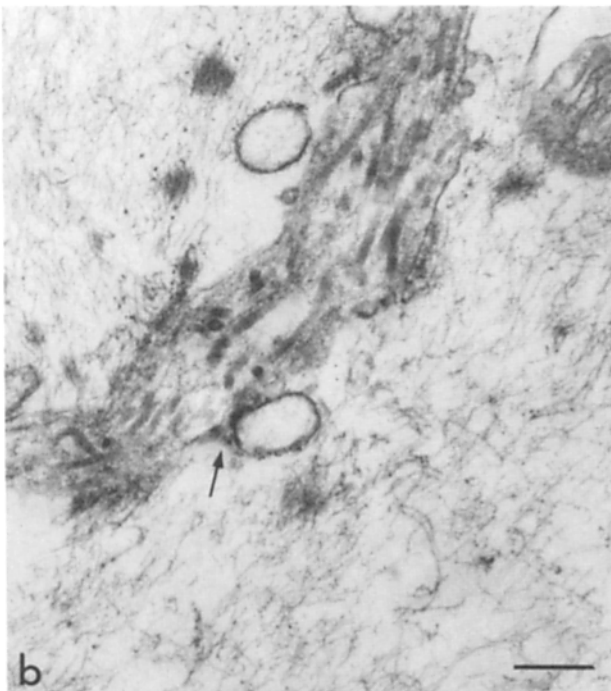
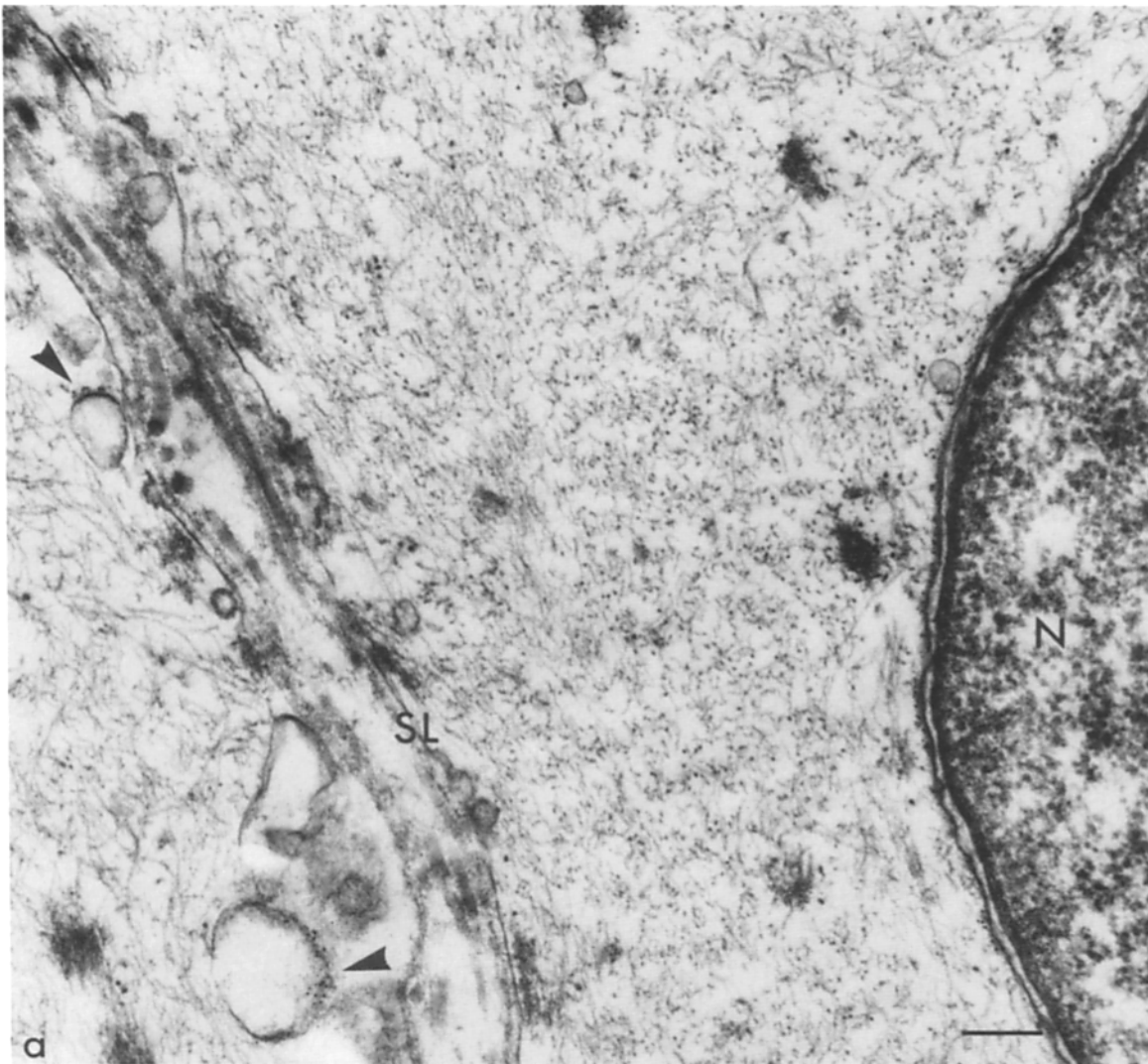
Antibodies to purified canine PL were raised in two rabbits, ~2 kg each. Both rabbits were injected subcutaneously with PL emulsified in Freund's complete adjuvant; 150 μg PL on day 1 and 120 μg PL on day 7. This was followed by intramuscular injections of 20 μg of PL in Freund's complete adjuvant on days 28 and 35. Serum was collected on day 45. The sera were purified on a PL-Affigel 10 (Bio-Rad Laboratories, Richmond, CA) affinity column and immediately concentrated and equilibrated with 20 mM Na phosphate, 150 mM NaCl, pH 7.2 (PBS) using centricon 30 membrane filters (Amicon Corp., Danvers, MA).

Western Blot Analysis

Antibody specificity was demonstrated by extracting 50 μl of the appropriate tissue sample in SDS, performing electrophoresis in 10% polyacrylamide gels, transferring the proteins to a nitrocellulose filter electrophoretically, blocking the nitrocellulose using PBS containing 10 mg/ml bovine hemoglobin, and incubating with the PL antibody. An alkaline phosphatase-conjugated second antibody was used to detect the PL antibody and it was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrate according to ProtoBlot instructions (Promega Biotech, Madison, WI).

Immunogold EM

Canine visceral smooth muscle was obtained from the ileum, ~15 cm proximal to the ileo-cecal junction. Canine vascular smooth muscle was obtained from the left common iliac artery, just proximal to the bifurcation



of the internal and external iliac arteries. Porcine vascular smooth muscle was obtained from the left anterior descending artery.

Gently disrupted smooth muscle tissues were immunogold labeled using preembedding procedures as previously described (30). Briefly, the muscle was cleaned of fat and connective tissue, minced, and disrupted by a single passage through a Dounce homogenizer. The tissue suspension above the pestle was withdrawn and washed several times in PBS containing 10 mg/ml bovine hemoglobin and 0.1 M sucrose (PBS/Hb/Sucrose). All solution changes were performed by sedimenting the tissue with a clinical centrifuge and resuspending the loose pellet in fresh solution. Samples were incubated for 12 h with affinity-purified antibody, washed with PBS/Hb/Sucrose, incubated for 12 h with 5-nm gold/goat anti-rabbit antibody (GAR) (Ted Pella Inc., Irvine, CA), washed with PBS/Hb/Sucrose, and prepared for routine thin-section EM. Control samples were incubated only with gold/GAR. In other control experiments, smooth muscle tissues were extracted with 1% Triton X-100 at 4°C for 12 h, washed thoroughly with PBS, and preincubated with the affinity-purified PL antibody. This antibody was then used to immunolabel gently disrupted, unextracted smooth muscle. Labeling of gently disrupted smooth muscle was also performed with PL antibody, which had been preincubated with purified desmin. Desmin was isolated from chicken gizzard (6) and it was generously provided by Dr. W. Ip, University of Cincinnati (Cincinnati, OH).

Enriched membrane fractions, isolated from porcine gastric muscle as previously described (24), were immunogold labeled using preembedding techniques. Briefly, the membranes were adsorbed to nitrocellulose paper (modified from reference 22), incubated with PL antibody for 3 h, washed with PBS/Hb/Sucrose, incubated with gold/GAR, washed again, and then the nitrocellulose paper was prepared for routine thin-section EM.

Results

The gentle disruption procedures used in this study left many areas morphologically intact. In the smooth muscle preparations, although the filament lattice was disturbed such that the interfibrillar spacings were moderately increased, the cytoplasm of virtually all the cells was filled with filaments, and dense bodies were distributed in a normal manner (Fig. 1 *a*). The visceral smooth muscle sample consisted of small bundles of cells with the central cells largely intact and the cells at the edges of the bundles only slightly disrupted. This allowed penetration of the antibodies and the gold/GAR throughout the cytoplasm of the cells at the periphery of the bundle. Although large portions of the sarcolemma (SL) of most cells remained intact, most of the subsarcolemmal tubules of SR appeared to have resealed as vesicles. In areas where both the cytoplasmic membranes and the SL are visible, a relatively uniform covering of gold particles was often observed on the outer surface of the vesicular SR, but the SL was not labeled (Fig. 1, *a* and *b*). In other areas, SR membranes had a much less uniform labeling of portions of the surface. The preparations from vascular smooth muscle tissue (Fig. 1 *c*) were more disrupted, perhaps due to the presence of more connective tissue, but the labeling pattern was identical to that seen with the visceral muscle. In general,

the degree of labeling of the cytoplasmic membranes of smooth muscle cells is less than that observed in similar preparations of canine cardiac muscle (unpublished observations) and it often forms small patches.

A striking feature of these cells is that the outer nuclear envelope is quite heavily labeled, indicating the presence of an abundant PL-like protein (Fig. 1, *a* and *c*). In mildly disrupted cell preparations, where the cells were morphologically intact, labeling of the outer nuclear envelope formed small patches (Fig. 1 *a*). However, in preparations in which the cells were more disrupted, labeling of the outer nuclear envelope appeared to be uniform (Fig. 1 *c*). These different labeling patterns may reflect differences in the degree of penetration of the immunoprobes.

In the smooth muscle fibers we occasionally observed low level labeling of the edges of dense bodies and intermediate filaments. Although the degree of labeling was close to background, we carried out the following studies to test whether the PL polyclonal antibody was specifically cross-reacting with an epitope of an intermediate filament polypeptide. (*a*) We preabsorbed the affinity-purified antibody with either Triton X-100-extracted smooth muscle tissue or with purified desmin and subsequently performed labeling experiments with gently disrupted smooth muscle. In these preparations, almost no gold particles were associated with intermediate filaments or dense bodies. However, the labeling of the cytoplasmic membranes and the outer nuclear envelope was not substantially altered (Figs. 2 and 3). (*b*) We performed an ELISA of the affinity-purified antibody with purified desmin. A low level of binding (17% above background) of the anti-PL polyclonal antibody to this intermediate filament protein was observed but the degree of binding was independent of the antibody concentration, suggesting that it was not specific. (*c*) Western blots of both visceral and vascular canine smooth muscle showed only a single band at ~22 kD in the unboiled state, which characteristically decreased to a lower molecular mass after boiling of the samples in SDS before gel electrophoresis (Fig. 4). These findings suggest that there is no specific cross-reactivity of the PL polyclonal antibody with intermediate filament or any other proteins. In addition, control studies with gold/GAR demonstrated that the label was not retained by either the membranes or the cytoplasmic filaments (data not shown).

Since the cells in these smooth muscle preparations were still largely intact, it was possible that the nonuniform distribution of labeling on the cytoplasmic membranes was a result of uneven penetration of the probes. To investigate this further we used enriched membrane fractions of SL and SR in which access to the antibody binding sites should not be

Figure 1. Disrupted canine smooth muscle. Visceral smooth muscle from ileum (*a* and *b*) and vascular smooth muscle from left common iliac artery (*c*). (*a*) This micrograph contains the peripheral portion of two fibers near the edge of a bundle of cells. The ultrastructure has remained relatively undisturbed by the disruption procedures. The SL of both cells is continuous at the left. The nucleus (*N*) of one of the cells is prominently displayed at the right of the micrograph. Filaments and dense bodies, which comprise the contractile apparatus, fill the majority of the cytoplasm. 5-nm gold particles are associated with the outer nuclear envelope and some of the cytoplasmic membranes (*arrowheads*). (*b*) Portions of two adjacent cells are visible in this micrograph. The extracellular space, containing collagen filaments of the endomysium, runs diagonally down from the top right. The SL of both cells is incomplete, but enough remains intact to determine that it is not labeled. The two subsarcolemmal vesicles have a uniform covering of gold particles on the surface away from the SL. One of the vesicles appears to have periodic densities spanning the gap between the vesiculated cytoplasmic membrane and the SL (*arrow*). These densities closely resemble junctional feet and are strong presumptive evidence that these cytoplasmic membranes are of SR origin. (*c*) This vascular smooth muscle preparation was somewhat more disrupted. The nuclei (*N*) of these disrupted cells characteristically have a heavy, uniform distribution of label on the outer membrane of the nuclear envelope. Bars: (*a*) 230 nm; (*b* and *c*) 270 nm.

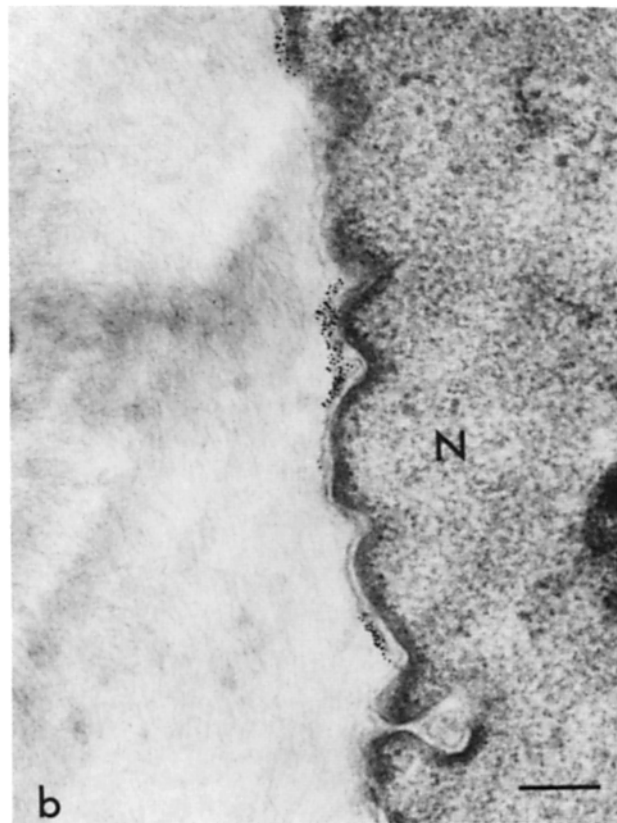
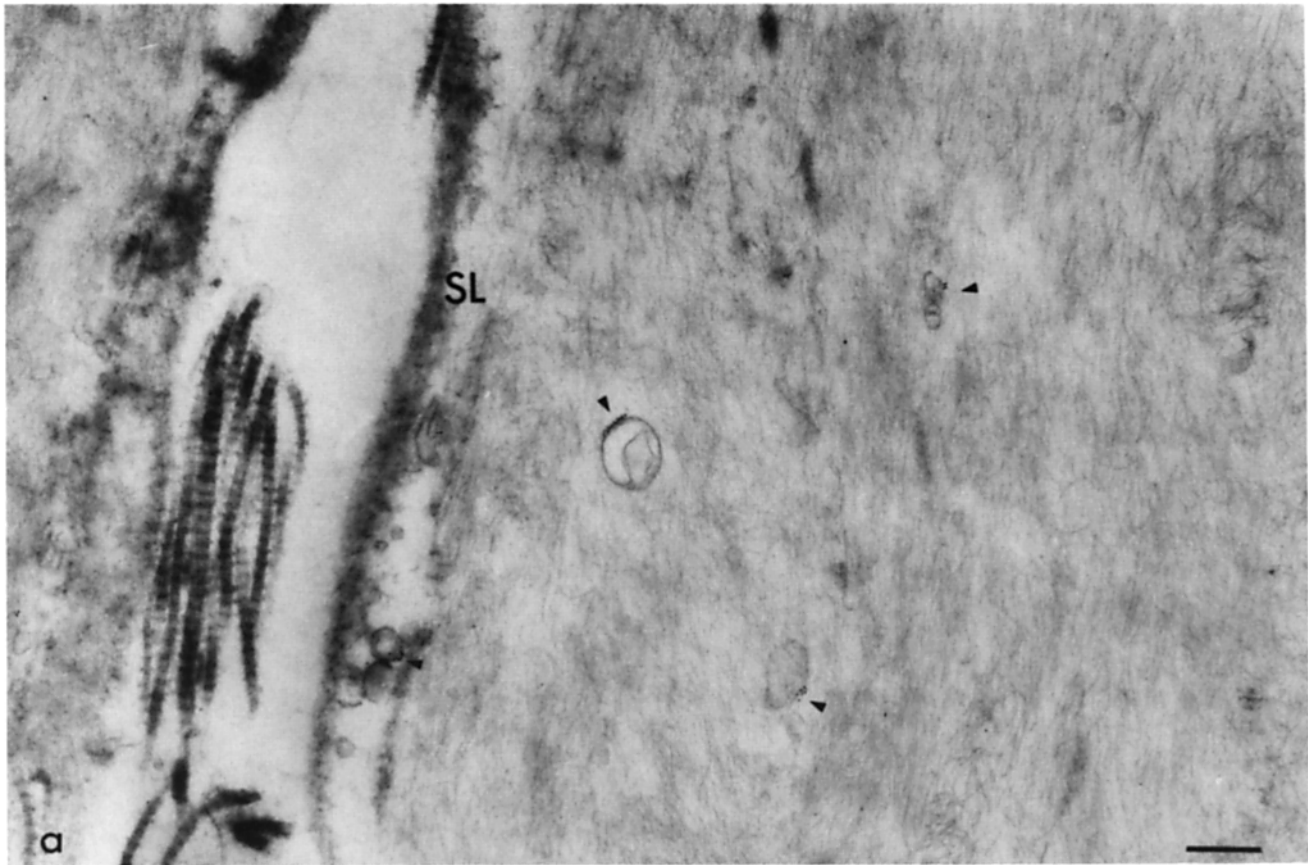


Figure 2. Disrupted porcine coronary arterial smooth muscle. The affinity-purified PL antibody was preincubated with Triton X-100-extracted smooth muscle. This antibody was then used to immunolabel gently disrupted, unextracted smooth muscle. (a) The contractile filaments of the cell depicted in this micrograph appear relatively well preserved. The cytoplasmic membranes are specifically labeled (*arrowheads*), while the SL is not labeled. The background labeling is low. (b) Label is also associated with the outer envelope of the nucleus (*N*) in these preparations. Bars: (a) 300 nm; (b) 250 nm.

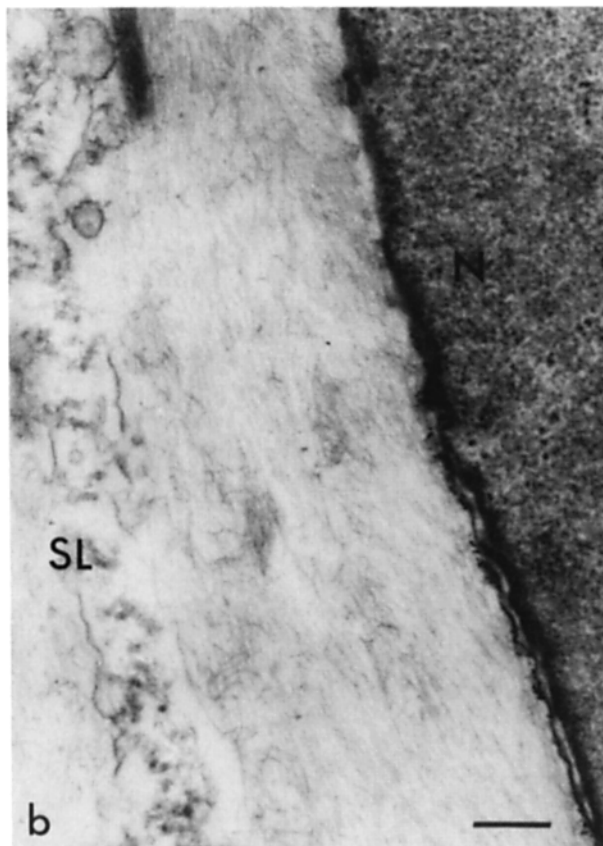
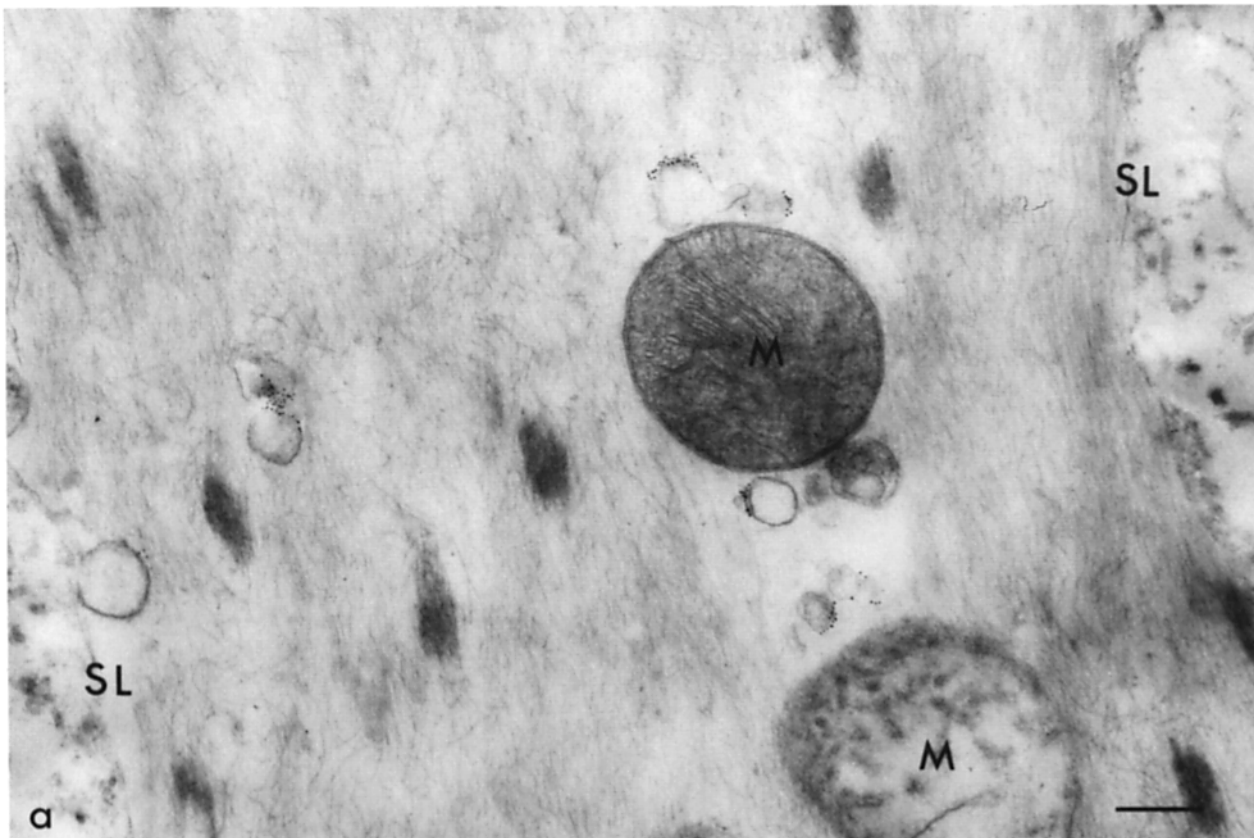


Figure 3. Disrupted canine visceral smooth muscle from ileum. The affinity-purified PL antibody was preincubated with purified desmin. This antibody was then used to immunolabel gently disrupted smooth muscle. (a) Label is associated only with SR membranes. M, mitochondria. (b) The outer nuclear envelope of the nucleus (N) is also labeled. Bars, 310 nm.

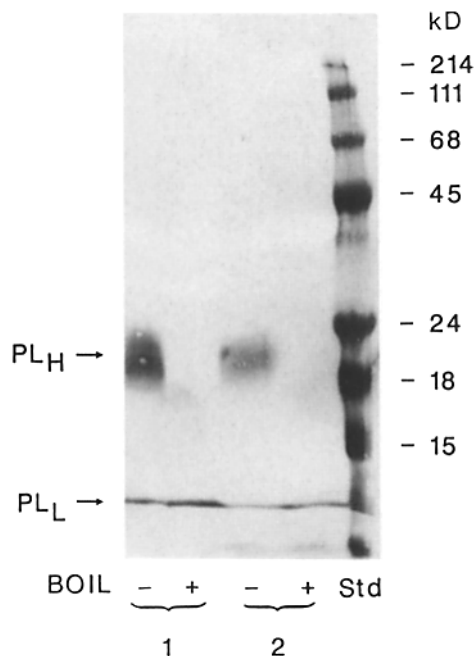


Figure 4. Immunoblot of PL. Canine visceral and vascular tissue extracts were prepared using SDS-PAGE (10%) and the gels were electroblotted onto nitrocellulose. The blot was treated with affinity-purified antibody raised against cardiac PL and developed using protoblot instructions (see Materials and Methods). Some samples were boiled (+) in SDS before gel electrophoresis. (*Std*) Prestained molecular mass standards: myosin (H-chain), 214 kD; phosphorylase b, 111 kD; BSA, 68 kD; ovalbumin, 45 kD; α -chymotrypsinogen, 24 kD; β -lactoglobulin, 18 kD; lysozyme, 15 kD. PL_H , phospholamban high M_r ; PL_L , phospholamban low M_r . Tissue extracts: (1) ileum, (2) left common iliac artery.

restricted by cytoplasmic structures. Enriched SR fractions from porcine gastric smooth muscle retained substantial amounts of label (Fig. 5, *c* and *d*). Some vesicles had a fairly uniform coating of gold particles. However, the label was distributed in patches on the surfaces of most membranes and this pattern was similar to the one observed with tissue preparations (Figs. 1–3). The enriched plasma membrane fraction also contained some vesicles that were labeled (Fig. 5, *a* and *b*). However, most of the vesicles are not labeled and those that have gold particles associated with them exhibit a labeling pattern similar to that seen in the enriched SR samples (Fig. 5, *c* and *d*). The most likely explanation is that these labeled membranes represent contamination of the SL fraction with SR. The degree of labeling is consistent with previous estimates suggesting that the SL fraction contains ~20–30% SR contamination (24). Control samples for both SL and SR fractions treated only with gold/GAR did not exhibit any retention of label (data not shown).

Discussion

In the mildly disrupted preparations used in this study, the major ultrastructural features (e.g., the SL, contractile filaments, mitochondria, and nucleus) were recognizable and retained their positional relationships. The micrographs are representative of the degree of structural preservation typi-

cally obtained using these procedures. In these circumstances, the preembedding immunogold technique was very effective. The improved penetration and washout of the probes greatly enhanced the specific labeling of membranes and the nonspecific or background labeling was virtually eliminated in most areas. Another advantage was the degree of sensitivity offered by this method. In the EM preparations single vesicles and small portions of membrane were examined and it was possible to observe labeling of proteins present in amounts too low to be detected by other procedures, such as Western blots.

Labeling of the SR in smooth muscle tissue appears to form distinct patches while the SR of canine cardiac tissue is uniformly labeled (unpublished data) in agreement with previous observations (8). A similar “patchy” labeling pattern was also observed when SR vesicles isolated from porcine gastric smooth muscle were used. This labeling pattern suggests that there may be a lower surface density of PL in smooth muscle SR than in canine cardiac SR. Previous reports have demonstrated that the membrane proteins are not uniformly dispersed in the SR of slow twitch fibers (4, 26, 28) and reconstituted SR with a high lipid-to-protein ratio (27). Thus, in membranes that have a reduced content of Ca^{2+} -ATPase the protein tends to aggregate leaving protein-free lipid patches (3, 27). The nonuniform distribution of label seen in this study suggests that this may also be the case with PL in the SR of smooth muscle.

It is particularly interesting that the outer nuclear envelope of smooth muscle cells was also labeled with the PL antibody. It remains to be seen whether or not the Ca^{2+} -ATPase is also a constituent of the outer nuclear envelope. However, the presence of a putative Ca^{2+} pump regulator in these membranes and the recently demonstrated high levels of Ca^{2+} in the nuclear region of smooth muscle cells (11, 29) suggest that the nucleus may be capable of controlling intranuclear Ca^{2+} levels.

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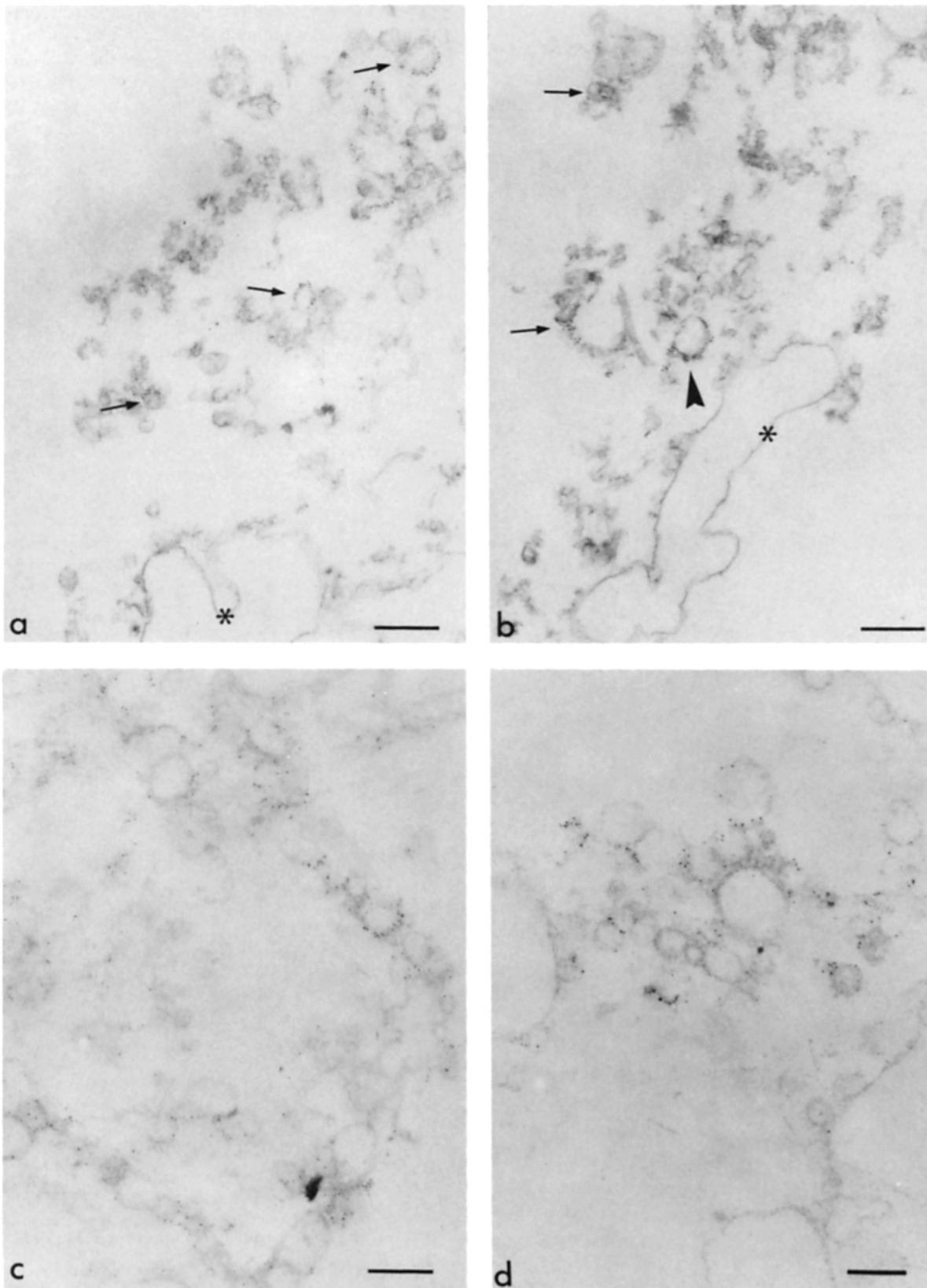


Figure 5. Membrane fractions from porcine gastric smooth muscle. Vesicles have been adsorbed to the surface of nitrocellulose paper and immunolabeled. (a and b) Enriched SL fraction. The micrographs are oriented such that the top surface of the nitrocellulose is just visible as relatively thick, dark strands (*) at the lower right. The vesicles form an irregular band running diagonally down from the top right to the bottom left. Most of the vesicles in this fraction are unlabeled but a few have patches of gold particles associated with them (arrows). One of the vesicles has large dense structures on its surface (arrowhead). These may be either junctional feet or ribosomes and thus this unlabeled portion of the membrane may be junctional SR or rough endoplasmic reticulum. The other vesicles that are labeled are also likely of SR origin. (c and d) Enriched SR fraction. The plane of section is obliquely through the surface so the vesicles are interspersed with nitrocellulose strands in all parts of the figure. Most of the membranes are labeled at a low level. The nonuniform labeling is consistent with the pattern of labeling observed in the disrupted tissue (compare to Fig. 1). Bars: (a and b) 310 nm; (c) 200 nm; and (d) 250 nm.

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