The Endoplasmic-Sarcoplasmic Reticulum of Smooth Muscle: Immunocytochemistry of Vas Deferens Fibers Reveals Specialized Subcompartments Differently Equipped for the Control of Ca^{2+} Homeostasis

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Abstract. Cryosection immunofluorescence and immunogold labeling with antibodies against specific markers were used in rat vas deferens smooth muscle fibers to reveal the molecular arrangement of the endomembrane system (referred to variously in the text as ER or sarcoplasmic reticulum [SR]; S-ER or ER/SR) known to participate in the control of Ca^{2+} homeostasis. The lumenal ER chaperon, immunoglobulin binding protein (BiP), as well as protein disulfide isomerase, and calreticulin, a Ca^{2+} binding protein expressed by most eukaryotic cells, appeared to be evenly distributed throughout the entire system (i.e., within [*a*] the nuclear envelope and the few rough-surfaced cisternae clustered near the nucleus; [*b*] single elements scattered around in the contractile cytoplasm; and [*c*] numerous, heterogeneous, mainly smooth-surfaced elements concentrated in the peripheral cytoplasm, part of which is in close apposition to the plasmalemma). All other structures, including nuclei, mitochondria, Golgi complex, and surface caveolae were unlabeled. An even distribution throughout the endomembrane system appeared also for the proteins recognized by anti-ER membrane antibodies. In contrast, calsequestrin (the protein that in striated muscles is believed to be the main actor of the rapidly exchanging Ca^{2+} storage within the lumen of the sarcoplasmic reticulum) was found preferentially clustered at discrete lumenal sites, most often within peripheral smooth-surfaced elements of moderate electron density. Within these elements dual labeling revealed intermixing of calsequestrin with the other lumenal ER proteins. Moreover, the calsequestrin-rich elements were enriched also in the receptor for inositol 1,4,5-trisphosphate, the second messenger that induces Ca^{2+} release from intracellular stores. These results document the previously hypothesized molecular heterogeneity of the smooth muscle endomembrane system, particularly in relation to the rapid storage and release of Ca^{2+}.

CONTROL of Ca^{2+} homeostasis is among the basic functions of eukaryotic cells, yet the intracellular structures that participate in the venture appear to vary considerably in both size and molecular organization. At the moment, detailed knowledge exists only for the striated fibers of both skeletal and cardiac muscles. These cells contain the sarcoplasmic reticulum (SR)[1], a highly developed, smooth-surfaced endomembrane system fully described at the ultrastructural level (Porter and Palade, 1957). The SR is composed by longitudinal tubules and fenestrated collars arranged as a water jacket around myofibrils, in direct continuity with the terminal cisternae. These latter structures are attached, via specialized feet composed of Ca^{2+} channels, the ryanodine receptors, with the transverse tubules of the sarcolemma to yield the triads and diads. This organization enables the SR to synchronously release Ca^{2+} to the contractile apparatus and to reaccumulate it quickly in each contraction–relaxation cycle (Fleischer and Inui, 1989). Based on the observation of membrane continuities during development (Porter and Palade, 1957; Ezerman and Ishikawa, 1967), the striated muscle SR was proposed as a specialized region of the ER, a hypothesis recently strengthened by the identification of common molecular markers (Fliegel et al., 1990; Michalak et al., 1992; Volpe et al., 1992). Within adult striated muscle fibers the few rough-surfaced ER cisternae are concentrated at one nuclear pole, in the proximity of the Golgi complex, while the SR pervades the contractile cytoplasm.

Knowledge of smooth muscle fibers is by far less detailed.

1. *Abbreviations used in this paper*: Ab, antibody; BiP, immunoglobulin binding protein; CR, calreticulum; CSQ, calsequestrin; Ins-P_3, inositol 1,4,5-triphosphate; pAb, polyclonal Ab; PDI, protein disulfide isomerase; SR, sarcoplasmic reticulum.

Although in these cells the role of Ca^{2+} stores is unquestioned (see Somlyo et al., 1988; Somlyo and Himpens, 1989; Endo et al., 1990; Chen and van Breemen, 1992), their identification is still debated. EM studies revealed an array of heterogeneous, membrane-bound elements concentrated primarily around the nucleus and beneath the plasmalemma, quantitatively variable in the fibers of different organs (from 1.8 to 5.1% of the cytoplasmic volume; Devine et al., 1972; Somlyo, 1980; Somlyo and Franzini-Armstrong, 1985). Rough-surfaced cisternae, predominant around the nucleus, also appear scattered throughout the cytoplasm and near the surface, in the proximity of, and often in obvious lumenal continuity with smooth-surfaced elements. The latter structures predominate in the peripheral rim of the cytoplasm, where part of them run in close apposition to the plasmalemma.

So far, the nomenclature used to designate these structures as a whole has been variable. In various laboratories the entire system has in fact been referred to as the SR (Devine et al., 1972; Forbes et al., 1979; Chen and van Breemen, 1992); in some others as the ER (Casteels et al., 1986; Raymaekers and Jones, 1986), while others have combined the two names and called the entire system the sarco(endo)-plasmic reticulum (S-ER, see Lytton et al., 1989) or defined the rough-surfaced portion as the ER and the smooth-surfaced as the ER/SR (Somlyo and Himpens, 1989). Because of these uncertainties, throughout this article we will use an even more vague nomenclature, i.e., the endomembrane system.

In the past, molecular and functional heterogeneity of the smooth muscle fiber endomembrane system was repeatedly hypothesized (see Devine et al., 1972; Somlyo and Franzini-Armstrong, 1985; Endo et al., 1990) but not yet investigated in detail. Highly specific antibodies (Abs) directed against either ER or striated muscle SR markers expressed also in the smooth muscle have now made these studies possible. Here we report immunocytochemical results obtained in the rat vas deferens fibers, chosen because of their rich endowment with two key components of intracellular Ca^{2+} control: the receptor for the Ca^{2+} releasing second messenger, inositol 1,4,5-triphosphate (Ins-P_3) (Mourey et al., 1990) and lumenal Ca^{2+} storage proteins of the calsequestrin (CSQ) family.

Materials and Methods

Materials

Characterization of most Abs used for the present study was reported in detail elsewhere. The two polyclonal Abs (pAbs) against the skeletal muscle CSQ, raised in a rabbit injected with the chicken protein and in a chicken with the rabbit protein, were affinity purified as described in Volpe et al., (1988 and 1990, respectively). The pAb against the dog heart CSQ was raised in a mouse by injection of the corresponding band cut out of the SDS-PAGE gel (Biral et al., 1992). The affinity-purified rabbit pAb against the rat cerebellar Ins-P_3 receptor was described by Peng et al. (1991); the rat mAb against BiP by Bole et al. (1986); the rabbit pAb against calreticulin (CR) by Perrin et al. (1991); the rabbit anti-microsomal membrane serum by Louvard et al. (1983). The rabbit pAb against the bovine liver protein disulfide isomerase (PDI) was raised by Dr. S. Fuller. Rhodamine- and fluoresceine-labeled donkey IgGs against rabbit and rat IgGs as well as rhodamine-labeled sheep IgGs against the same antigens, unlabeled goat Fab fragments and goat serum were purchased from Technogenetics (Milan, Italy). Colloidal gold particles (5 and 15 nm), coated with goat IgGs against either rabbit (large and small particles) or rat (small particles only) IgGs, were purchased from Biocell (Cardiff, UK). Before each labeling experiment the colloidal gold preparations were carefully checked in the electron microscope for adherence to specifications in terms of both size uniformity and absence of aggregates. Only the preparations exhibiting >95% of single particles and no aggregates larger than two particles were used in the present work. ^{125}I-protein A was purchased from Amity-Amersham (Milan, Italy). Other chemicals were analytical or the highest grade available.

Subcellular Fractionation

Male Sprague Dawley rats were sacrificed by decapitation, and their vasa deferentes were rapidly collected. Homogenates were prepared in 10 vol of 0.32 M sucrose from pools of 10 finely minced organs by 30 strokes in a Dounce-type homogenizer. Microsomes were isolated from homogenates first centrifuged at 10,000 rpm for 10 min (Spinco 60Ti rotor; Beckman Instruments, Inc., Fullerton, CA). The collected supernatants were spun at 60,000 rpm for 60 min, and the microsomal pellets resuspended to a final protein concentration of ~3 mg protein/ml.

SDS-PAGE and Western Blotting

Protein was determined by the BCA procedure (Pierce, Rockford, IL). SDS-PAGE was carried out as described by Volpe et al. (1992). Transfer of proteins onto nitrocellulose membranes (blots) was carried out at 220 mA for 16–18 h in a buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol. Staining the gels by the Stains-All procedure and labeling the blots with ^{45}Ca (overlay) were as by Volpe et al. (1988).

Western blots were processed (60-min incubations at room temperature) first with PBS containing 5% dry, defatted milk, then with Abs dissolved in PBS containing 1% BSA and 0.05% Tween 20. After washing four times for 10 min with PBS, 5% milk and 0.05% Tween 20, bound Abs were revealed with ^{125}I protein A in the same solution. In the case of BiP (recognized by a rat mAb) this treatment was preceded by an incubation with rabbit anti-(rat Ig) IgGs (60 min, room temperature). For further details see Volpe et al. (1992). Densitometry of the relevant antigen bands of immunoblots was carried out using a Molecular Dynamics Imagequant apparatus chromatoscanner CS-380.

Conventional and Immuno Microscopy

Small transverse vas deferens sections, rapidly cut with razor blades, were immersed at 4°C in a mixture of 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.25% glutaraldehyde in 125 mM phosphate buffer, pH 7.4. 2 h later the samples for conventional EM were washed extensively with the phosphate buffer, postfixed with 1% OsO4 in 125 mM cacodylate buffer, dehydrated in ethanol, block stained with uranyl acetate and embedded in Epon. Thin sections were doubly stained with uranyl acetate and lead citrate.

The samples for cryosections were infiltrated with concentrated sucrose, frozen in a 3:1 (vol/vol) mixture of propane and cyclopentane cooled with liquid nitrogen, and transferred to an Ultracut ultramicrotome equipped with a FC_4 cryosection apparatus (Reichert Jung, Vienna, Austria). The sections for immunofluorescence (~1 μm thick) were flattened over glass slides and covered with 2% liquid gelatin in 125 mM Na phosphate buffer, pH 7.4. After a short treatment with 1% Na borohydrate (to eliminate glutaraldehyde fluorescence) they were washed and exposed for 30 min to a solution containing 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer, pH 7.4. After washing, the sections were exposed (1 h at 37°C or overnight at 4°C) to either one of the various primary Abs diluted in the above Triton X-100 and goat serum–containing solution. They were then washed thoroughly and treated with the appropriate rhodamine-labeled sheep Abs (1:20–1:40 in the Triton X-100, goat serum solution, 30–60 min, 37°C), washed again and mounted in glycerol to be examined in a Zeiss Photomicroscope III apparatus (Carl Zeiss, Inc., Oberkochen, Germany). Controls in which the primary Ab was either omitted or replaced by preimmune or nonimmune IgGs yielded completely negative results.

For dual labeling, the sections reacted with the rhodamine-labeled Abs as described above were saturated with sheep IgG Fab fragments against rabbit IgGs (20 μg/ml, 30 min, room temperature), then washed thoroughly and exposed to anti-CR Abs (1 h, diluted in the buffer used before). After another wash, the sections were exposed to fluoresceine-labeled donkey Abs (1 h, 37°C, buffer and concentrations as for the rhodamine labeling), then washed, mounted in glycerol, and examined as described above. During sample observation, dual photographs of the same field were taken, first for fluoresceine and then for rhodamine. With the narrow-band fluorescence

filters used omission of either the first or the second primary Ab, or their substitution with nonimmune IgGs, resulted in the lack of appreciable signal for rhodamine or fluoresceine, respectively. These results exclude not only cross-reactivity of the various primary Abs, but also cross-labeling of the secondary Abs, spill-over of the signals across the filters and thus bleed-through artifacts.

For immunogold labeling, ultrathin cryosections (50–100 nm thick) were collected over nickel grids and covered with 2% gelatin. After treatment with 125 mM Na phosphate buffer, pH 7.4, supplemented with 0.1 M glycine, they were exposed for 1 h at 37°C to the first Ab diluted in phosphate–glycine buffer, then washed with the buffer and decorated with anti-IgG–coated gold particles (5 or 15 nm, dilution 1:80 in the same buffer). For dual labeling the sections were exposed in sequence to the two Abs followed by the corresponding gold particles. In all cases the anti-CSQ Ab and the large gold particles were applied second. For details about washes and quenching (with goat Ig Fab fragments against rabbit IgGs) see Volpe et al. (1991). The immunodecorated grids were then processed as recommended by Keller et al. (1984). Both conventional sections and cryosections were examined in a Hitachi H-7000 electron microscope. Pictures were usually taken at a magnification of 24,000.

With nonimmune serum, the 5-nm gold particle labeling over the nucleus and cytoplasm of smooth muscle fibers and adjacent cells was very low and uniformly distributed. The average labeling, calculated under standard conditions in a group of randomly chosen pictures, i.e., the background (see Villa et al., 1991), was 2.2 gold particles/μm^2. With 15-nm particles background values were slightly higher (5.3/μm^2). When the specific Abs were used, labeling over the cell structures devoid of the antigen, such as nuclei and mitochondria, was not significantly different from the background.

Results

Western Blotting

The specificity of the Abs used in the present work was analyzed by Western blotting of rat vas deferens homogenate and microsomal proteins. Fig. 1 illustrates part of the results we obtained with microsomes. The two bands, one major, the other minor, revealed by the pAb raised against the chicken muscle CSQ (Fig. 1, lane *a*, 55 and 63 kD) comigrated with the dog heart and rabbit skeletal muscle protein standards, respectively (not shown). An identical pattern, with the two, major and minor bands, was obtained with the anti–dog skeletal muscle CSQ pAb raised in chicken (not shown). The major CSQ band of 55 kD was also recognized by the anti–rabbit heart CSQ pAb (Fig. 1, lane *b*), while the minor, muscle-type band was hardly visible, as expected (Fliegel et al., 1987; Scott et al., 1988). This Ab also recognized another, unidentified band of the vas deferens microsome pattern (~66 kD) and therefore was not used for immunocytochemistry. Fig. 1, lane *c* illustrates the 60-kD band recognized by the anti–rat liver CR pAb. This band comigrated with the upper component of the CR doublet purified by Treves et al. (1990) from the rat liver. The subsequent two lanes, *d* and *e*, illustrate the two ER lumenal proteins, BiP and PDI. Abs against these two proteins labeled single bands at 78 and 55 kD, respectively. Fig. 1, lane *f* shows the four microsomal marker bands recognized by the rabbit anti-ER membrane pAb of Louvard et al. (1982) at ~91, 62, 58, and 29 kD, respectively, while Fig. 1, lane *g* shows the high M_r Ins-P_3 receptor (260 kD). When total homogenates were analyzed, no additional labeled bands were revealed with respect to microsomes (not shown).

The identification of the major SDS-PAGE band labeled by anti-CSQ Abs as a member of the Ca^{2+} binding protein family expressed by striated muscles was further strengthened by its metachromatic blue staining with the Stains-All procedure and by the Ca^{2+} binding of the corresponding

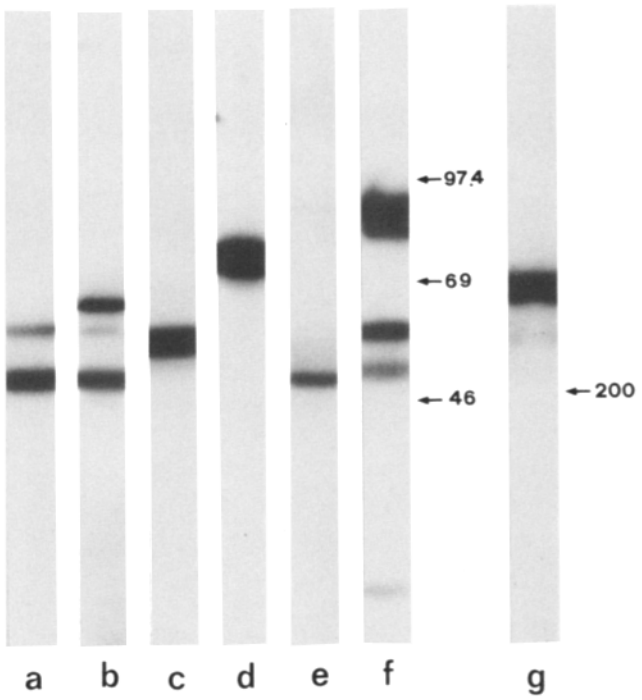


Figure 1. Western blots of rat vas deferens microsomes decorated with Abs against ER and SR markers. 100 μg of microsomal proteins were resolved on either 10 (lanes *a–f*) or 6% (*g*) SDS-PAGE slabs, then transferred to nitrocellulose. Parallel blots were challenged with Abs against chicken skeletal muscle CSQ (*a*), dog heart CSQ (*b*), rat liver CR (*c*), plasma cell BiP (*d*), bovine liver PDI (*e*), pancreatic microsomal membrane proteins (*f*), and rat cerebellar Ins-P_3 receptor (*g*). Bound Abs were reacted with ^{125}I-protein A and revealed by radioautography.

blot band revealed by the ^{45}Ca overlay technique (not shown). Expression of CSQ and CR in the vas deferens was quantitated by microdensitometry, making reference to purified protein standards (Volpe et al., 1990; Treves et al., 1990), scales of which were run and immunodecorated in parallel to the microsomal samples. The calculated values for microsomes are the following: CSQ, 12 and CR, 32 μg/mg total protein.

Immunofluorescence

These experiments were carried out with the various Abs (for CSQ with either one of the Abs raised against the skeletal muscle protein) using 1-μm-thick cryosections of the vas deferens smooth muscle. Considerable heterogeneity in the labeling intensity was observed, even among adjacent fibers (see Fig. 2). The pattern of cross sections was punctate in all cases, often with stronger and more consistent labeling near the cell surface. In longitudinal sections puncta were accompanied by apparently semi-continuous, irregular short lines. Comparison of the samples decorated with the various primary Abs used revealed similar patterns of labeling at the cell periphery. In the central areas, however, especially in the proximity of the nucleus, the patterns diverged. With Abs against CR (Fig. 2 *A*), BiP, PDI, and ER membranes (not shown) single or multiple, intensely fluorescent dots were often observed. In contrast, with anti-CSQ and Ins-P_3 recep-

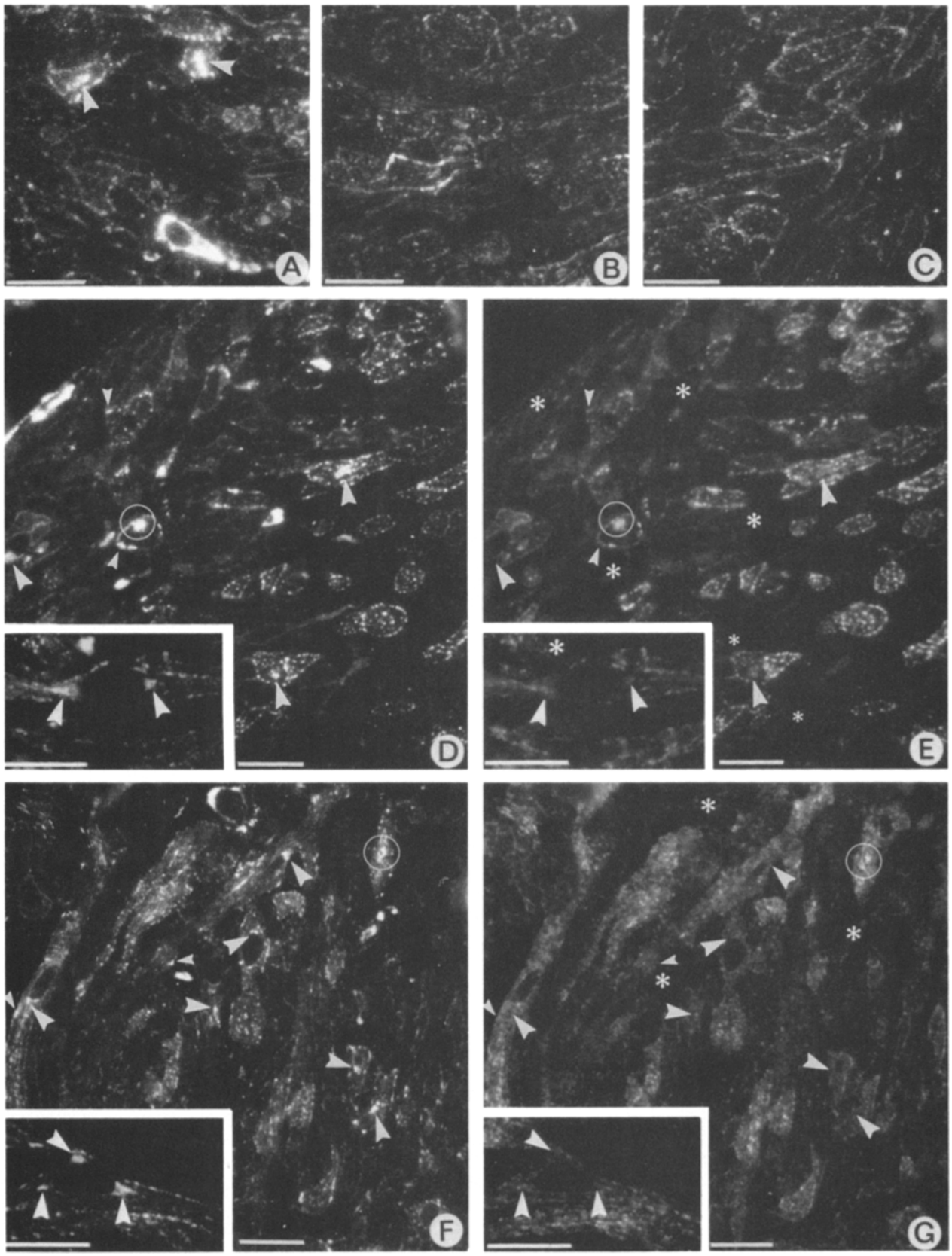


Figure 2. Immunofluorescence images of 1-μm-thick vas deferens cryosections decorated for CR (*A*, *D*, and *F*), CSQ (*B* and *E*) or IP_3 receptor (*C* and *G*). *A–C* are single labeled. Notice that the fibers shown are all similarly labeled at their periphery by numerous puncta. Within the fibers, however, the CSQ and Ins-P_3 receptor labeling (*B* and *C*) is often weaker and remains punctate, whereas with CR (*A*) larger, intensely fluorescent dots appear in two fibers (*arrowhead*). The intensely fluorescent cell visible in the lower portion of *A* is not a smooth muscle fiber but a fibroblast. *D–G* illustrate the results obtained with individual sections dually labeled for CR (fluoresceine,

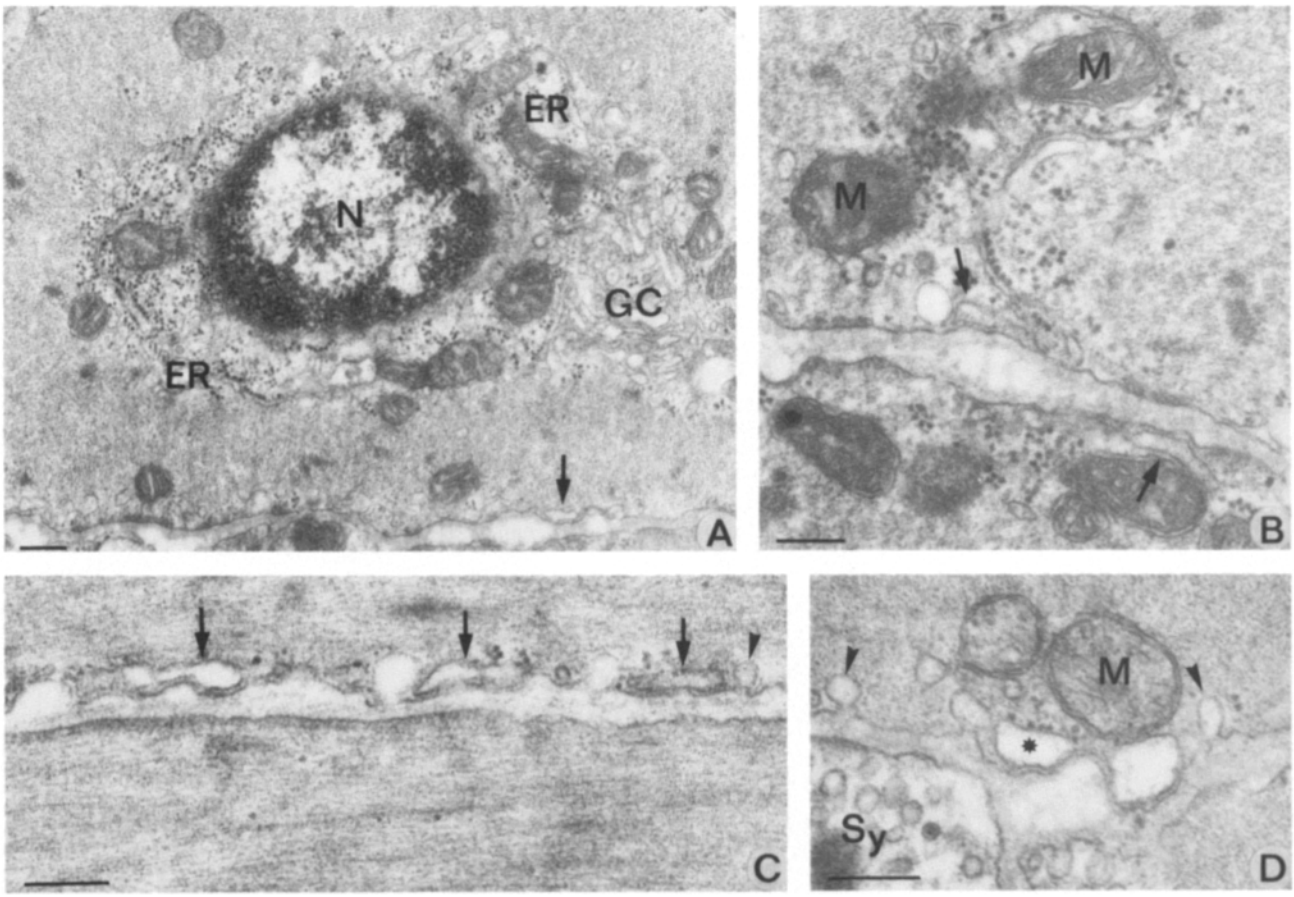


Figure 3. Thin sections of Epon-embedded vas deferens smooth muscle fibers. *A* illustrates the distribution of the Golgi complex (*GC*) and numerous rough-surfaced ER cisternae around the nucleus, in the central area of the fiber. A few additional cisternae, rough as well as smooth surfaced, are scattered throughout the cytoplasm and concentrated in the proximity of the plasmamembrane. Details of the subplasmalemma region are shown in *B–D*. Notice in particular the plasmalemma-coupled cisternae in *C* (*arrows*); the cisternae covered with ribosomes on only one face in *B* and *D*. Such a cisterna in *D* is swollen and apparently coupled to the plasmalemma (*asterisk*); the caveolae (*arrowheads*) in *D*. In the latter panel, the structure labeled *Sy* is a presynaptic terminal. *M*, mitochondrion; *N*, nucleus. Bars, 0.25 μm.

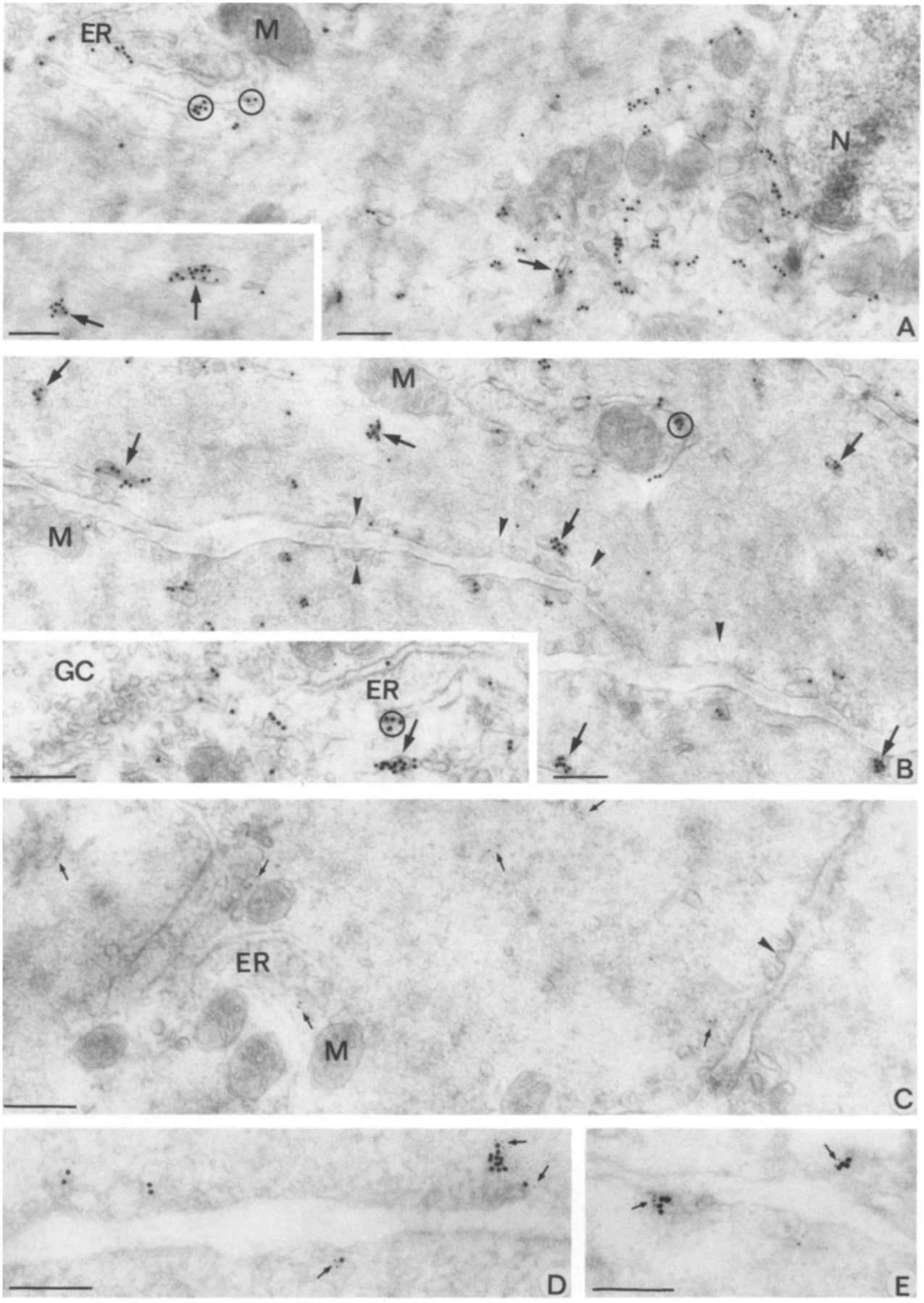
tor Abs the central areas were usually less fluorescent than the periphery of the fibers (Fig. 2, *B* and *C*).

To further reveal the observed differences among the labeling patterns of the various Abs, dual fluoresceine–rhodamine labeling of individual sections was carried out, using in all experiments the fluoresceine-labeled CR for reference (see Fig. 2, *D* and *F*). With the ER markers (BiP, PDI, and ER membrane proteins, not shown) no appreciable differences with respect to CR, but precise matching of both peripheral puncta and lines and perinuclear dots was observed. In contrast, with anti-CSQ (Fig. 2 *E*) and -Ins-P_3 receptor (Fig. 2 *G*) Abs, the CR-positive perinuclear dots were usually either less fluorescent or inappreciable. At the cell periphery most of the puncta appeared colabeled for both CR-CSQ and CR–Ins-P_3 receptor, and only a few were positive for CR only. Scattered among the smooth muscle fibers a small population of cells, recognized as fibroblasts by phase-contrast microscopy (not shown), exhibited a peculiar labeling pattern: strong for CR and the ER markers, very weak for the Ins-P_3 receptor and completely negative for CS (Fig. 2, *D–G*).

Conventional EM

Thin sections of Epon-embedded vas deferens and other smooth muscles were already described in previous studies (see Devine et al., 1971, 1972; Forbes et al., 1979; Somlyo, 1980; Somlyo and Franzini-Armstrong, 1985). The few im-

D and *F*) and CSQ or Ins-P_3 receptor (rhodamine, *E* and *G*, respectively). The main immunolabeling differences between the twin panels concern the perinuclear area, where intensely fluorescent dots of the CR pattern correspond to moderate or low fluorescence areas in the CSQ and IP_3 receptor patterns (*large white arrowheads*). The few perinuclear dots fluorescent both in the CR and (at least in part) in the CSQ or IP_3 receptor images are circled. In the subplasmalemma area the labeling patterns observed in the double-labeled sections are similar, i.e., most puncta appear intensely positive for both CR and CSQ or Ins-P_3 receptor. Discrepancies are only a few, part of which are marked by small white arrowheads. Asterisks in *E* and *G* label fibroblasts, cells interposed between with the smooth muscle fibers, which react positively for CR and appear negative for CSQ and IP_3 receptor. Bars, 10 μm.



ages of Fig. 3 are shown to help the understanding of the immunogold images of the following figures. Please note the predominance of rough-surfaced cisternae in the perinuclear area (Fig. 3 *A*), adjacent to the Golgi complex. Smaller rough-surfaced cisternae were seen distributed, apparently at random, throughout the cytoplasm. At the cell periphery cisternae were often aligned parallel to, and at a constant distance (~25 nm) from the plasmalemma (coupled cisternae, Fig. 3, *A–D*). Other cisternae did run perpendicular or obliquely to the cell surface (Fig. 3 *B*). Many of these peripheral cisternal profiles appeared smooth surfaced, others however were covered with ribosomes, often irregularly or over only one of their surfaces (Fig. 3, *B* and *D*). Caveolae were visible in most fields (Fig. 3, *B–D*), however apparently less numerous than in the fibers of different smooth muscles (Devine et al., 1972; Somlyo, 1980). The other cell organelles were as previously described (see Devine et al., 1972; Somlyo, 1980).

Immunogold

Labeling of ultrathin cryosections with gold particles of two sizes (5 and 15 nm) addressed to the various antigens revealed peculiar distribution patterns. Fig. 4, *A* and *B*, illustrates the distribution of CSQ in both the peripheral and central cytoplasm. The immunolabeling was particularly strong over small structures with ovoidal or irregularly elongated profile, often characterized by a moderately dense content (Fig. 4, *A, B,* and *insets*). In contrast, much less intensely labeled, or completely unlabeled, were the long and narrow cisternae, most often localized near the nucleus (compare Figs. 4 *A* and 3 *A*) but present also in the rest of the cytoplasm, that correspond to the rough-surfaced ER profiles visible in the Epon-embedded preparations. Expansions in apparent, direct membrane continuity with these cisternae, or discrete sites of the cisternal segregated lumen, both characterized by moderately dense content, could however be intensely immunolabeled for CSQ (Fig. 4 *A*). In contrast, the plasma membrane caveolae (recognized by their direct surface opening) and the nuclear envelope were consistently negative (Fig. 4, *A* and *B*). Also negative were the cisternae and most of the vesicles of the Golgi complex area (Fig. 4 *B, inset*) as well as the fibroblasts scattered around in the tissue (not shown).

Among the other Abs used for immunogold labeling, only that specific for the Ins-P_3 receptor yielded results similar to CSQ. In spite of the low labeling obtained with this Ab (approximately threefold the background), a clear general picture did emerge (Fig. 4, *C–E*). The labeling was in fact higher over a population of relatively small, often moderately dense profiles, that dual labeling revealed to be rich in CSQ. In these structures the immunogold particles addressed to the Ins-P_3 receptor were often aligned around the CSQ signal, as expected for a membrane and a content antigen, respectively (Fig. 4, *D* and *E*). Labeling for the Ins-P_3 receptor of structures negative or poor of CSQ, including the plasmalemma surface caveolae, the Golgi complex and the long, probably rough-surfaced ER cisternae, was not above background levels (Fig. 4 *C* and not shown). Apparently negative were also the fibroblasts (not shown).

Compared with CSQ and the Ins-P_3 receptor, the immunogold patterns revealed with the other investigated Abs (against CR, Fig. 5 *A–D*; and against the typical ER markers: PDI, Fig. 5, *E–H*, and BiP, not shown) appeared more largely and uniformly distributed to the endomembrane system. No consistent differences in labeling density were observed between the cisternae localized near the surface or towards the center of the fibers, no matter how long and apparently rough-surfaced (Fig. 5 *C*); moderately dense-cored and rich of CSQ, revealed by dual labeling (Fig. 5, *D* and *F–H*); and flat and coupled to the plasmalemma (Fig. 5, *A, E,* and *G*). Also, the nuclear envelope was most often positive for these markers (Fig. 5 *A* and not shown). Within the CSQ-rich cisternae the signal for the latter protein and those for either CR or the ER content markers, PDI and BiP, were at least partially intermixed (Figs. 5, *D–H* and not shown). In contrast, the Golgi complex (not shown), surface caveolae (Fig. 5, *A, E,* and *F*) and other structures (nuclei, mitochondria) were consistently negative. Fibroblasts were heavily labeled, however only in their well developed ER cisternal system (Fig. 5 *B*).

To further confirm the differences in the distribution of the various lumenal markers discussed so far, their labeling ratios in the superficial and perinuclear ER cisternae were established by direct particle counting in randomly selected, double-labeled pictures (see Satoh et al., 1990). The results obtained (Table I) confirm the subjective conclusions reported above. The ratio of CSQ to both CR and PDI was in fact much (4–5-fold) higher in the peripheral than in the perinuclear cisternae.

Discussion

In spite of the numerous studies carried out during the last three decades, the endomembrane system of smooth muscle fibers has not yet achieved a precise cell biological definition. Similar to the SR of striated muscles the system is known to play a key role in the regulation of the Ca^{2+} uptake and release events that underlie the relaxation–contraction cycle. Its general architecture is however different from that of the striated muscle SR, which is arranged orderly and

Figure 4. Ultrathin cryosections immunolabeled for CSQ (*A* and *B*), Ins-P_3 receptor (*C*) and the two antigens together (*D* and *E*). The CSQ-labeling of the images shown in this and the following figures (large gold) have been all obtained with the rabbit anti-chicken Ab of Volpe et al. (1990). Immunolabeling is concentrated over membrane-bound elements containing moderately dense material (*arrows*), some apparently discrete (*A* and *B* and *insets*), others in apparent direct continuity with typical ER cisternae (*circles* in *A* and *inset B*). These CSQ-rich elements are distributed preferentially near the plasmalemma (*B*) and present also in the deep cytoplasm. The distribution of the Ins-P_3 receptor (small gold, *small arrows*, *C–E*) resembles that of CSQ. In the double-labeled images (*D* and *E*) the Ins-P_3 receptor labeling lies around the subplasmalemma cisternae, near the sites corresponding to the limiting membrane, whereas the CSQ labeling (large gold) is located over the cisternal content. Caveolae are indicated by arrowheads. Other symbols as stated in Fig. 3. Bars, 0.25 μm.

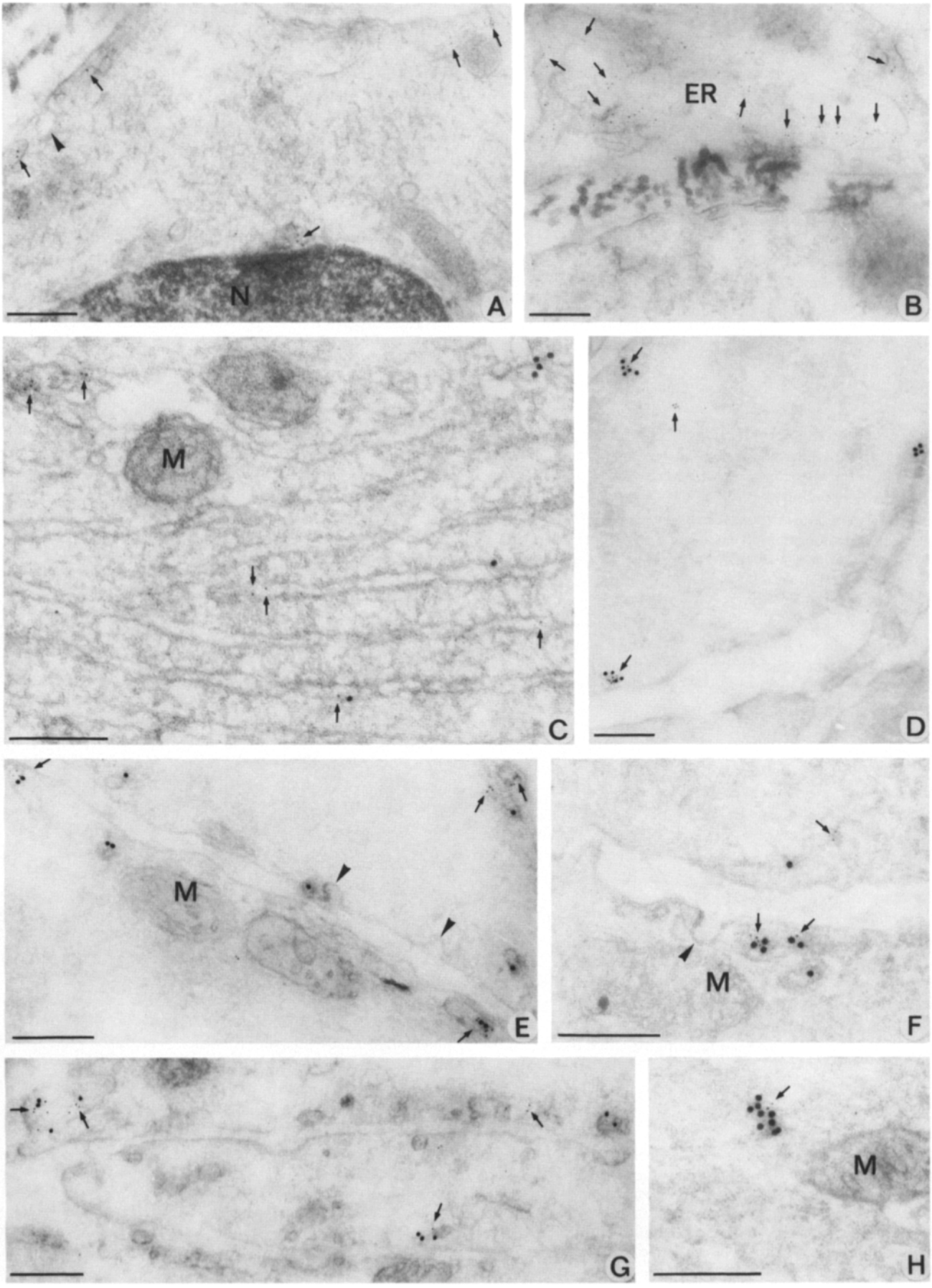


Table I. CR and PDI to CSQ Immunolabeling Ratios in the Perinuclear (Mostly Rough Surfaced) and Subplasmalemma Cisternae of the Endomembrane System

	CSQ/CR	CSQ/PDI
Central cisternae	1.28 ± 0.23	0.43 ± 0.10
Peripheral cisternae	5.26 ± 0.55	2.13 ± 0.59

Results (averages ± SD) were obtained from direct countings of random pictures (37–50/group) taken from cryosections double labeled under standard conditions.

coordinately with the contractile apparatus. The smooth muscle system is in contrast composed by a wealth of elements, heterogeneous already at the level of conventional EM, distributed primarily in the proximity of the nucleus and the plasmalemma.

In the present work, we have initiated a molecular characterization of these various components, using immunocytochemistry at the light and electron microscope level. Our experimental model has been the smooth fiber of the vas deferens, chosen because of its appreciable expression of two main components of Ca^{2+} stores, the Ins-P_3 receptor (Mourey et al., 1990), and CSQ. Of the latter, two isoforms have been detected, the major comigrating with the heart, the minor with the skeletal muscle protein.

Admittedly, the markers that we have investigated do not exhaust the list of proteins involved in the control of Ca^{2+} homeostasis. The Ca^{2+} ATPase of smooth muscle fibers is however of the so-called SERCA 2b, housekeeping type (Lytton et al., 1989), that appears to be widely distributed throughout the entire ER (see Villa et al., 1991, 1992). As far as the ryanodine receptor, periodic transverse bridges, similar to those described in striated muscles, were shown to be concentrated in the faces of the smooth muscle cisternae directly coupled to the plasmalemma (Somlyo and Franzini-Armstrong, 1985). Since in thin sections and ultrathin cryosections of vas deferens tissue (in contrast to our previous results in skeletal muscle and Purkinje neurons) (Volpe et al., 1992; Satoh et al., 1990) bridges were not easily distinguished, they will not be further discussed in this work.

Specialization in the Endomembrane System

The two well known general markers of the ER lumen, PDI and BiP (Sitia and Meldolesi, 1992), investigated here for the first time in smooth muscle fibers, appeared distributed at a similar concentration to all types of elements of the system and excluded from the other structures, including the Golgi complex and surface caveolae. Previous studies in other cell types had revealed concentration within specialized ER areas of another lumenal protein, CR (Treves et al., 1990; Arber et al., 1992; see, however, Peter et al., 1992; Michalak et al., 1992). In our smooth muscle fibers a discrete distribution within the lumen was observed, however, not for CR which appeared widely distributed, but for CSQ. The latter therefore diverged from the other lumenal proteins because of its concentration within smooth-surfaced elements, most often localized in the peripheral cytoplasm, characterized by the moderately higher electron density of their content. Such a distribution was not exclusive since CSQ was detected, although with low concentration and irregular distribution, also within the rest of the system, including the rough-surfaced cisternae. This is at variance with striated muscles, where CSQ is restricted almost exclusively to the specialized SR terminal (in the heart also the corbular) cisternae (see Campbell, 1986). The striated muscle CSQ does not include the KDEL COOH-terminal sequence (Fliegel et al., 1987; Scott et al., 1988) and therefore its localization does not depend on the retrieval KDEL receptor, used by BiP, PDI, and CR to accumulate within the ER lumen (Pelham et al., 1990). CSQ seems rather trapped by its interaction with specific membrane proteins (Franzini-Armstrong et al., 1987; MacLennan and Phillips, 1992), a mechanism that might account also for the preferential localization we have observed in smooth muscle fibers.

The four lumenal proteins discussed so far—BiP, PDI, CR and CSQ—are all known to bind Ca^{2+} with moderate affinity (K_d ~1 mM) (Campbell, 1986; Macer and Koch, 1988; Michalak et al., 1992) as expected within a Ca^{2+} store. The Ca^{2+} capacity of these proteins, i.e., the number of Ca^{2+} moles bound per mole, is particularly high for CSQ and CR (~50 and 25–50, respectively) (Campbell, 1966; Michalak et al., 1992). Because of the wide distribution of CR together with BiP and PDI, the entire endomembrane system appears therefore competent for Ca^{2+} storage. However, in the specialized elements rich in CSQ the combination of the latter with the other Ca^{2+} binding proteins can only result in a marked increase of the Ca^{2+} storage capacity. Moreover, the colocalization of the Ins-P_3 receptors, revealed by dual labeling, strongly suggests the CSQ-rich elements correspond to rapidly exchanging Ca^{2+} stores sensitive to the second messenger. Our results, which in essence resemble those recently reported in chicken Purkinje neurons (Villa et al., 1991; Volpe et al., 1991; Takei et al., 1992), document the heterogeneity of the smooth muscle fiber endomembrane system in relation to Ca^{2+} homeostasis. This possibility had been previously hypothesized based on both ultrastructural data (Devine et al., 1972; Somlyo and Franzini-Armstrong, 1985) and the identification by x-ray microprobe analysis of calcium-rich spots in the juxtamem-

Figure 5. Ultrathin cryosections immunolabeled for CR or PDI (small gold), alone (CR, *A* and *B*) or together with CSQ (large gold, *C* and *D* and *F–H* for CR and PDI, respectively). CR (*small arrows*) appears widely distributed to the entire endomembrane system of the smooth muscle fibers, including the nuclear envelope (*A*), the long, rough-surfaced cisternae (*C*), the small cisternae adjacent or coupled to the plasmalemma (*A* and *D*). Double labeling reveals CR positivity of most CSQ-rich elements, where the two Ca^{2+} binding proteins are apparently intermixed in the segregated content (*D*). *B* shows the intense CR immunolabeling in the well developed ER system of a fibroblast. The section of *D* was left unstained. *E–G* show dual PDI (small gold, *small arrows*)-CSQ (large gold) immunolabeling of juxtaplasmalemma structures; *H* of a structure scattered in the fiber cytoplasm. Notice the intense immunolabeling of the two antigens that appear at least partially intermixed within the lumen of cisternae both plasmalemma attached (*E*) or located at some distance from the surface (*F–H*). Arrowheads mark caveolae, which appear negative for the three antigens. Other labels as in Fig. 3. Bars, 0.25 μm.

brane and central regions of other types of smooth muscle fibers (Bond et al., 1984; Kowarski et al., 1985). However, the molecular components responsible for the accumulation of calcium had never been identified.

Whether, and to what extent, the additional CSQ-poor elements of the endomembrane system also participate in Ca^{2+} exchange cannot be established at the present time. The sensitivity of the immunogold labeling is in fact too low to exclude the presence of a small concentration of Ins-P_3 receptors. In these elements, however, Ca^{2+} could have another important function; i.e., the regulation of protein interactions and thus of protein targeting within the exocytic pathway of the cell (Wada et al., 1991; Suzuki et al., 1991).

Conclusions

In previous studies, the smooth muscle endomembrane system had been referred to with various terms – SR, ER, S-ER and ER/SR (see Chen and van Breemen, 1992; Casteels et al., 1986; Lytton, 1989; Somlyo and Himpens, 1989) – based on a variety of results (biochemistry, physiology, conventional EM, x-ray microanalysis) that could not, however, reveal the distribution of specific markers. The present demonstration that the whole system contains BiP and PDI leaves no doubts as to its ER nature. On the other hand, the enrichment of CSQ and the Ins-P_3 receptor indicates the smooth-surfaced, moderately electron-dense elements localized preferentially towards the periphery to be specialized in Ca^{2+} storage and release. Thus, these elements might be proposed to correspond to the SR. At the moment, the above proposal concerns exclusively the vas deferens. In fact, the information about the composition of the endomembrane system in smooth muscle fibers of other organs has been obtained only by mRNA and protein analyses of homogenates or total microsome fractions. These data indicate the CSQ concentration to be lower than in the vas deferens, moderately (stomach) or very much (uterus, aorta, and urinary bladder) (Wuytack et al., 1987; Milner et al., 1991; and unpublished results). The expression of Ins-P_3 receptor mRNA and protein, on the other hand, seems to be more even among the various smooth muscles (see Marks et al., 1990; Mourey et al., 1990; Furuichi et al., 1990; and unpublished results). The existence of an ER subcompartment specialized in Ca^{2+} regulation is therefore possible in other smooth muscles, but its composition and cellular distribution will not be known until additional studies of the type reported here (for the vas deferens) are carried out.

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