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Ca^{2+} Stores in Purkinje Neurons: Endoplasmic Reticulum Subcompartments Demonstrated by the Heterogeneous Distribution of the $InsP_3$ Receptor, Ca^{2+}-ATPase, and Calsequestrin

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The nature of second messenger–responsive intracellular Ca^{2+} stores in neurons remains open for discussion. Here, we demonstrate the existence in Purkinje cells (PCs) of endoplasmic reticulum (ER) subcompartments characterized by an uneven distribution of three proteins involved in Ca^{2+} storage and release: the inositol 1,4,5-trisphosphate ($InsP_3$) receptor, Ca^{2+}-ATPase, and calsequestrin. Ca^{2+}-ATPase and the $InsP_3$ receptor have a widespread, although not identical, distribution throughout the ER. Calsequestrin is localized throughout the smooth ER and is particularly concentrated in pleiomorphic vesicles with a moderately electron-dense core, which appear to represent a subcompartment of the smooth ER. In double-labeling experiments many of these vesicles were unlabeled by $InsP_3$ receptor antibodies. These results suggest a key role of the ER as an intracellular Ca^{2+} store and demonstrate a possible structural basis for distinct intracellular Ca^{2+} pools regulated by different second messengers.

Cytosolic Ca^{2+} ions play an important role in cellular regulation. While Ca^{2+} is present at millimolar concentration in extracellular media and within the lumen of some intracellular organelles, it is present at submicromolar concentration in the cytosol of resting cells. This large concentration gradient is used to generate intracellular signals. The plasmalemma and intracellular membranes that separate Ca^{2+}-rich compartments from the cytosol contain Ca^{2+} channels gated by either physical or chemical signals. Channel opening leads to a rise in cytosolic Ca^{2+}, which in turn modulates a variety of cellular functions by acting on Ca^{2+}-binding proteins. Eventually, the rise in cytosolic Ca^{2+} is reversed by the action of Ca^{2+} transport systems, which extrude Ca^{2+} from the cytosol. Extracellular signals may affect the concentration of cytosolic Ca^{2+} by acting, directly or indirectly (e.g., via G-proteins or intracellular second messengers), on gated Ca^{2+} channels present in the plasmalemma. In addition, via intracellular second messengers, they may induce a release of Ca^{2+} from intracellular Ca^{2+}-rich compartments (Ca^{2+} stores) (for reviews, see Berridge and Irvine, 1989; Meldolesi et al., 1990; Tsien and Tsien, 1990).

The best characterized intracellular compartment involved in the regulated release of Ca^{2+} is the sarcoplasmic reticulum of muscle cells, an organelle generally viewed as a specialized form of endoplasmic reticulum (ER) (Porter and Palade, 1957; Fleischer and Inui, 1989). Three proteins that play a crucial role in Ca^{2+} dynamics, namely, a Ca^{2+} channel (the ryanodine receptor), a Ca^{2+} pump (a vacuolar Ca^{2+}-ATPase), and a low-affinity/high-capacity intralumenal Ca^{2+}-binding protein (calsequestrin), are present in this organelle. They are organized in a special array at the so-called triadic junction (Franzini-Armstrong et al., 1987), a structure specially suited to mediate a rapid and reversible Ca^{2+} signal in response to depolarization of the transverse tubule (Fleischer and Inui, 1989).

The precise identity of second messenger–responsive intracellular Ca^{2+} stores in nonmuscle cells has been the focus of considerable attention over the last several years (see Meldolesi et al., 1990). The ryanodine receptor (Ellisman et al., 1990; McPherson and Campbell, 1990), vacuolar Ca^{2+}-ATPases (Gunteski-Hamblin et al., 1988; Lytton and MacLennan, 1988; Kaprielian et al., 1989; Plessers et al., 1991) as well as calsequestrin (Volpe et al., 1990a) or proteins functionally analogous to calsequestrin (Booth and Koch, 1989; Fliegel et al., 1989; Treves et al., 1990) have been identified in nonmuscle cells. In addition, the Ca^{2+} channel gated by the second messenger inositol 1,4,5 trisphosphate ($InsP_3$), the $InsP_3$ receptor, has been identified (Supattapone et al., 1988) and thoroughly characterized (Furuichi et al., 1989; Mignery et al., 1989; Mignery and Südhof, 1990). However, the precise subcellular localization of these proteins in nonmuscle cells is still debated (Meldolesi et al., 1990; Volpe et al., 1990b).

Immunocytochemical studies carried out in nonmuscle cells had suggested that a calsequestrin-like, low-affinity Ca^{2+}-binding protein and a vacuolar Ca^{2+}-ATPase were localized in a population of vesicular structures distinct from other well characterized intracellular compartments including the ER (Hashimoto et al., 1988; Volpe et al., 1988; Treves et al., 1990). These results raised the possibility that many, and perhaps all, cells contained a specialized vesicular compartment, defined as the calciosome, related in function to the sarcoplasmic reticulum (Hashimoto et al., 1988; Volpe et al., 1988; Treves et al., 1990).

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On the other hand, studies on the resident lumenal proteins of the ER have demonstrated that the property to bind Ca^{2+} with low affinity and high capacity is a general characteristic of the most abundant proteins of the ER lumen (Booth and Koch, 1989; Sambrook, 1990). These findings suggested that the ER in all cells may contain a high Ca^{2+} concentration and act as an intracellular Ca^{2+} store.

The molecular characterization of two intracellular second messenger–gated Ca^{2+} channels, the ryanodine receptor (Fleischer and Inui, 1989; Takeshima et al., 1989; Zorzato et al., 1990) and the $InsP_3$ receptor (Supattapone et al., 1988; Furuichi et al., 1989; Mignery et al., 1989; Mignery and Südhof, 1990), and the availability of antibodies specific for these proteins made possible the further investigation of the nature of second messenger–responsive intracellular Ca^{2+} stores in nonmuscle cells (Mignery et al., 1989; Ross et al., 1989; Ellisman et al., 1990; Otsu et al., 1990; Satoh et al., 1990; Walton et al., 1991). These studies have focused on Purkinje cells (PCs) of the cerebellum because PCs contain particularly high concentrations of both Ca^{2+} channels. They demonstrated that both the $InsP_3$ receptor and the ryanodine receptor are localized in the ER, further confirming a central role of the ER in Ca^{2+} regulation. However, the distribution of the two proteins within the ER appeared to be heterogeneous and different (Mignery et al., 1989; Ellisman et al., 1990; Satoh et al., 1990; Walton et al., 1991). A further investigation of intracellular Ca^{2+} stores is therefore important and timely.

Recently, a vacuolar Ca^{2+}-ATPase (SERCA2, and primarily SERCA2b) has been found to be highly concentrated in chicken PCs (Kaprielian et al., 1989; Plessers et al., 1991). In addition, a protein identical to muscle calsequestrin by a variety of criteria has been identified in chicken cerebellum (Volpe et al., 1990a), and pilot experiments demonstrated that this protein was also selectively localized in PCs (Takei et al., 1990). These findings opened the possibility of carrying out a comparative analysis in a neuronal cell of the localization of proteins that play different roles in the physiology of intracellular Ca^{2+} stores.

In this study we have used immunocytochemistry to compare the subcellular localization in PCs of the $InsP_3$ receptor, Ca^{2+}-ATPase, and calsequestrin. We have found that both the $InsP_3$ receptor and Ca^{2+}-ATPase have a widespread distribution within the ER, but we also found regional differences in their localization. Calsequestrin was present throughout the smooth ER of perikarya, dendrites, and axons but was particularly concentrated in vesicular structures that may be specializations of the ER or in functional continuity with the ER. Its distribution was clearly different from that of the ER-resident protein Bip (Bole et al., 1986). Calsequestrin-rich vesicles may be related to organelles previously defined as "calciosomes" (Hashimoto et al., 1988; Volpe et al., 1988). In summary, the present study raises the possibility that typical ER elements may function in parallel with specialized structures (calciosomes?) in the homeostasis of cytosolic Ca^{2+}. It also provides new insights into the structural organization of the ER in neurons.

These results were reported previously in a preliminary form (Takei et al., 1990).

Materials and Methods

Animals

Male Sprague–Dawley rats (approximately 150–200 gm) were obtained from Charles River (Wilmington, MA), and female White Leghorn chickens (approximately 750 gm), from Kutsco (Voluntown, CT). Bovine brains were obtained from a local slaughterhouse.

Antibodies and gold conjugates

Affinity-purified rabbit polyclonal antibodies directed against the 19-mer C-terminal peptide of the mouse $InsP_3$ receptor were prepared and characterized as previously described (Mignery et al., 1989). These antibodies recognize mammalian as well as chicken $InsP_3$ receptor. Monoclonal antibodies raised against the cardiac/slow-twitch isoform of chicken Ca^{2+}-ATPase and that recognize both SERCA2a and SERCA2b were the kind gift of D. Campbell and D. Fambrough (Johns Hopkins University, Baltimore, MD) (Kaprielian et al., 1989). These antibodies do not recognize the fast-twitch isoforms of the protein (SERCA1). Affinity-purified mouse polyclonal antibodies directed against chicken skeletal muscle calsequestrin were obtained and characterized as previously described (Volpe et al., 1990a). Rat monoclonal antibodies directed against the ER-resident protein Bip were kind gift of Dr. D. Bole (University of Michigan, Ann Arbor, MI) (Bole et al., 1986). Rhodamine- and fluorescein isothiocyanate (FITC)-antibody conjugates and nonimmune IgGs were from Cappel (Cochranville, PA). Protein A–gold particles were prepared as described by Slot and Geuze (1985). Gold particle–conjugated goat anti-mouse IgGs (5 nm) were from Janssen (Beerse, Belgium).

Immunofluorescence

Rat and chicken brains were fixed by perfusion (4% formaldehyde in 0.12 M Na-phosphate buffer). Preparation of frozen sections and immunostaining were carried out essentially as described (De Camilli et al., 1983a) except that all labeling steps were carried out at room temperature. At the end of the immunolabeling, sections were mounted in *p*-phenylendiamine/glycerol (1 mg/1 ml *p*-phenylendiamine in 70% glycerol).

Electron microscopy

Agarose-embedded tissue fragments. Tissue fragments were embedded in agarose using the procedure described by De Camilli et al. (1983b) with some modifications. Fresh brain tissue was quickly cut in small pieces, which were mildly homogenized in about sixfold (w/v) ice-cold homogenation medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM Na-phosphate buffer, pH 7.4) by passing them two or three times through a loose-fitting glass–Teflon homogenizer. The homogenate was then mixed with large volume of ice-cold isotonic fixative (0.25 M sucrose, 3% formaldehyde, 0.25% glutaraldehyde in 5 mM phosphate buffer). Fixed cell fragments were pelleted by centrifugation (19,000 × *g* for 45 min) in a Sorvall SS-34 rotor at 4°C. The pellet was resuspended in 0.3 ml of 0.12 M Na-phosphate buffer and homogenized in a small glass–Teflon homogenizer. This material was then mixed 1:1 with fluid 2% agarose (54°C) to prepare agarose blocks containing tissue fragments as described (De Camilli et al., 1983b). Immunogold labeling, postfixation, and plastic embedding of the agarose blocks were performed essentially as described (De Camilli et al., 1983b) with the exception that gold particles rather than ferritin conjugates were used as secondary reagents. In single-labeling experiments, rabbit antibodies were revealed with Protein A–gold conjugates (6 and 8 nm), and mouse antibodies, with gold-conjugated goat anti-mouse IgGs (5 or 10 nm). For double-labeling experiments, agarose-embedded blocks previously labeled for the $InsP_3$ receptor (4 nm Protein A–gold conjugates) were then processed for calsequestrin immunoreactivity (10 nm gold-conjugated goat anti-mouse IgGs) by the ultrathin frozen section technique. To this purpose, agarose blocks processed up to the washing step preceding OsO_4 postfixation (De Camilli et al., 1983b), were infiltrated overnight in phosphate-buffered saline (PBS) containing 50% polyvinyl pyrrolidone and 2.3 M sucrose and finally rapidly frozen in liquid nitrogen. Ultrathin frozen sections were prepared and labeled for calsequestrin immunoreactivity as described below for ultrathin frozen sections of intact tissue.

In pilot experiments it was found that the level of immunolabeling for Ca^{2+}-ATPase was higher if specimens had been pretreated with low concentrations of nonionic detergent. Therefore, blocks to be stained for the Ca^{2+}-ATPase were incubated with 0.05% saponin in Na-phosphate buffer for 10 min before the primary antibody incubation.

Ultrathin frozen sections. Preparation of ultrathin frozen sectioning was performed essentially as described by Keller et al. (1984) and Tokuyasu (1989). Rat or chicken cerebella were fixed by perfusion (4% paraformaldehyde in 0.12 M Na-phosphate buffer) at 4°C. Tissues were cut into small pieces, infiltrated overnight in PBS containing 50% polyvinyl pyrrolidone and 2.3 M sucrose, and finally rapidly frozen in liquid ni-

trogen. Ultrathin frozen sections were cut with an Ultracut E microtome equipped with FC4E cryo-attachment (Reichert, Vienna) and collected on formvar carbon-coated nickel grids. Grids were then incubated in 1% bovine serum albumin (BSA) in PBS followed by incubation with primary antibodies in the same buffer for 1.5 hr. After a wash with 0.1% BSA in PBS, grids were incubated with gold conjugates diluted in 1% BSA/PBS. Gold conjugates were the same as those used for the labeling of agarose-embedded specimens (see above). After a short wash, bound antibodies were fixed with 2% glutaraldehyde in PBS for 5–10 min. Grids were then postfixed, counterstained, and embedded through the following steps: brief rinse in water, 15 min in 2% OsO_4, brief rinse in water, 30 min in 2% uranyl acetate, 2 × 2.5 min in 2.3% polyvinylalcohol containing 15 μg/ml lead citrate. Finally grids were air dried after removing excess fluid.

Conventional plastic sections

Rat and chicken brains were fixed by perfusion with 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) at various temperatures (4°C, 20°C, and 37°C), cut into small pieces, fixed for additional 4 hr by immersion in the same fixative, and postfixed with 1% OsO_4 in 0.1 M Na-cacodylate buffer (pH 7.4). They were then stained en bloc with 2% uranyl acetate, dehydrated with a series of graded ethanol and propylene oxide, and embedded in Epon 812. Ultrathin sections were cut with a Reichert ultratome, counterstained with uranyl acetate and lead citrate, and observed with a Philips 301 electron microscope.

Results

Mouse monoclonal and polyclonal antibodies directed against the cytoplasmic domain of the chicken cardiac/slow-twitch Ca^{2+}-ATPase (SERCA2) (Kaprielian et al., 1989) and against chicken calsequestrin (Volpe et al., 1990a), respectively, have been previously described and characterized. Since the antibodies are specific for the chicken isoforms of these molecules, chicken cerebella were used for the study of these proteins. Rabbit polyclonal antibodies directed against the cytoplasmic 19-mer C-terminus of the mouse $InsP_3$ receptor have also been previously characterized (Mignery et al., 1989; De Camilli et al., 1990). These antibodies recognize both the chicken and the mammalian protein. Thus, chicken as well as mammalian cerebella were used for the study of the $InsP_3$ receptor.

Light microscopy studies

Frozen sections of chicken cerebella were stained by immunofluorescence or immunoperoxidase with antibodies directed against the $InsP_3$ receptor, Ca^{2+}-ATPase, or calsequestrin. Immunoreactivity for each of the three proteins was detected at very high concentration in all Purkinje cells (Fig. 1*a–d*). However, some differences among the staining patterns for the three proteins were noticeable.

As previously shown for the mammalian cerebellum, $InsP_3$ immunoreactivity was detectable only in Purkinje cells (Mignery et al., 1989; Ross et al., 1989; Satoh et al., 1990) and, within Purkinje cells, was very intense throughout all cytoplasmic compartments including somata, dendrites, dendritic spines, axons, and axon terminals (Fig. 1*a,e,i*).

The distribution of Ca^{2+}-ATPase immunoreactivity was not specific for PC neurons, but in the cerebellar cortex, PC were by far the most intensely immunoreactive neurons (Fig. 1*c,g*). Within PCs, the distribution of Ca^{2+}-ATPase immunoreactivity was similar to that of the $InsP_3$ receptor in perikarya, dendrites, dendritic spines, and initial segments of axons visible in the granular layer (Fig. 1*c,g*). Ca^{2+}-ATPase immunoreactivity, however, was only barely detectable, although present, in the distal segments of PCs axons, including axons in the cerebellar white matter of the folia and of the cerebellar medulla and axon terminals in the deep cerebellar nuclei (Fig. 1*k*). Presence of Ca^{2+}-ATPase in neurons of the deep cerebellar nuclei is visible in Figure 1*k*. A lower concentration of Ca^{2+}-ATPase immunoreactivity in axons was characteristic of all neurons of the brain, so that in all regions of the neuropil, including the deep cerebellar nuclei (Fig. 1*k*), a perikaryal-dendritic pattern of staining predominated.

Calsequestrin immunoreactivity was restricted to PCs and was present in perikarya and dendritic trees as well as in the entire axonal arbors (Fig. 1*b,f,j*). However, spines of the dendritic tree were virtually unstained. For a comparison, the distribution of a well characterized resident protein of the ER, Bip (Bole et al., 1986), was examined. Bip immunoreactivity was present at high concentration in all cellular elements of the cerebellum (Fig. 1*d,h,l*) and of the whole CNS (not shown) and was particularly concentrated in PCs (Fig. 1*d,h*). Bip immunoreactivity was intense throughout the perikaryon, the dendritic tree including spines, and the proximal axon, but only barely detectable in the distal axon and in axon terminals. Note that while Bip and Ca^{2+}-ATPase immunoreactivities are present at similar intensity throughout the dendritic tree of PC, they are present at low concentration in the distal dendrites of the deep cerebellar nuclei (Fig. 1*k,l*). A similar observation was made in

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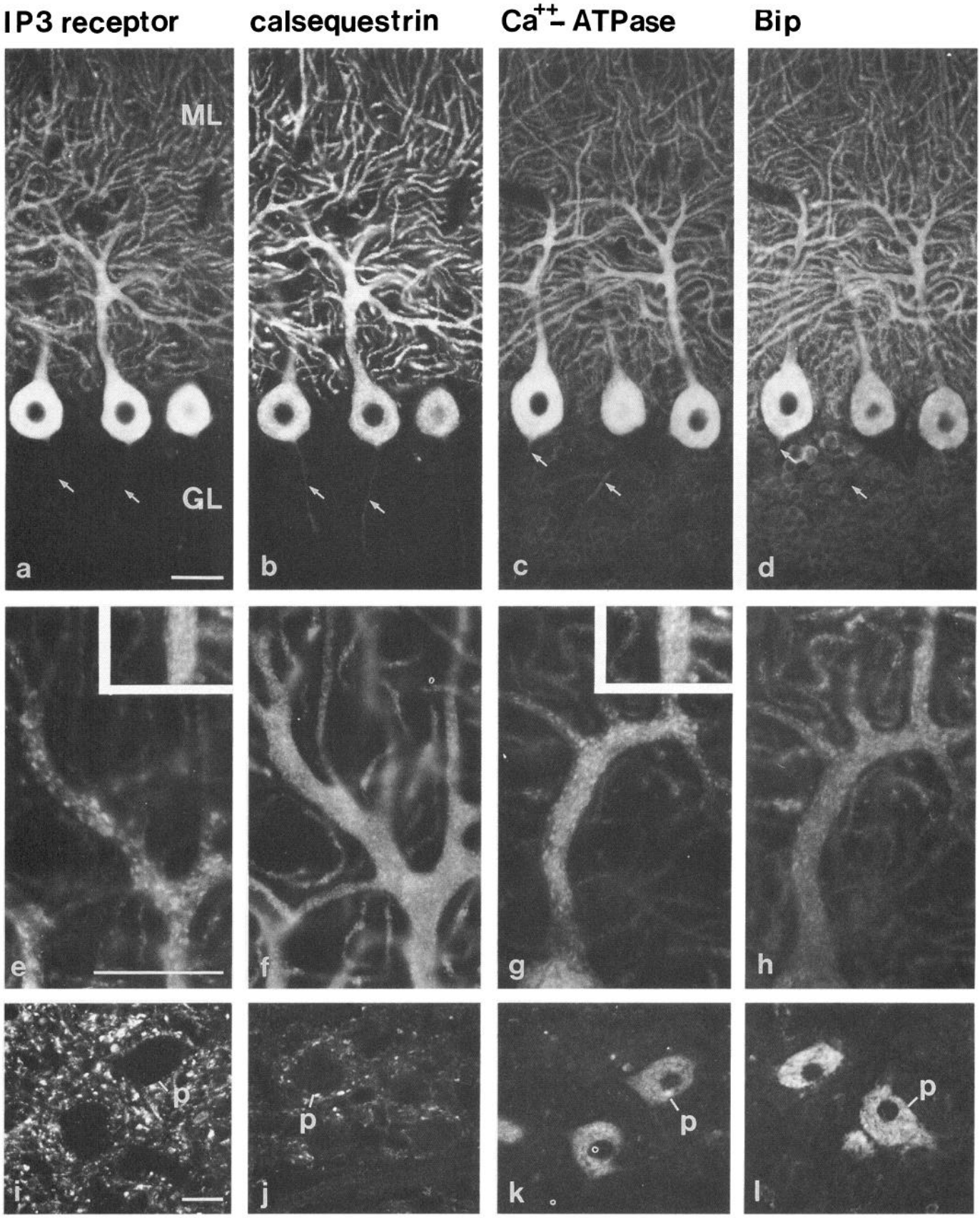
Figure 1. Immunofluorescence localization of $InsP_3$ receptor, calsequestrin, Ca^{2+}-ATPase, and Bip in frozen sections of chicken cerebellum. *a–d,* Sagittal sections of the cerebellar cortex showing portions of the PC layer, the molecular layer (*ML*), and the granule cell layer (*GL*). *a* and *b* were double labeled for the $InsP_3$ receptor (*a*) and for calsequestrin (*b*). *c* and *d* were double labeled for Ca^{2+}-ATPase (*c*) and Bip (*d*). All four proteins are concentrated in PCs. Ca^{2+}-ATPase and Bip are also present at lower levels in other cell types (note granule cells). *Arrows* point to initial segments of PC axons in the granule layer. *e–h,* High-power views of the molecular layer double stained for the $InsP_3$ receptor and calsequestrin (*e* and *f*) and for Ca^{2+}-ATPase and Bip (*g* and *h*). Note in large dendrities the very different texture of the immunostain for the $InsP_3$ receptor and for calsequestrin, and the identical texture of the stain for Ca^{2+}-ATPase and for Bip. The two *insets* of *e* and *g* show dendrites of PCs double stained for the $InsP_3$ receptor (*e*) and Ca^{2+}-ATPase (*g*), demonstrating the similarity of the two staining patterns in a large dendritic trunk and in a small dendrite studded with spines. *i–l,* Deep cerebellar nuclei stained for the $InsP_3$ receptor (*i*), calsequestrin (*j*), Ca^{2+}-ATPase (*k*), and Bip (*l*). $InsP_3$ receptor and calsequestrin immunoreactivity visible in this region corresponds to axons and axon terminals of PCs that innervate the perikarya (*p*) of the nuclei. Ca^{2+}-ATPase and Bip immunoreactivities are virtually undetectable in PC axon terminals, bur are present in the perikarya of this region. Scale bars, 20 μm.

Figure 2. Localization of the $InsP_3$ receptor in dendrites of PCs. *a–e,* Fragments of rat cerebellar cortex embedded in agarose and labeled for the $InsP_3$ receptor by an immunogold procedure. Immunogold is selectively localized on smooth-surfaced tubulovescicular profiles; the overwhelming majority of such elements are immunoreactive. The main shafts of large dendritic segments are shown in *a* and *b*. The plasmalemma (*pm*) and an adjacent glial cell process (*gc*) can be seen in *b*. Note in *b* presence of labeling on subplasmalemmal cisternae (*sc*) but not on the PC plasmalemma. *c* shows a labeled cisterna in close apposition to a mitochondrion (note dense projections connecting the cisterna to the outer mitochondrial membrane). *d* and *e* show labeling of tubulovesicular elements in a spine. *f* shows $InsP_3$ immunoreactivity in the spine of a chicken PC in an ultrathin frozen section. *psd,* postsynaptic density; *m,* mitochondria. Scale bars, 200 nm.


IP3 receptor
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Ca++- ATPase
Bip
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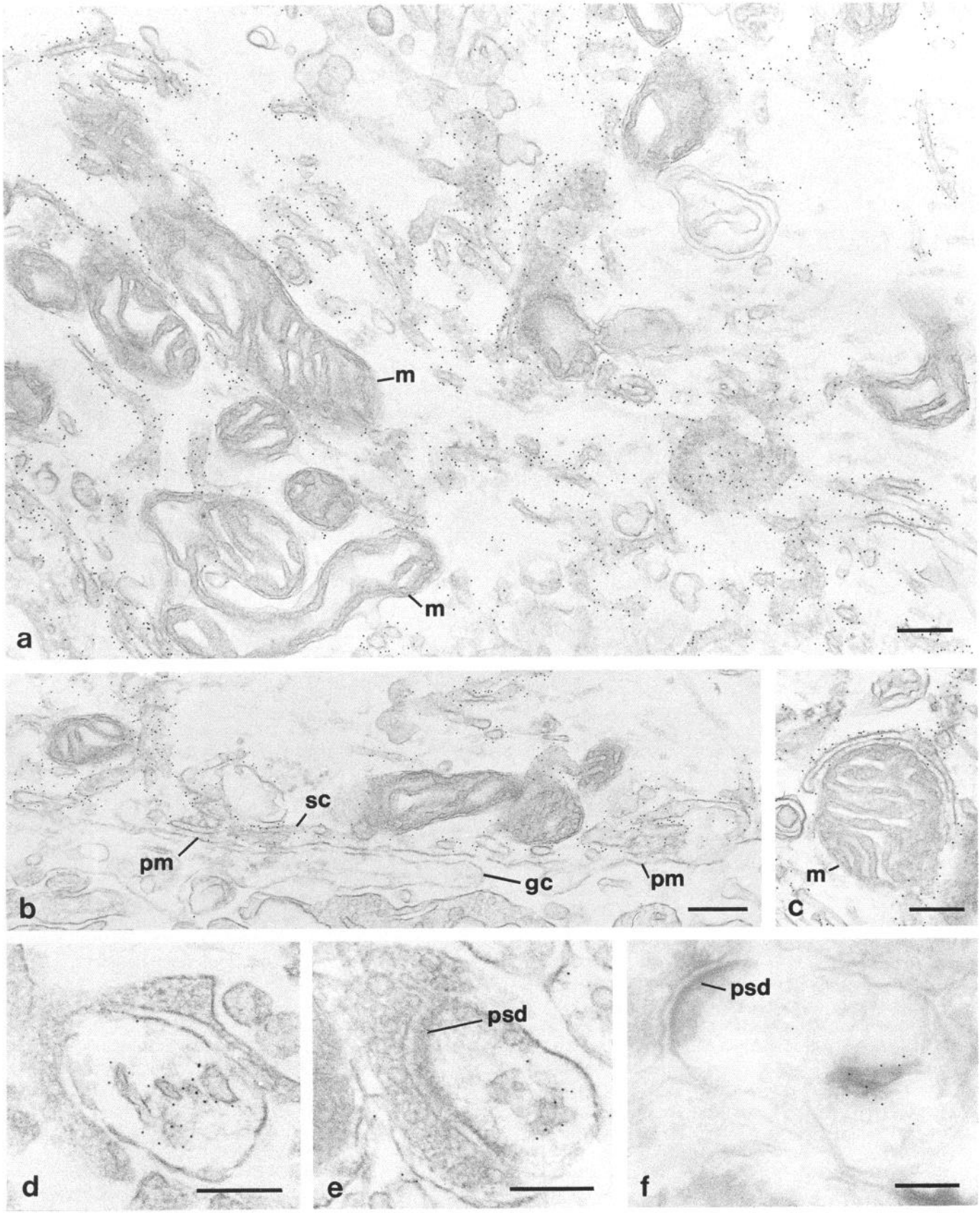
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virtually all neurons except PCs (not shown). The intense staining of the whole PC dendritic arbor with antibodies to ER proteins may reflect a more extensive development of the smooth ER in dendrites of PCs than in dendrites of other neurons.

When cerebellar sections stained by immunofluorescence for the four antigens were examined at high magnification in a standard (Fig. 1*e–h*) or confocal microscope (not shown), differences in the fine texture of the immunostain were observed. As shown by the double-immunofluorescence picture shown in Figure 1, *e* and *f*, $InsP_3$ receptor immunoreactivity had a patchy reticular texture (Fig. 1*e*), very different from the finely punctate appearance of calsequestrin immunoreactivity (Fig. 1*f*). Ca^{2+}-ATPase and Bip immunoreactivities had an identical reticular pattern (see double-immunofluorescence micrographs shown in Fig. 1*g,h*). This pattern was extremely similar to that of $InsP_3$ receptor immunoreactivity as demonstrated by double-immunofluorescence for the $InsP_3$ receptor and Ca^{2+}-ATPase (Fig. 1*e,g,* insets).

No staining of PCs was observed when primary antibodies were substituted by control sera (not shown).

Electron microscopy studies

Electron microscopy immunocytochemistry was performed by using two different and complementary procedures. One procedure involves the immunolabeling of tisue and cell fragments previously embedded in an agarose matrix. This is designed at maximizing labeling of cytoplasmic epitopes of cellular membranes because the surrounding cytomatrix is eluted by the cell lysis preceding fixation (De Camilli et al., 1983b). The peculiar morphology of PCs, and the structure of surrounding neuropil, allows the identification of PC fragments. Optimal labeling of the $InsP_3$ receptor and of Ca^{2+}-ATPase was obtained with our antibodies by this technique. The other procedure involves the surface labeling of ultrathin frozen sections (Keller et al., 1984; Tokuyasu, 1989). This was the procedure of choice for calsequestrin immunolabeling because both cytoplasmic and lumenal epitopes of intracellular organelles are accessible to antibodies by this technique. Labeling of the $InsP_3$ receptor and the Ca^{2+}-ATPase was less efficient by this procedure, probably due to the presence of fixation cross-linked cytomatrix around their cytoplasmic epitopes.

$InsP_3$ receptor

When agarose-embedded preparations of chicken or mammalian cerebellar tissue were labeled for the $InsP_3$ receptor, very intense immunogold labeling was observed within all fragments of PCs. Identical results were obtained with avian and mammalian cerebella. In dendrites as well as in axons, the overwhelming majority of intracellular membranes other than mitochondria were intensely positive for the $InsP_3$ receptor. The abundance and the morphology of labeled elements were consistent with their identification as elements of the ER, in particular of the smooth ER. Ribosomes were poorly preserved in these preparations. Membranes that had ribosomal remnants at their surface were labeled much less intensely. Figure 2*a* and *b* shows details of proximal dendritic segments. In the two fields, most tubuloreticular elements are intensely labeled. Labeled elements in dendrites and perikarya included cisternae closely apposed to the plasmalemma (Fig. 2*b*) and to mitochondria (Fig. 2*c*) as well as tubules and cisternae in dendritic spines (Fig. 2*d,e*).

Figure 3 shows $InsP_3$ receptor immunoreactivity in fragments of axons and axon terminals. In the myelinated axon shown in Figure 3*a*, the great majority of smooth membranous profiles (but not mitochondria) are $InsP_3$ receptor positive. In axon terminals, labeling is present on tubular and cisternal elements but not on synaptic vesicles, large dense-core vesicles (Fig. 3*b*), or in a subpopulation of tubular and vacuolar elements (Fig. 3*c*). The latter may represent the endosome-like structures previously demonstrated in nerve endings by the use of extracellular tracers (Heuser and Reese, 1973) and by immunolabeling for synaptic vesicle proteins (Navone et al., 1986). No $InsP_3$ immunoreactivity was observed on the plasmalemma (Fig. 2*b,d,e*) or on membranes of the Golgi complex (not shown).

In all compartments (dendrites, perikarya, and axons), labeled cisternae closely apposed to each other to form peculiar stacks (Fig. 4*a,b*) were observed. Cisternae appeared to be connected by dense material that, in favorable sections, appeared to have a periodic structure. In some cases, appositions similar to those observed in large stacks were seen between pairs of ER elements (Fig. 3*a*). $InsP_3$ immunolabeling was concentrated at regions of the cisternal membranes adjacent to sites of cisternal appositions, while regions farther away from these sites were less intensely labeled or unlabeled (Figs. 3*a,* 4*a,b*). The regions directly involved in membrane appositions were also unlabeled (Figs. 3*a,* 4*a,b*). If antigen was present in these regions, it was probably inaccessible to antibodies and to gold particles due to the immunocytochemical procedure used, which involves labeling before sectioning. In fact, Satoh et al. (1990), who observed similar stacks in PCs fixed *in situ* and processed by the ultrathin frozen section technique, found that the $InsP_3$ receptor was particularly concentrated at sites of membrane apposition and was present only at lower concentration on other ER regions. Similar results were obtained by us when the ultrathin frozen section procedure (Figs. 2*f*; 3*a,* inset; 4*c*) was used. In conclusion, the complementary results obtained with the two labeling techniques are consistent with a special accumulation of $InsP_3$ receptor at cisternal stacks, but clearly demonstrate a high concentration of the $InsP_3$ receptor throughout most of the smooth ER. The depletion of $InsP_3$ receptor from membrane regions directly adjacent to membrane appositions may be due to clustering of the receptor at apposition sites.

Stacks of ER cisternae such as those described in this study and in the study of Satoh et al. (1990) had already been described in the late 1950s as a peculiar characteristic of PCs (Fernandez-Moran, 1957). Subsequent studies suggested that stacks represent fixation artifacts that are particularly abundant when fixation is performed at low temperature or are the expression of PC damage (Herndon, 1964; Karlsson and Schultz, 1966; Van Nimwegen and Sheldon, 1966; Bestetti and Rossi, 1980). To investigate this issue further, we examined plastic sections of cerebellar tissue fixed by a formaldehyde-glutaraldehyde mixture at either 4°C, 20°C, or 37°C. At low temperature, stacks were much more evident because cisternae were highly dilated and distorted (not shown). They also appeared to be more numerous and to be more randomly located in the PC cytoplasm.

However, cisternal stacks were clearly visible also in specimens optimally fixed at 37°C (Fig. 5). In these preparations, cisternae were extremely flattened, with opposite lumenal faces almost touching each other. In dendrites they were often located in the cortical cytoplasm (Fig. 5*a,c,e*), with the cisternae aligned parallel to the plasmalemma. Stacks frequently included cisternae that had one side tightly apposed to the plasmalemma (Fig. 5*a,e*) (Rosenbluth, 1962) or to mitochondria (e.g., Fig. 5*a,b,f*)

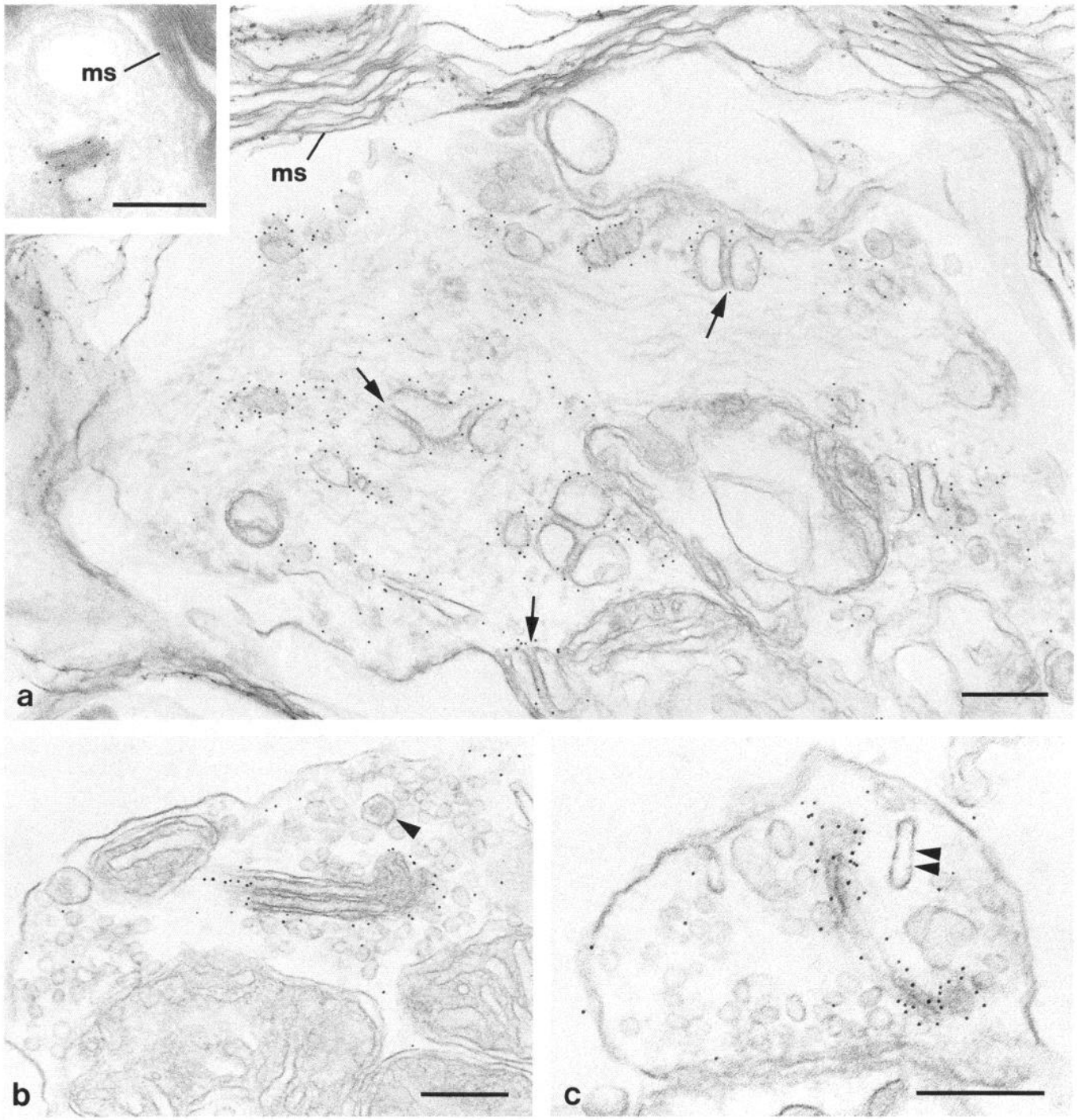

Figure 3. Localization of the $InsP_3$ receptor in axons and axon terminals of PCs: fragments of cerebellar tissue embedded in agarose and labeled for the $InsP_3$ receptor by immunogold. A myelinated portion of a PC axon is shown in *a*. Most vesicular profiles visible in the axon are immunoreactive. Note that some of these immunoreactive elements are apposed to each other (*arrows*) and that dense material is visible at sites of apposition. Lack of gold labeling of membrane portions participating in these appositions is probably due to accessibility problems because they can be labeled in ultrathin frozen sections (*inset*). *ms,* myelin sheath. *b* and *c* show PC axon terminals. A stack of cisternae and a tubulovesicular element are labeled in *b* and *c,* respectively. Note lack of labeling on other membranous profiles including synaptic vesicles, large dense-core vesicles (*single arrowhead*), and tubulovesicular elements that may represent endosome-like structures (*double arrowhead*). *a* and *b* are from bovine cerebella; *c* is from rat cerebellum. Scale bars, 200 nm.

(Herndon, 1963). Periodic dense projections were seen not only between cisternae, but also between cisternae and the plasmalemma and between cisternae and mitochondria (Fig. 5). Peculiar, thin mitochondrial segments completely surrounded by smooth ER cisternae were sometimes visible (Fig. 5*a*). Occasionally, transitions from side views to en face views of membrane appositions between adjacent cisternae were observed (Fig. 5*e,f*). In such cases, dense projections appeared as large particles similar in size to ribosomes, but less electron dense (Fig. 5*e,f*). Similar structures could be seen on isolated ER elements

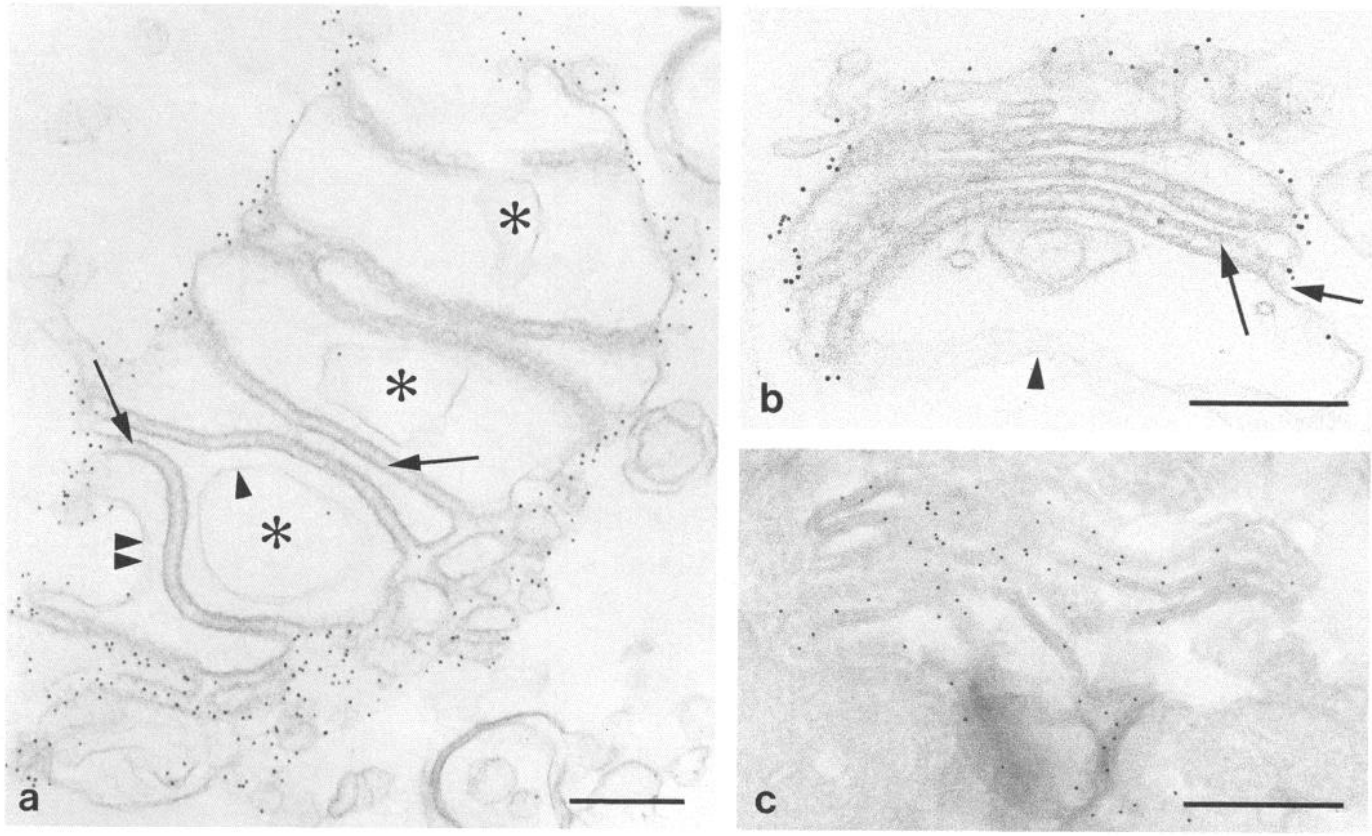

Figure 4. $InsP_3$ receptor immunoreactivity on the membranes of PC cisternal stacks. *a* and *b*, Immunogold labeling of agarose-embedded fragments of cerebellar cortex from bovine cerebella. Cisternal lumena are highly dilated in these preparations. Gold labeling is concentrated on membrane regions directly contiguous to sites of membrane apposition, while some membrane regions more distant from these sites are completely unlabeled (*arrowheads*). The latter regions have a smooth surface and form infoldings (*double arrowhead*), which can appear as intracisternal vacuoles (*asterisks*). Regions of membrane apposition are not accessible to antibodies in these preparations (see text). *c*, Ultrathin frozen section of the perikaryon of a chicken PC showing that, by this technique, $InsP_3$ receptor immunoreactivity can be demonstrated at sites where cisternal membranes are apposed to each other. Scale bars, 200 nm.

(Fig. 5*e*, arrowheads). As previously observed (Herndon, 1964; Karlsson and Schultz, 1966; Van Nimwegen and Sheldon, 1966; Satoh et al., 1990), cisternal stacks were continuous with the rough ER (Fig. 5*b*) and were clearly different in morphology from cisternae of the Golgi complex (Fig. 5*d*).

Ca^{2+}-ATPase

The subcellular distribution of Ca^{2+}-ATPase, as determined by the immunogold labeling of chicken PC fragments embedded in agarose, was found to be similar to the subcellular distribution of the $InsP_3$ receptor. In perikarya as well as in dendrites, abundant immunoreactivity was seen on the great majority of tubulovesicular structures with the characteristics of the ER (Fig. 6*a*). Labeled membranes included ER elements present in spines (Fig. 6*f*) and cisternal stacks (Fig. 6*c,d,e*). On all these membranes, gold particles often appeared less homogeneously distributed than in preparations labeled for the $InsP_3$ receptor. No label was seen on the plasmalemma (Fig. 6*a,c,f*) or on elements of the Golgi complex (Fig. 6*b*). Very little immunolabeling was seen in endomembranes of axons (not shown). Double-labeling of the same cerebellar fragments for the $InsP_3$ receptor and for Ca^{2+}-ATPase using gold particles of different size confirmed that most membranous elements in perikarya and dendrites were positive for both proteins (not shown).

The localization of Ca^{2+}-ATPase in the chick cerebellum was also investigated by the ultrathin frozen section procedure. Only an extremely low level of labeling was obtained by this technique (not shown). No accumulation of immunoreactivity was observed at sites of membrane apposition in cisternal stacks.

Calsequestrin

Ultrathin frozen sections of chicken cerebellum immunolabeled for calsequestrin are shown in Figures 7*a–e* and 8. Gold particles were observed on the majority of smooth-surfaced tubular and cisternal elements present in somata, dendrites, and axons of PCs. These included cisternal stacks (Fig. 7*a*). In addition, a

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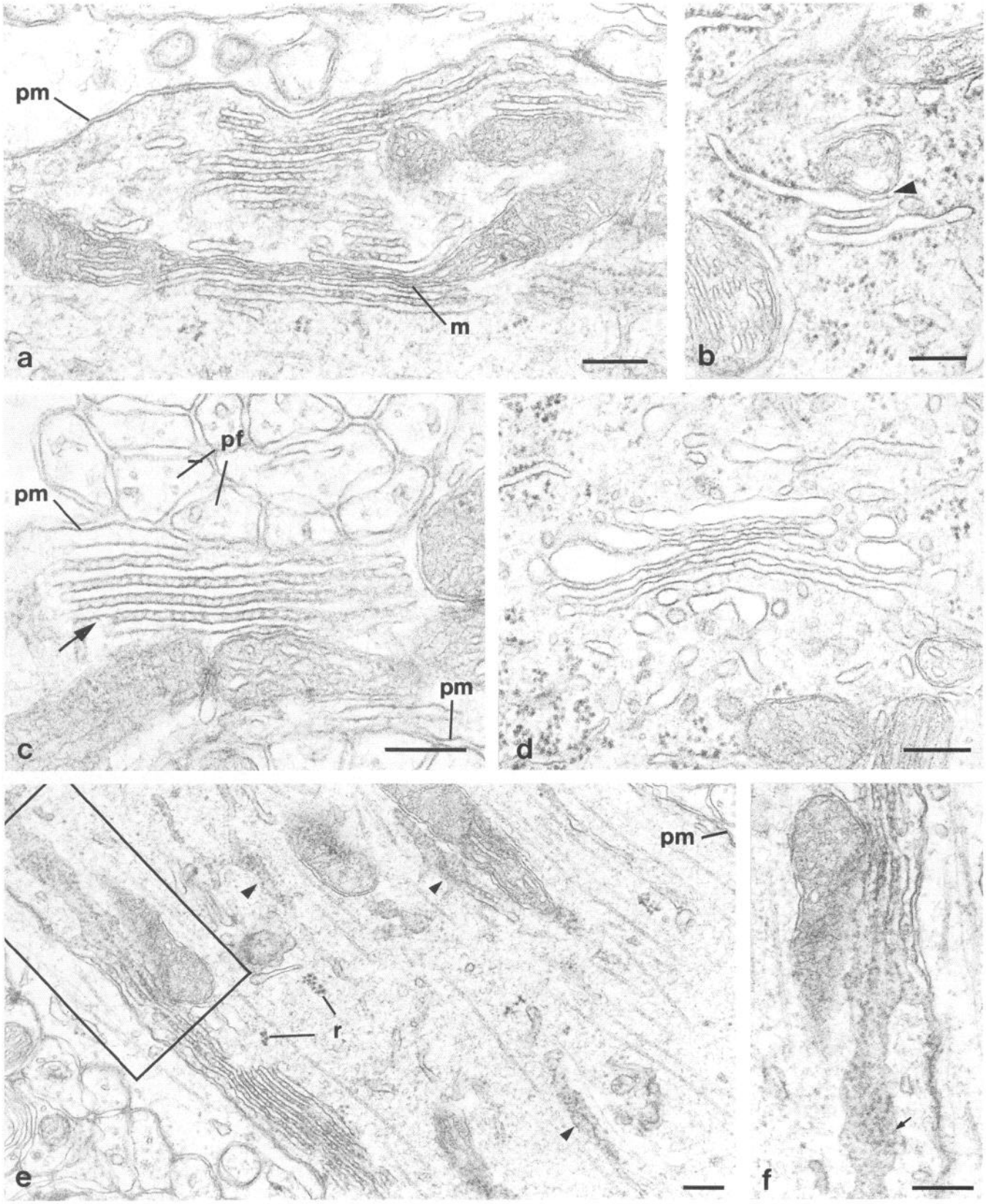
Figure 5. Conventional plastic sections of rat cerebellar cortex showing cisternal stacks in dendrites (*a, c, e,* and *f*) and perikaryon (*b*) of PCs. Fixation was performed at 37°C. *a–c,* Piles of cisternae connected to each other by dense projections with a periodic structure (*arrow*) are visible. Some cisternae in the piles are closely connected to either the PC plasmalemma (*pm*) or outer mitochondrial membranes. Note the peculiar arrangement of some of these membranes around a thin mitochondrial segment (*m*) in *a*. The continuity between cisternal stacks and the rough



ER is shown in *b*. *pf*, cross sections of parallel fiber axons. *d*, Golgi complex of a PC. Note, by a comparison between *c* and *d*, that the Golgi complex is clearly different in morphology from cisternal stacks. *e*, Low-power micrograph showing cisternal stacks in the subplasmalemmal region of Purkinje cell dendrites. *f* shows at higher magnification the area enclosed by a *rectangle* in *e*. It shows a transition from a cross section to a tangential section of cisternal membranes. Note, in the tangential view, the presence at the surface of these cisternae of dense particles (*f*, *arrow*), which may correspond to dense projections seen en face. Similar particles, which are slightly smaller than ribosomes (*r*) and are less electron dense, can also be observed in tangential sections of isolated tubulovesicular elements in *e* (*arrowheads*). Scale bars, 200 nm.

higher concentration of gold particles was observed on vesicles with a moderately dense core (Figs. 7*a–e*, 8). These structures were often irregularly shaped, but several round vesicles with a diameter of about 100–150 nm were also seen (Fig. 7*d,e*). They were observed in all regions of the PC cytoplasm, including somata, dendrites, axons, and axon terminals (Figs. 7*a–e*, 8), but not in spines (not shown). Such organelles are likely to correspond to the fine calsequestrin-positive puncta visible by immunofluorescence. Thus, their absence from spines is consistent with results of immunofluorescence studies. In some experiments, agarose-embedded fragments of PCs previously labeled for the $InsP_3$ receptor by small-sized immunogold particles (4 nm) were processed by the ultrathin frozen section technique for calsequestrin immunoreactivity using larger gold particles (10 nm). In these experiments, calsequestrin immunoreactivity was seen in $InsP_3$ receptor–containing vesicles and tubules (Fig. 7*h*). However, the profiles of many of the larger calsequestrin-rich vesicles were often completely devoid of $InsP_3$ receptor immunoreactivity (Fig. 7*f,g*). The rough ER (Fig. 7*a*) and cisternae of the Golgi complex (Fig. 7*b*) were also virtually negative for calsequestrin, although calsequestrin-positive vesicles were frequently observed next to the Golgi complex (Fig. 7*b*).

When ultrathin frozen sections of the cerebellar cortex including PCs were immunolabeled for Bip, this protein was found to have a widespread localization both in the rough, as well as in the smooth ER (not shown). Since calsequestrin was not detected by our immunolabeling protocol in the rough ER, this finding demonstrates a heterogeneous distribution of lumenal proteins within the ER.

Discussion

The results reported in this study suggest a key role of the ER in cytosolic Ca^{2+} regulation in PCs and support the hypothesis (Henkart et al., 1976; Duce and Keen, 1978; Henkart, 1980) that the ER functions as an intracellular Ca^{2+} store also in nonmuscle cells. They also demonstrate the presence of regional specializations of the ER, which may be relevant to cytosolic Ca^{2+} regulation and suggests a possible structural basis for the existence of distinct intracellular Ca^{2+} stores regulated by different second messengers.

Our findings indicating a selective localization of the $InsP_3$ receptor in the ER, and primarily in the smooth ER, extend previous results obtained by us (Mignery et al., 1989; De Camilli et al., 1990) and by others (Ross et al., 1989; Satoh et al., 1990; Walton et al., 1991). Furthermore, we demonstrate the presence of $InsP_3$ receptor immunoreactivity in a tubulovesicular network that extends into nerve terminals. This observation adds an important new piece of evidence to the hypothesis that the axonal ER represents a direct extension of the smooth ER of perikarya and dendrites (Broadwell and Cataldo, 1984; Lindsay and Ellisman, 1985) and is consistent with a role of the $InsP_3$ receptor in the regulation of neurotransmitter release.

Satoh et al. (1990), using the ultrathin frozen section technique, reported that the $InsP_3$ receptor is mainly concentrated at peculiar stacks of ER cisternae. Using the same technique, we confirmed the extremely high concentration of the $InsP_3$ receptor at cisternal stacks. In addition, our results obtained with agarose-embedded tissue and cell fragments indicated a very high density of the receptor also on smooth ER surfaces which are not involved in membrane appositions. In favorable sections of cisternal stacks, regularly spaced dense projections appeared to connect apposed cisternae. These connections are reminiscent in appearance and dimensions (width of about 20 nm) of the regularly spaced connections observed in skeletal muscle between terminal cisternae of the sarcoplasmic reticulum and the T tubule (Franzini-Armstrong and Nunzi, 1983; Franzini-Armstrong et al., 1987). The latter structure is thought to represent the large cytoplasmic N-terminal portions of ryanodine receptor tetramers (Franzini-Armstrong et al., 1987; Fleischer and Inui, 1989; Takeshima et al., 1989; Zorzato et al., 1990). As previously suggested by Satoh et al. (1990), the structural similarity and scattered primary sequence homology between the ryanodine receptor and the $InsP_3$ receptor (Furuichi et al., 1989; Mignery et al., 1989; De Camilli et al., 1990), together with the immunocytochemical evidence for a concentration of $InsP_3$ receptor immunoreactivity at sites of cisternal apposition, raise the possibility that dense projections connecting ER cisternae of the stacks may represent the large N-terminal cytoplasmic domain of the $InsP_3$ receptor. The dimension of these projections corresponds to the dimensions of the $InsP_3$ receptor as determined by negative staining studies (Chadwick et al., 1990).

It is also possible that at least some of the dense projections represent the ryanodine receptor itself, because the ryanodine receptor has been detected at high concentration in PCs (see below) (Ellisman et al., 1990; Walton et al., 1991). However, cisternal stacks were not described in the immunocytochemical studies of Ellisman et al. (1990) and Walton et al. (1991). Furthermore, the feet of the muscle ryanodine receptor connect the sarcoplasmic reticulum to the plasmalemma rather than to other intracellular membranes (Franzini-Armstrong and Nunzi, 1983). Perhaps some of the dense connections between ER and the plasmalemma may be represented by ryanodine receptors. Walton et al. (1991) reported the presence of ryanodine receptors in subplasmalemmal cisternae, but from their images it was not possible to conclude whether ryanodine receptors are concentrated at sites of apposition between the two membranes.

Dense projections connecting adjacent ER cisternae appeared as 20 nm particles when seen in tangential views. Similar particles were also visible in en face views of ER tubules and cisternae apparently not involved in membrane appositions and were present at very high density on these membranes as well (Fig. 5*a,b*). Such observation is another piece of evidence suggesting a high concentration of the $InsP_3$ receptor on most smooth ER membranes, although these particles cannot be conclusively identified as the $InsP_3$ receptors. If they do represent $InsP_3$ receptors, their close spacing appears mutually exclusive with the presence of ribosomes (see Fig. 5*e*). This is in agreement with the selective accumulation of the $InsP_3$ receptor in the smooth ER.

Cisternal stacks visible in PCs are generally considered fixation artifacts or the expression of PC damage (Herndon, 1964; Karlsson and Schultz, 1966; Van Nimwegen and Sheldon, 1966; Bestetti and Rossi, 1980; Hansson 1981). Massive formation of these stacks has been observed under certain experimental conditions (Herndon, 1964; Karlsson and Schultz, 1966; Bestetti and Rossi, 1980; Hansson, 1981). Even in some of our preparations, virtually all smooth ER elements visible in a field appeared to be involved in the formation of membrane appositions with one or more other ER elements (see, e.g., Fig. 3*a*). However, we consistently observed at least some ER stacks in all Purkinje cells, including cells that appeared optimally fixed.

We suggest that the entire smooth ER of PCs has the special property to form cisternal aggregates and that the number of

ER stacks present *in situ* may be the results of a dynamic equilibrium between this property and other factors that tend to disperse the ER. Whatever the nature of ER stacks, the hypothesis that intercisternal connections may be represented by $InsP_3$ receptor molecules provides a possible explanation for the selective occurrence of these structures in PCs. It will be of interest to determine whether the $InsP_3$ receptor itself is directly involved in linking apposed membranes, for example, by establishing homophilic interactions with $InsP_3$ receptor molecules located on opposite cisternae.

As previously noted, ER stacks have some resemblance to stacks that constitute the spine apparatus (Gray, 1959) of a variety of neurons. The intercisternal space of the spine apparatus, however, is somewhat different in structure (Spacek, 1985). In addition, a typical "spine apparatus" is not seen in dendrites of PCs (Spacek, 1985). These considerations, as well as the selective concentration of the $InsP_3$ receptor in PCs, speak against the hypothesis that the spine apparatus visible in many neurons might be the precise equivalent of cisternal stacks seen in PCs. However, it may represent an homologous structure.

There are no clues regarding the possible function of ER stacks. If they do occur *in vivo,* the very flat lumena of the cisternae and the abundance of Ca^{2+} release channels on their membranes raise the possibility that efflux of Ca^{2+} from these structures mediated by $InsP_3$ might be very prominent but rapidly self-limiting due to lack of a large Ca^{2+} storage compartment directly adjacent to the channels. Satoh et al. (1990) proposed that $InsP_3$ receptor molecules localized at sites of membrane attachment may not be functional. If $InsP_3$ receptor molecules at cisternal appositions were nonfunctional, an increased number of the stacks in damaged cells (Herndon, 1964; Karlsson and Schultz, 1966; Van Nimwegen and Sheldon, 1966; Bestetti and Rossi, 1980) might reflect an adaptive change aimed at minimizing the possibility of a devastating Ca^{2+} efflux from the ER.

In addition to the $InsP_3$ receptor, a SERCA2 Ca^{2+}-ATPase (Gunteski-Hamblin et al., 1988; Kaprielian et al., 1989; Plessers et al., 1991), which is probably represented primarily by SERCA2b (Plessers et al., 1991), was found to have a widespread distribution within the ER of chicken Purkinje cells, including ER elements present in the spines and cisternal stacks. In many cases the uneven distribution of immunogold at cisternal surfaces suggested a clustered distribution of Ca^{2+}-ATPase molecules. Interestingly, a clustered distribution has been described for Ca^{2+}-ATPase of muscle sarcoplasmic reticulum (Franzini-Armstrong and Ferguson, 1985). We did not find any evidence for the presence of Ca^{2+}-ATPase at sites of membrane apposition in cisternal stacks, consistent with the possibility that $InsP_3$ receptors at these locations might form crystal-like aggregates. An interesting difference between the distribution of the $InsP_3$ receptor and that of the Ca^{2+}-ATPase was the much lower concentration of the Ca^{2+}-ATPase in axons (beyond their initial portion traveling in the granule cell layer). A different ratio between the two molecules in axons and dendrites may reflect different patterns of Ca^{2+} signaling in the two compartments.

Chicken cerebellum is the only nonmuscle tissue where bona fide calsequestrin has been demonstrated (Volpe et al., 1990a). We have shown here that within chicken cerebellum, calsequestrin is selectively localized in PCs. The subcellular localization of calsequestrin was of special interest. This protein was present at low concentration throughout the ER, but was undetectable in the rough ER. This was in contrast to the presence of another low-affinity/high-capacity Ca^{2+}-binding protein, Bip (Bole et al., 1986), in the lumena of both the rough and the smooth ER. In addition, calsequestrin was particularly concentrated in short tubules and vesicles with a moderately dense core. Many of these structures appeared to be part of the smooth ER, but some appeared to be disconnected from the ER network.

In double-labeling experiments the profiles of many of these isolated calsequestrin-rich vesicles were found to be devoid of $InsP_3$ receptor immunoreactivity. However, structures with the morphological characteristics (size and core electron density) of these vesicles were heavily immunoreactive in preparations labeled for Ca^{2+}-ATPase, suggesting that they contain the Ca^{2+} pump (see Fig. 6*d*). The relationship of calsequestrin-rich organelles to the ER proper is at present unclear. Several hypotheses, which include the following, can be considered.

The first possibility, which we favor, is that they may form by direct budding from the ER, such budding being completely distinct from the budding of carrier vesicles destined to the Golgi complex. If so, they may be in dynamic continuity with the ER via repeated cycles of fusion and budding. Alternatively, they may represent an intermediate compartment (Pelham, 1989) between the ER and the Golgi. This hypothesis seems unlikely because these vesicles, as well as the smooth ER, are present in nerve terminals, while the Golgi complex is restricted to perikarya and dendrites (De Camilli et al., 1986). A third possibility is that they may represent organelles distal to the Golgi complex or at least to the cis-Golgi complex. Even this possibility seems unlikely because calsequestrin would be the first example of a lumenal protein concentrated in both a pre-Golgi and a post-Golgi compartment. The elucidation of the carbohydrate structure covalently attached to calsequestrin may provide some insight into the biogenesis of calsequestrin-rich vesicles. In chicken skeletal muscle, calsequestrin is endo-H sensitive, yet about one-

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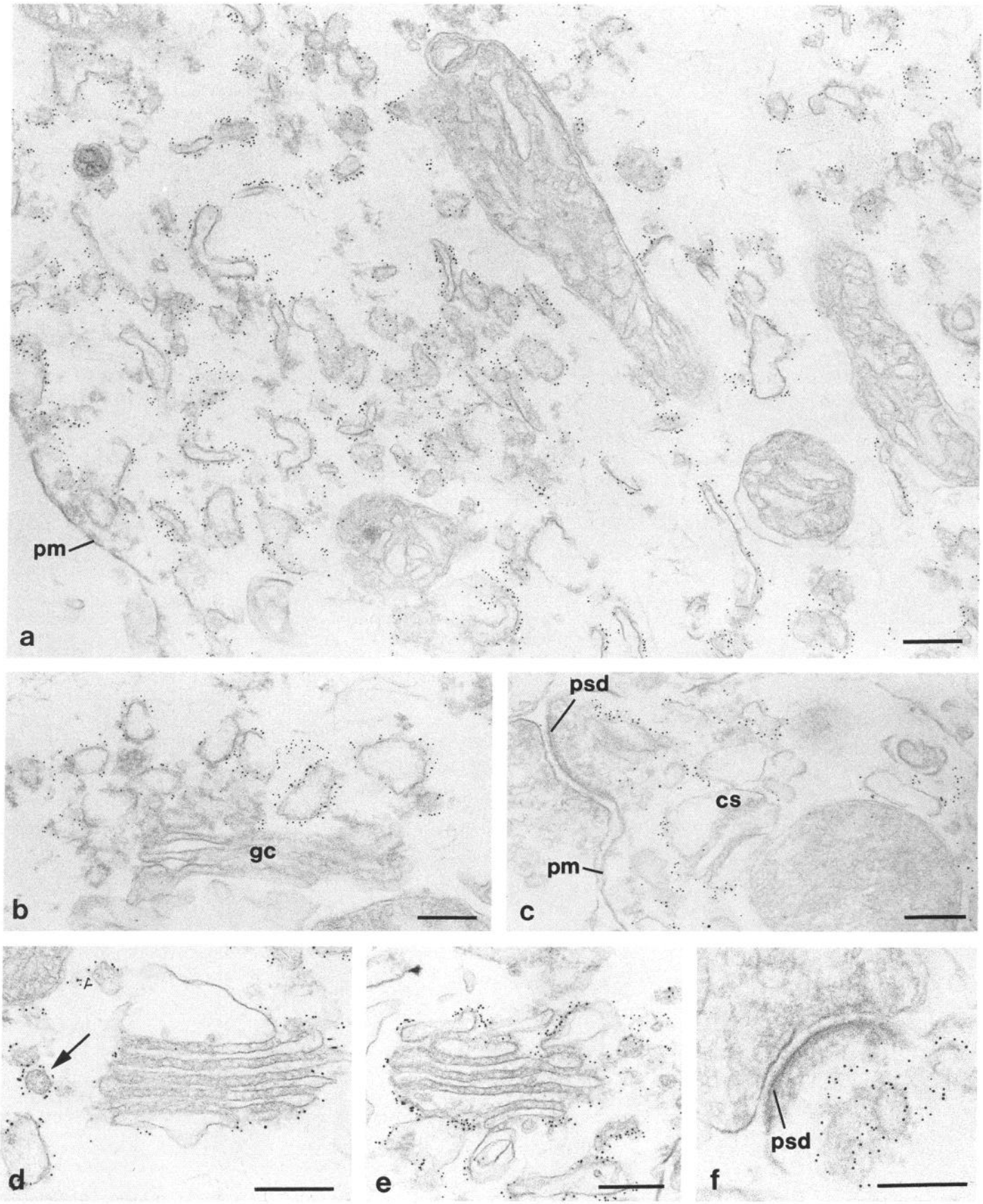
Figure 6. Localization of Ca^{2+}-ATPase in dendrites of chicken PCs: fragments of cerebellar cortex embedded in agarose and labeled for Ca^{2+}-ATPase by an immunogold procedure. *a,* Proximal PC dendrite. Most intracellular membranous profiles visible in the field, but not the plasmalemma (*pm*), are immunoreactive. Gold labeling appears unevenly distributed on the surface of these membranes. *b,* Golgi complex (*gc*) (unlabeled), surrounded by labeled vesicular structures. *c,* Portions of PC dendrites including a cisternal stack (*cs*) and a postsynaptic density (*psd*). *d* and *e,* Cisternal stacks: patches of gold particles are visible on free surfaces of the cisternae. The *arrow* in *d* points to a vesicle with moderately dense content reminiscent of vesicles that contain calsequestrin (see Fig. 8). *f,* Presence of gold labeling on internal membranes of a dendritic spine. Scale bars, 200 nm.

Figure 7. Localization of calsequestrin immunoreactivity in ultrathin frozen sections. *a–e,* Perikaryal (*a* and *b*) and dendritic (*c–e*) regions of PCs in sections of chicken cerebellum fixed *in situ.* Gold particles are visible on elements of the smooth ER and are particularly concentrated in vesicular structures with a moderately dense content (*arrowheads*). In preparations labeled with small gold particles (5 nm), some label is seen on cisternal stacks (*cs*). No label is present on cisternae of the Golgi complex (*gc*) or on elements of the rough ER (*rer*). *f–h,* Cerebellar fragments (chicken) were first immunolabeled for the $InsP_3$ receptor by the agarose-embedding procedure (4 nm gold particles) and then processed for calsequestrin immunoreactivity by the ultrathin frozen section procedure (10 nm gold particles). Some (*f* and *g*), but not all (*h*) calsequestrin-positive vesicles are completely devoid of $InsP_3$ immunoreactivity. Scale bars, *a–c,* 200 nm; *d–h,* 100 nm.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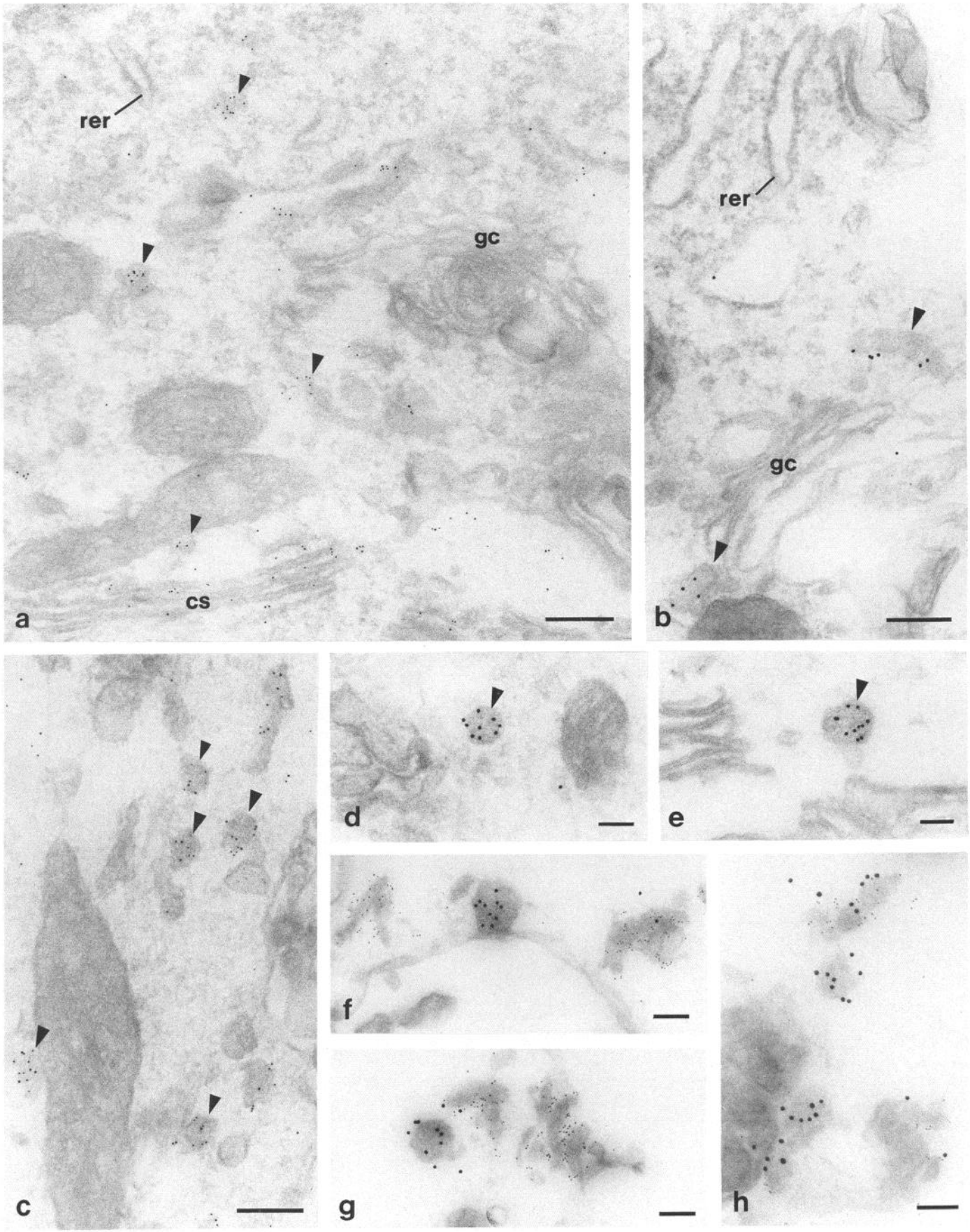
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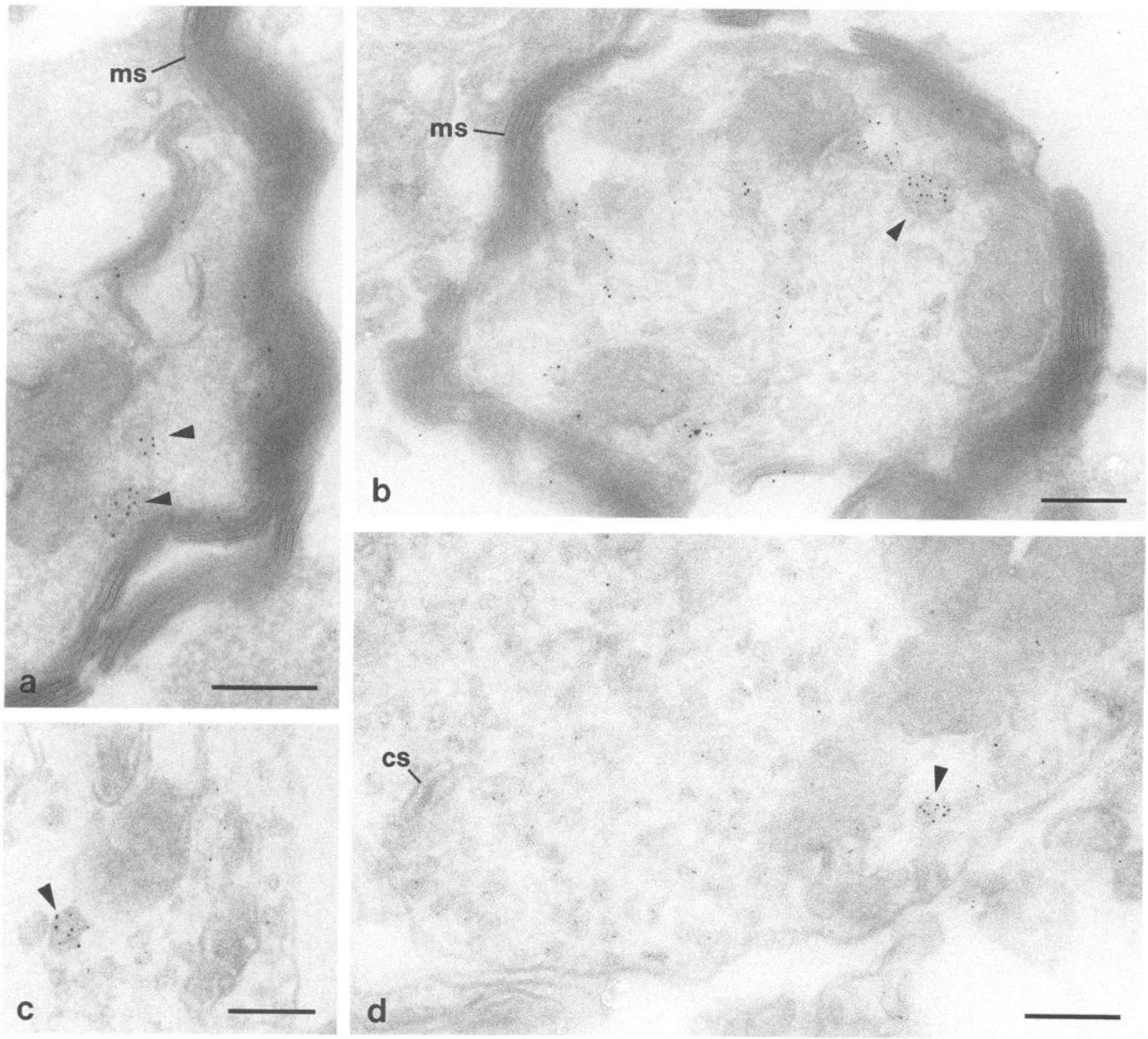

Figure 8. Localization of calsequestrin immunoreactivity in ultrathin frozen sections of axons (*a* and *b*) and axon terminals (*c* and *d*) of chicken PCs. *a* and *b*, Gold particles are localized on elements of the smooth ER and seem to be more concentrated on vesicular structures with a moderately dense content (*arrowheads*). Note lack of labeling of synaptic vesicles (very small particles within synaptic vesicles are postfixation artifacts) and on a cisternal stack (*cs*). Low-level staining of cisternal stacks could be observed only when very small gold particles were used. *ms*, myelin sheath. Scale bars, 200 nm.

third of calsequestrin binds WGA (Thomas et al., 1989). This observation, together with the absence of the ER retention sequence KDEL (Pelham, 1989) in muscle calsequestrin (Fliegel et al., 1987; Scott et al., 1988), led to the suggestion that calsequestrin travels through the cis-Golgi before reaching its final destination (Thomas et al., 1989). It should be noted that connections between the cis-Golgi and the smooth ER in neurons have been described (Lindsey and Ellisman, 1985).

It is also highly unlikely that vesicles heavily labeled by anti-calsequestrin antibodies might represent neuropeptide-containing sensory granules [the so-called large dense-core vesicles (De Camilli and Jahn, 1990)] stained because of an immunological cross-reactivity between calsequestrin and proteins of the "granin" family. The granins, like calsequestrin, are low-affinity Ca^{2+}-binding proteins extremely rich in acidic amino acids (Huttner et al., 1991). First, the anti-calsequestrin antibodies used in this study were not found to cross-react with the granins in western blots (Volpe et al., 1990). Second, immunoreactivity recognized by these antibodies was restricted to PCs, while the granins have a widespread distribution in the brain (Huttner et al., 1991). Third, calsequestrin-rich vesicles were similarly observed at very high concentrations in all neuronal compartments. Large dense-core vesicles may be present in dendrites, but are in general much more concentrated in axon terminals (De Camilli and Jahn, 1990).

Recently, the presence of the ryanodine receptor was dem-

onstrated in cerebellar chicken PCs and the distribution of this protein was investigated by immunofluorescence and by electron microscopy using the ultrathin frozen section technique. The ryanodine receptor was demonstrated in ER elements of dendrites and axons but not in dendritic spines (Ellisman et al., 1990; Walton et al., 1991). The absence of both the ryanodine receptor and calsequestrin from spines is intriguing if one considers the close spatial relationship of the two proteins in skeletal muscle. In skeletal muscle calsequestrin forms an insoluble lumenal matrix within the terminal cisternae of the sarcoplasmic reticulum. This matrix is directly connected to the membrane regions that contain the ryanodine receptor (Saito et al., 1984; Franzini-Armstrong et al., 1987). An attractive possibility is that calsequestrin-rich vesicles may be enriched in ryanodine receptors, although the ryanodine receptor clearly appears to be localized in other portions of the ER as well (Ellisman et al., 1990; Walton et al., 1991). This possibility has been supported by subcellular fractionation experiments that have indicated a different migration of the ryanodine receptor and of the $InsP_3$ receptor in isopicnic sucrose density gradients and a sedimentation of calsequestrin similar to that of the ryanodine receptor in the denser fractions of these gradients (P. Volpe, E. Damiani, A. Villa, P. Podini, and J. Meldolesi, unpublished observations).

While this work was in progress, a parallel study on the distribution within chicken PCs of calsequestrin, Ca^{2+}-ATPase, and Bip was carried out by Villa et al. (1991) using the ultrathin frozen section technique. The results of the two studies are in general good agreement. Villa et al. (1991) also reported that calsequestrin is concentrated in a subpopulation of smooth-surfaced vacuoles and tubules and is present at lower concentration in the ER. They demonstrated that calsequestrin-rich vesicles are Bip negative and suggested that calsequestrin may form aggregates that exclude other proteins. In contrast to our finding, they did not observe any apparent difference between calsequestrin content of rough and smooth ER. This discrepancy remains to be further investigated. Our study complements the study by Villa et al. (1991) by reporting a comparison between the distribution of the $InsP_3$ receptor and the distribution of calsequestrin using double-labeling techniques. Conversely, Villa et al. (1991), who had available antibodies directed against Ca^{2+}-ATPase and calsequestrin from different animal species, reported some immunocytochemical evidence for the presence of Ca^{2+}-ATPase on calsequestrin-rich vesicles.

On the basis of our present results, and of the results of Villa et al. (1991) and Walton et al. (1991), we suggest that the $InsP_3$ receptor, the ryanodine receptor, Ca^{2+}-ATPase, and calsequestrin are all ER-resident proteins, which at least in PCs are unevenly segregated in ER subcompartments that may be anatomically discontinuous. Such an hypothesis is consistent with the presence in some cell types of two pools of intracellular Ca^{2+} differentially responsive to physiological stimuli and pharmacological manipulations (Burgoyne et al., 1989; Malgaroli et al., 1990; Wakui et al., 1990). The two ER subcompartments may be functionally interconnected by fusion and budding, thus allowing a dynamic communication between the two pools. It is of interest that GTP, which has been shown to be required for the fusion of intracellular membranes, was found to promote communication between distinct Ca^{2+} stores in cell-free systems (Ghosh et al., 1989). The possibility can be considered that vesicles positive for calsequestrin-like and Ca^{2+}-ATPase immunoreactivities previously described in other cell types and defined as "calciosomes" (Hashimoto et al., 1988; Volpe et al., 1988; Treves et al., 1990) represent compartment homologs to the calsequestrin-rich vesicles observed now in PCs.

The novel information emerging from the study of intracellular Ca^{2+} stores demonstrates that the ER of a neuron is more heterogeneous than previously thought. In addition to the rough and smooth ER, other ER subcompartments can be identified within the smooth ER of PCs. Furthermore, the relative abundance of specific molecules is different in the smooth ER of dendrites and axons. As suggested by the distribution of calsequestrin and Bip, even the ER lumenal protein matrix varies in composition from one region of the reticulum to another in spite of anatomical continuity. It will be of interest to identify the mechanisms responsible for creating and maintaining these different ER microdomains.

In conclusion, our findings provide new clues on the functions of the ER as an intracellular Ca^{2+} store regulated by second messengers. In addition, they suggest a structural base for the existence of distinct and differentially regulated Ca^{2+} pools. PCs are clearly highly specialized cells. Chicken PCs may be even more specialized because, so far, chicken PCs are the only nonmuscle cells in which presence of calsequestrin has been demonstrated (Volpe et al., 1990a), yet the organization of intracellular Ca^{2+} stores in these cells is likely to be somehow related to the organization of these stores in other nonmuscle cells.

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