# Intracellular Ca²⁺ Stores in Chicken Purkinje Neurons: Differential Distribution of the Low Affinity-high Capacity Ca²⁺ Binding Protein, Calsequestrin, of Ca²⁺ ATPase and of the ER Lumenal Protein, Bip

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*Abstract.* To identify intracellular Ca²⁺ stores, we have mapped (by cryosection immunofluorescence and immunogold labeling) the distribution in the chicken cerebellar cortex of an essential component, the main low affinity-high capacity Ca²⁺ binding protein which in this tissue has been recently shown undistinguishable from muscle calsequestrin (Volpe, P., B. H. Alderson-Lang, L. Madeddu, E. Damiani, J. H. Collins, and A. Margreth. 1990. *Neuron.* 5:713–721). Appreciable levels of the protein were found exclusively within Purkinje neurons, distributed to the cell body, the axon, and the elaborate dendritic tree, with little labeling, however, of dendritic spines. At the EM level the protein displayed a dual localization: within the ER (rough- and smooth-surfaced cisternae, including the cisternal stacks recently shown [in the rat] to be highly enriched in receptors for inositol 1,4,5-trisphosphate) and, over 10-fold more concentrated, within a population of moderately dense, membrane-bound small vacuoles and tubules, identified as calciosomes. These latter structures were widely distributed both in the cell body (∿1% of the cross-sectional area, partic-

ularly concentrated near the Golgi complex) and in the dendrites, up to the entrance of the spines. The distribution of calsequestrin was compared to those of another putative component of the Ca²⁺ stores, the membrane pump Ca²⁺ ATPase, and of the ER resident lumenal protein, Bip. Ca²⁺ ATPase was expressed by both calciosomes and regular ER cisternae, but excluded from cisternal stacks; Bip was abundant within the ER lumena (cisternae and stacks) and very low within calciosomes (average calsequestrin/Bip immunolabeling ratios were ∿0.5 and 36.5 in the two types of structure, respectively). These results suggest that ER cisternal stacks do not represent independent Ca²⁺ stores, but operate coordinately with the adjacent, lumenally continuous ER cisternae. The ER and calciosomes could serve as rapidly exchanging Ca²⁺ stores, characterized however by different properties, in particular, by the greater Ca²⁺ accumulation potential of calciosomes. Hypotheses of calciosome biogenesis (directly from the ER or via the Golgi complex) are discussed.

**D**URING the past decades, continuous attention has been focused on intracellular, rapidly exchanging Ca²⁺ stores, first of muscle fibers (the sarcoplasmic reticulum [SR]¹), and more recently also of nonmuscle cells (for reviews see Campbell, 1986 and Meldolesi et al., 1990). The latter cell types express structures sensitive to the receptor-generated second messenger, inositol 1,4,5-trisphosphate (Ins-P₃, Berridge and Irvine, 1989; Meldolesi et al., 1990). In addition, at least some of them release Ca²⁺ when treated

with caffeine, apparently from stores functionally, and probably also structurally, distinct from those sensitive to Ins-P₃ (see Thayer et al., 1988; Malgaroli et al., 1990; Wakui et al., 1990; Burgoyne et al., 1989). The nature of the Ca²⁺ stores in nonmuscle cells remains undefined. Initially, the recovery of the Ins-P₃–induced release activity with the microsome fraction was taken as a proof for the ER (Streb et al., 1984). Subsequent experiments demonstrated, however, recovery not with the whole but with discrete subfractions, in some cases of higher, in others of lower buoyant density compared to average microsomes (Henne et al., 1987; Guillemette et al., 1988; Volpe et al., 1988; Thévenod et al.,

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1. *Abbreviations used in this paper:* Ab, antibody; CR, calreticulin; CS, calsequestrin; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

1989; Alderson and Volpe, 1990). These results were interpreted as indications that the store resides either in an ER subcompartment or in another, non-ER component of the microsome fraction.

In order for the $Ca^{2+}$ storage structures to be functionally competent they need to possess not only $Ca^{2+}$ uptake and release, but also the ability to accumulate large amounts of calcium in a state that permits rapid exchange. In striated muscle fibers (see Campbell, 1986) such an activity is subserved by calsequestrin (CS), a protein binding $Ca^{2+}$ with low affinity (kD ~1 mM) and high capacity (44 mol/mol). In the SR, CS is strategically concentrated within terminal cisternae, close to the large caffeine-sensitive release channels, the ryanodine receptors (RyRs). Another protein, calreticulin (CR), with properties similar to CS, was identified also in nonmuscle cells (Waismann et al., 1985; Fliegel et al., 1989; Smith and Koch, 1989) and shown (initially by immunostaining with cross-reactive anti-CS, and later with specific antibodies [Abs]) to be concentrated within peculiar structures, equipped also with a $Ca^{2+}$ ATPase, which were given the name of calciosomes (Volpe et al., 1988; Hashimoto et al., 1988; Treves et al., 1990).

Recent developments in this field have taken place by the study of cerebellar Purkinje neurons. These cells were found to express unusually high concentrations of both the Ins-P₃ receptor (Ins-P₃R; Ross et al., 1989; Maeda et al., 1989; Mignery et al., 1989, 1990) and the RyR (Ellisman et al., 1990). The Ins-P₃R, a ligand-gated channel composed of four identical 313-kD subunits (Ferris et al., 1989; Furuichi et al., 1989; Mignery et al., 1990), is now known to be concentrated primarily in a population of smooth-surfaced cisternae, often arranged in stacks, in direct lumenal continuity with the rest of the ER, distributed in all regions (soma, dendrites, axons, and terminals) of rat Purkinje neurons. Lower (~1:10) concentrations were found also in the ER and nuclear envelope (Satoh et al., 1990; Otsu et al., 1990). As far as the RyR (also a homotetramer, however, of ~500-kD subunits; Takeshima et al., 1989; Zorzato et al., 1990), its distribution appears widespread within Purkinje neurons, with exclusion, however, from the dendritic spines. The precise intracellular distribution of this receptor has not been established yet (Ellismann et al., 1990).

In our previous studies with rat Purkinje neurons (Satoh et al., 1990), the functional role of the stacked Ins-P₃R–rich cisternae could not be clarified because the low affinity-high capacity $Ca^{2+}$ binding protein expressed by those cells differs from both CS and CR and has not been characterized yet (Satoh et al., 1990; Meldolesi et al., 1990). In the chicken cerebellum, however, the main such protein was recently shown to be very similar (if not identical) to muscle CS (Volpe et al., 1990) and will thus be herewith designated with the same name. Using Abs against the muscle protein we demonstrate here cerebellar CS to be restricted to Purkinje neurons, with high concentrations, however, not within the stacked cisternae but within structures corresponding to the previously described calciosomes. Combined with immunolabeling results obtained with other Abs (against the cerebellar $Ca^{2+}$-ATPase; Kaprielian et al., 1989 and the ER
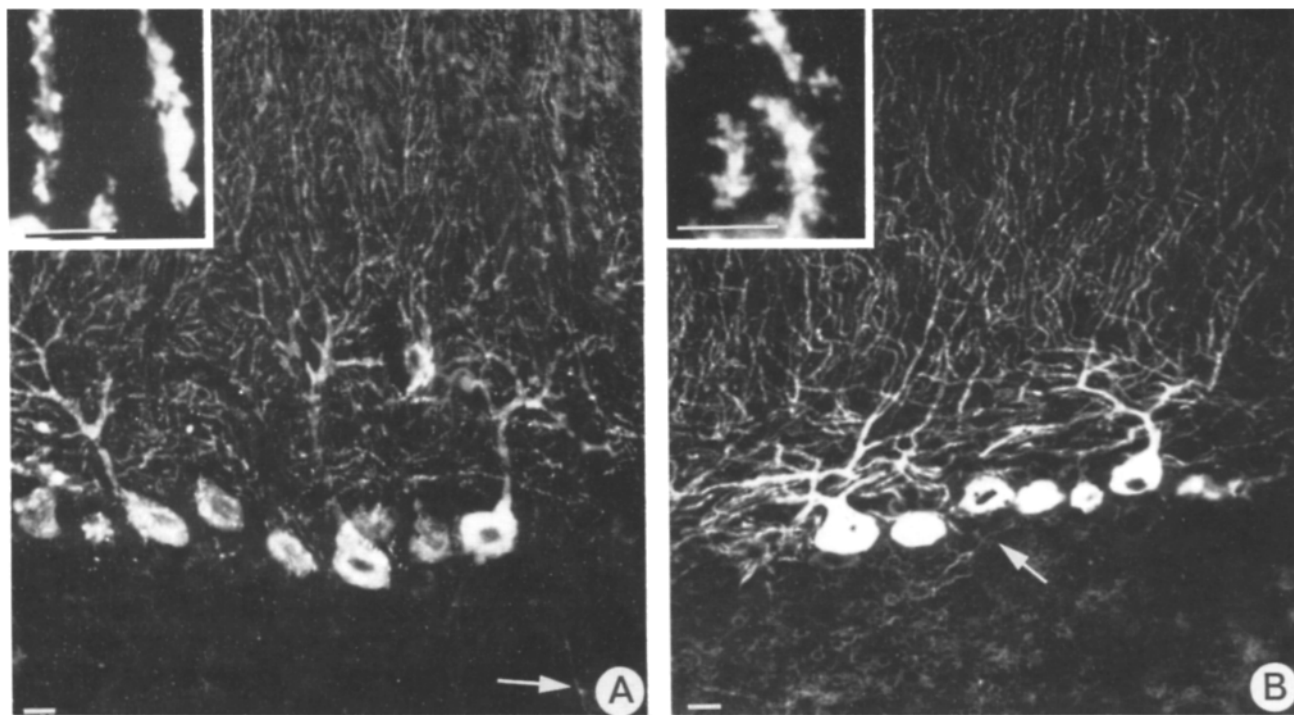


*Figure 1.* Calsequestrin (*A*) and $Ca^{2+}$ ATPase (*B*) immunofluorescence in chicken Purkinje neurons. The general picture for the two antigens is similar, with marked immunolabeling of both the large cell body and the dendritic tree. Axons traveling through the molecular layer of the cerebellar cortex are also labeled (*arrows*). The 1-$\mu$m-thick section, high power insets reveal that dendritic spines are strongly positive for $Ca^{2+}$ ATPase and most often negative for CS. Of the non-Purkinje cells of the cerebellar cortex, granule neurons (located in the granular layer, lower third of the panels) appear CS negative and exhibit a weak labeling for $Ca^{2+}$ ATPase. Bars: (*main panels*) 10 $\mu$m; (*insets*) 5 $\mu$m.
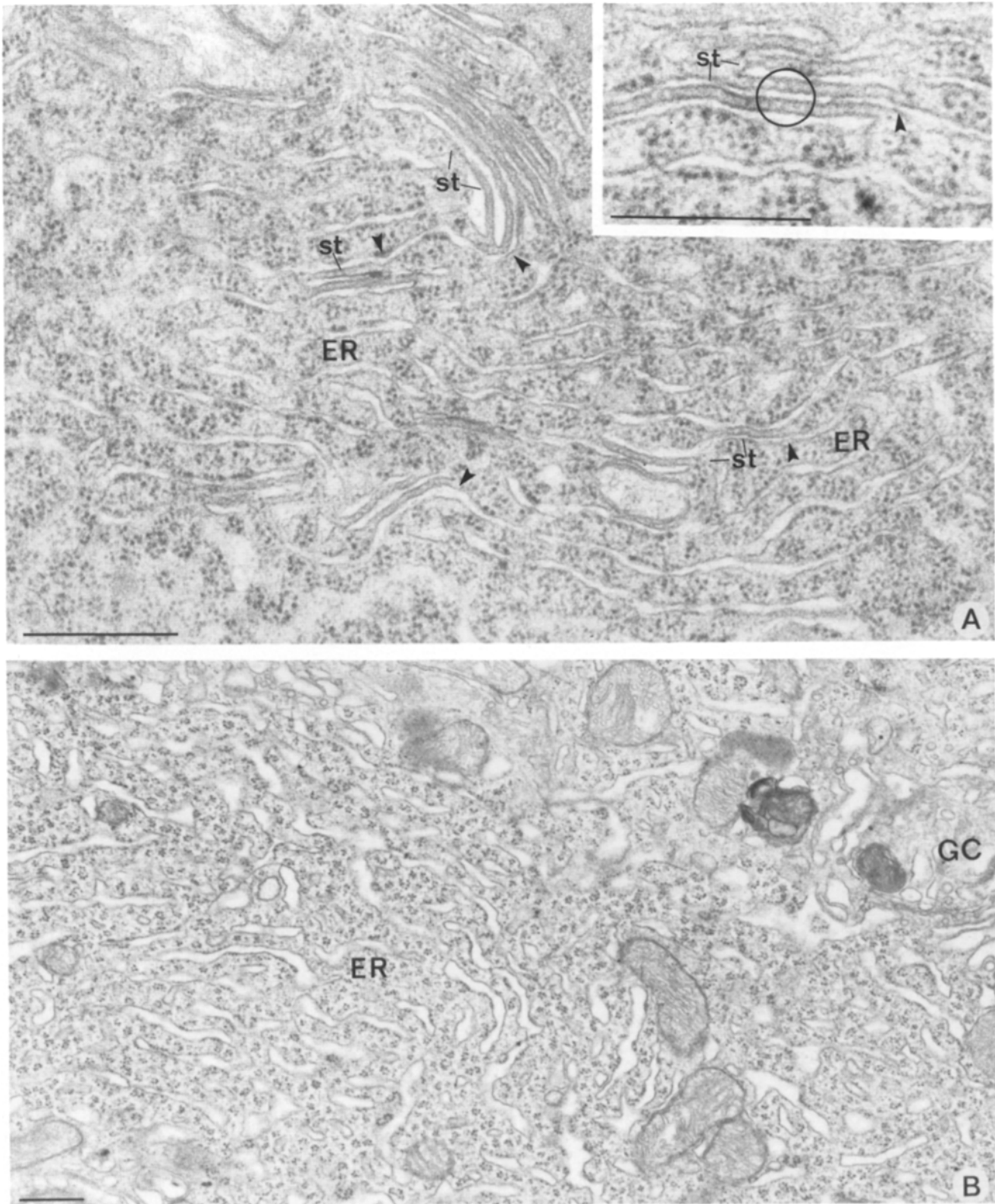
*Figure 2.* Conventional EM of chicken Purkinje neurons. Two cytoplasmic areas are illustrated. In *A*, numerous ER cisternae, running individually (mostly rough surfaced) or arranged in parallel stacks (in all cases smooth surfaced, *st*), are shown. Continuities between the two types of cisternae are marked by arrowheads. The evenly distributed bridges in between stacked cisternae are illustrated in the inset. *B* shows part of the Golgi area (*GC, upper right*) including large, dense bodies, numerous vesicles, and heterogeneous tubules and vacuoles. The rest of the field is occupied primarily by irregularly running ER cisternae, part of which are rough surfaced, intermingled with heterogeneous, smooth-surfaced elements of different size and shape. Bars, 0.5 μm.
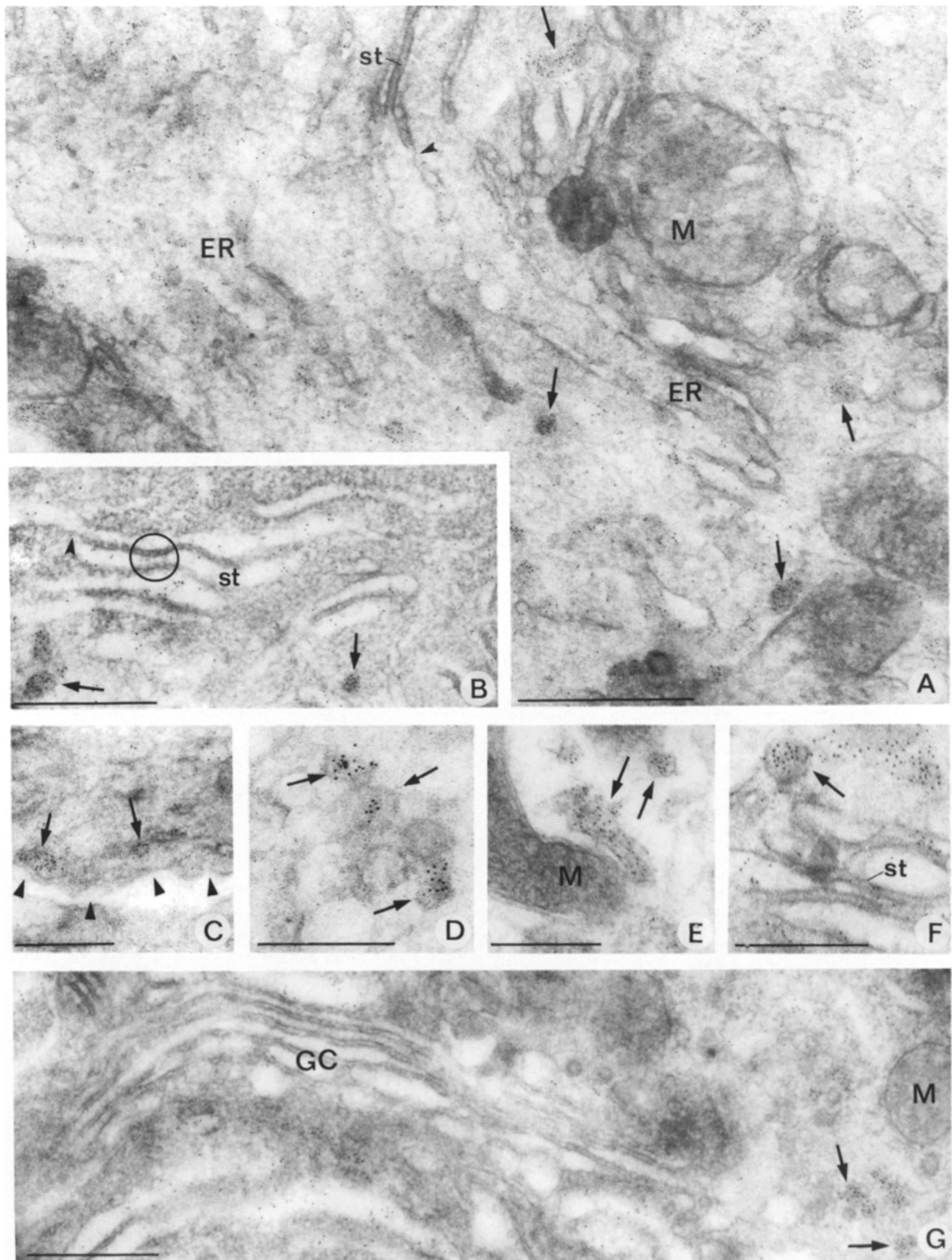
*Figure 3.* CS immunogold labeling of the Purkinje neuron body. High labeling occurs over calciosomes, a population of heterogeneous membrane-bound structures often, but not always, exhibiting a moderately dense content, part of which are marked by arrows. ER cisternae,

lumenal protein, Bip; Bole et al., 1986), these data provide clues about the nature, function, and biogenesis of intracellular $Ca^{2+}$ stores in Purkinje neurons.

## Materials and Methods

### Materials

Rabbit polyclonal antichicken muscle CS Abs were prepared and purified as described by Choi and Clegg (1990; see also Volpe et al., 1990). The mouse monoclonal anticardiac/slow twitch SR $Ca^{2+}$ ATPase 3H2 Ab and the rat monoclonal anti-Bip Ab were those described by Kaprielian et al. (1989) and Bole et al. (1986). The rabbit serum against rat ER membrane proteins was described by Louvard et al. (1982) and the polyclonal Ab against the rat cerebellar Ins-$P_3$R by Supattapone et al. (1988) and Ross et al. (1989). Additional polyclonal Abs against the latter protein were raised by injecting mice with the synthetic $NH_2$-terminal, 19 amino acid peptide, coupled to keyhole hemocyanin as described by Mignery et al. (1989). Pools of sera obtained from the injected animals were analyzed by both ELISA and Western blotting. Polyclonal Abs against the rabbit fast twitch muscle RyR are described by Zorzato et al. (1990). Rhodamine-labeled anti-rabbit, rat, and mouse IgGs were purchased from Technogenetics (Milan, Italy); and 5- and 15-nm colloidal gold particles, coated with goat IgGs against either rabbit (large and small particles), mouse, or rat (small particles only) IgGs were purchased from Biocell (Cardiff, UK). Other chemicals were analytical or the highest grade available.

### Conventional and Immunomicroscopy

A total of six adult hens, obtained from Incubatoio Bergamasco (Bergamo, Italy), were used. Two of them were anesthetized with tribromoethanol and then perfused through the heart, first with 500 ml of PBS at 4°C and then with a mixture of 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.25 glutaraldehyde in 125 mM phosphate buffer, pH 7.4 (500 ml at 4°C). The remaining hens were killed by decapitation. Cerebella were rapidly removed and immediately immersed in the fixative (4°C). Then ~1-mm-thick frontal slices were prepared and sectioned into squares. Those including the boundary between the molecular and granular layers of the cortex were further fixed in the same mixture for 2 additional h and then processed for both conventional microscopy and immunocytochemistry. Samples for conventional EM were washed extensively with the phosphate buffer, postfixed with 1% $OsO_4$ in 125 mM cacodylate buffer, dehydrated in ethanol, block stained with uranyl acetate, and embedded in Epon. Thin sections were double stained with uranyl acetate and lead citrate. The samples for cryosections were infiltrated with concentrated sucrose, frozen in a 3:1 mixture of propane and cyclopentane cooled with liquid nitrogen, and transferred to either a conventional cryostat or an Ultracut ultramicrotome equipped with a FC4 cryosection apparatus (both from Reichert Jung, Vienna, Austria) (section thickness ~15 and 1 $\mu m$, respectively). The sections were flattened over glass slides and covered with 2%

liquid gelatin in phosphate buffer. After a short treatment with 1% Na borohydrate, they were washed and exposed for 30 min to a normotonic solution containing 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer, pH 7.4. After being washed, the samples were exposed (1 h at 37°C or overnight at 4°C) to either one of the various primary Abs listed above, diluted in the Triton X-100, goat serum–containing solution. Sections were then washed again thoroughly and treated with the appropriate rhodamine-labeled goat 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.

For immunogold, ultrathin cryosections (50–100 nm) were collected over nickel grids and covered with 2% gelatin. After treatment with 125 mM phosphate buffer, pH 7.4, supplemented with 0.1 M glycine, they were exposed to the first Ab for 1 h at 37°C, (anti-CS, -$Ca^{2+}$ ATPase, -Bip, or nonimmune, diluted in phosphate-glycine buffer), and then washed with phosphate-glycine buffer and decorated with anti-IgG (rabbit, rat or mouse)-coated gold particles (5 nm, dilution 1:80 in the same buffer). For dual labeling the rabbit anti-CS polyclonal Abs were applied together with either the anti-$Ca^{2+}$ ATPase or the anti-Bip monoclonals, and the same was eventually done for the large (15 nm, coated with Abs anti–rabbit IgGs) and small (5 nm, coated with Abs anti-mouse or -rat IgGs) gold particles. The immunodecorated grids were then washed and processed as recommended by Keller et al. (1984). For additional details see Hashimoto et al. (1988). Both conventional sections and cryosections were examined in a Hitachi H-7000 electron microscope. Pictures were usually taken at 24,000×.

### Specificity and Quantitative Evaluation of Immunogold Labeling

With nonimmune serum the 5-nm gold particle labeling of cryosections was very low and uniformly distributed over the nucleus and cytoplasm of Purkinje and adjacent cells. The average labeling, calculated under standard conditions in a group of randomly chosen pictures, i.e., the background, was 4.2 gold particles/$\mu m^2$. With 15-nm particles background values were slightly higher (7.3/$\mu m^2$). When specific Abs were used, labeling over some of the Purkinje cell structures, such as nuclei and mitochondria, was not significantly different from the background.

For quantitative evaluation of immunolabeling (Griffiths and Hoppeler, 1986), particles laying over the lumen and limiting membranes of ER individual and stacked cisternae and calciosomes were counted in groups of randomly selected pictures obtained from sections processed under standard conditions. To express the data in terms of relative density, they were normalized to the cross-sectional areas of the analyzed structure, measured by means of a Zeiss MOP1 apparatus.

## Results

### Immunofluorescence

Fig. 1 illustrates the immunofluorescence patterns revealed in chicken cerebellar cortex slices (~15 $\mu m$ in thickness) by the use of Abs raised against the chicken muscle CS (an affinity-purified polyclonal Ab; Choi and Clegg, 1990; Fig. 1 A), and the cardiac/slow twitch isoform of the SR $Ca^{2+}$ ATPase (the 3H2 monoclonal Ab; Kaprielian et al. 1989; Fig. 1 B). As can be seen, in all cases immunoreactivity was found to be highly concentrated within Purkinje neurons where it was distributed to all regions: the cell body cytoplasm, the elaborate dendritic tree, and also the axon. A similar pattern was observed with an mAb against another protein, the ER lumenal chaperonin, Bip (Bole et al., 1986, not shown).

The localization of CS in the dendrites was further investigated in subsequent 1-$\mu m$-thick cryosections processed in

running individually (A) or in parallel stacks (st; A, B and F), are consistently but moderately labeled. Mitochondria (M; A, E, and G) are unlabeled, and the same occurs with Golgi cisternae (GC), recognized in G adjacent to labeled, moderately swollen cisternae of ER nature. Arrowheads in C label the cell surface. Bars: (A, B, and G) 0.5 $\mu m$; (C–F) 0.25 $\mu m$.
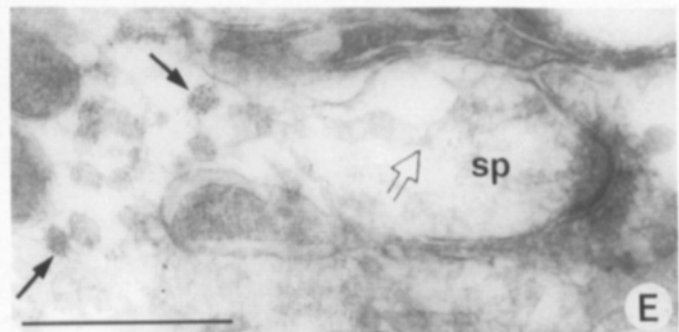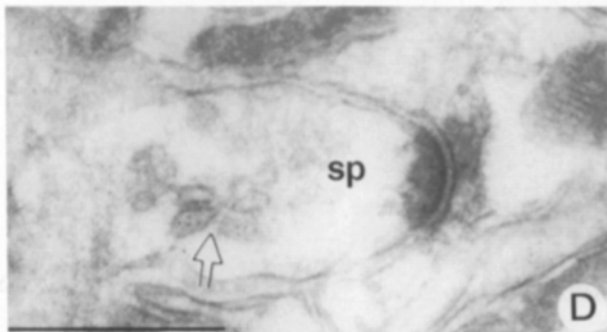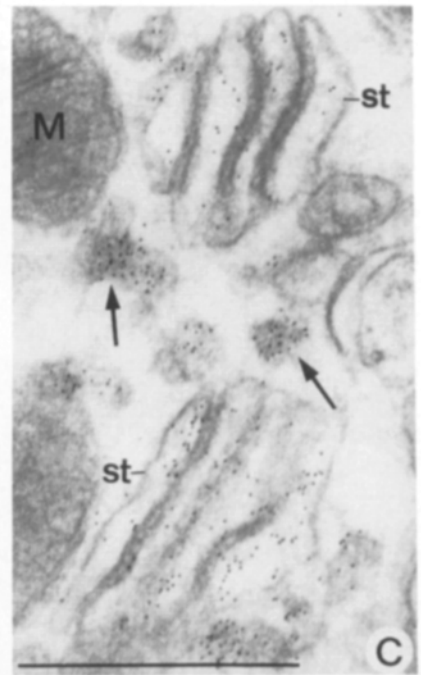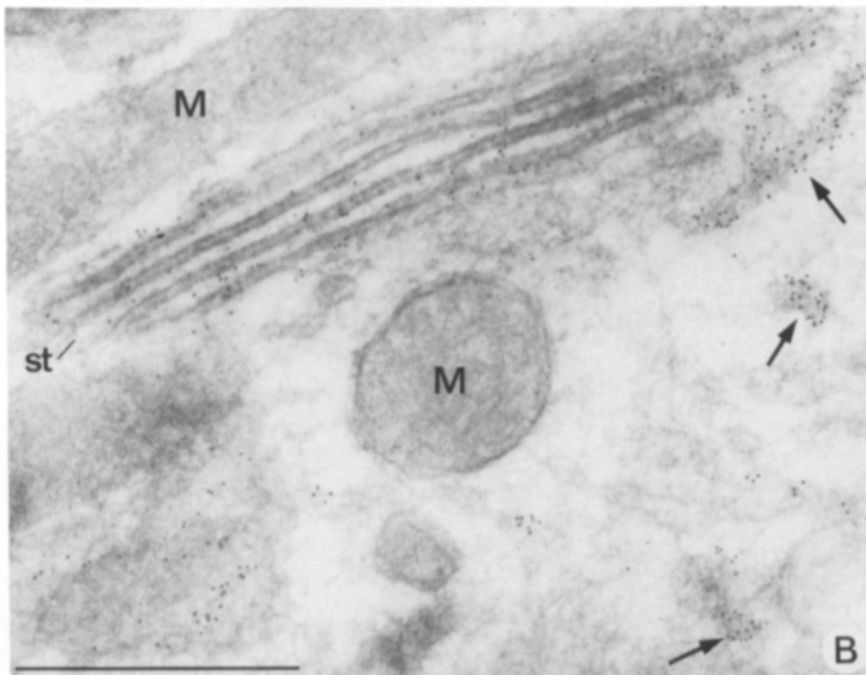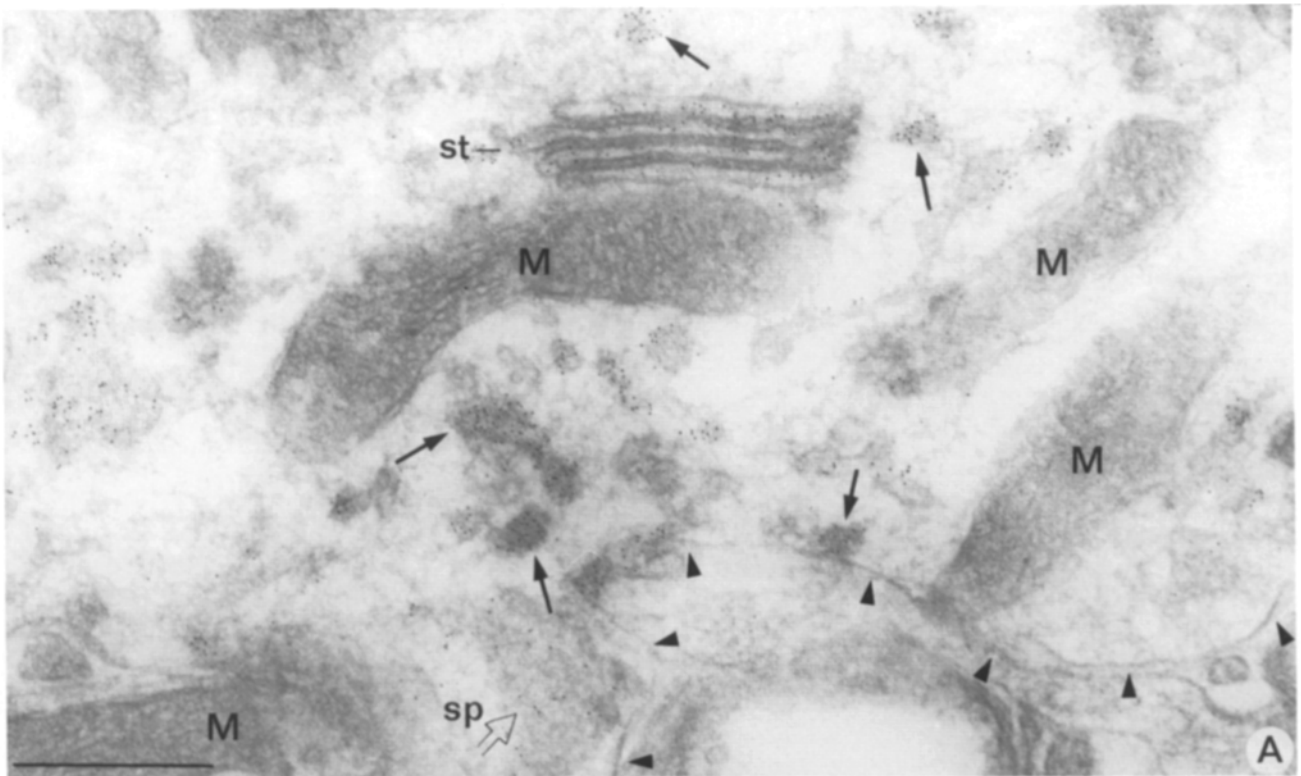
parallel with Abs against that protein and the $Ca^{2+}$ ATPase. The latter is known to be almost uniformly distributed in both the shaft and the spines (Michelangeli et al., 1991). In contrast, with the anti-CS Ab (Fig. 1, *A, inset*) the distribution was often irregular or granular in the shafts, and the number of clearly positive spines was only a small fraction with respect to that observed for $Ca^{2+}$ ATPase (Fig. 1 *B, inset*). With anti-CS Abs the other elements of the cerebellar cortex (in particular, granule and Golgi neurons of the granular layer; stellate and basket cells of the molecular layer; and glial cells) appeared negative (Fig. 1 *A*). In contrast, with anti-$Ca^{2+}$ ATPase, distinct, although weaker, signals were revealed in the cytoplasm of cell bodies, particularly in granule cells (Fig. 1 *B*; see also Kaprielian et al., 1989), while with anti-Bip, stellate and basket cells were also labeled (not shown).

An additional group of Abs was also tested. Weak signals in both Purkinje and granule neurons were obtained with a serum against ER membrane proteins (Louvard et al., 1982), whereas no response was seen with two anti-Ins-$P_3R$ Abs: the rabbit IgGs (Supattapone et al., 1988) previously employed with success in the rat cerebellum (Ross et al., 1989; Satoh et al., 1990) and a pool of sera obtained from mice injected with the 19 amino acid $NH_2$-terminal sequence of the receptor coupled to keyhole hemocyanin as described by Mignery et al. (1989). Likewise, no specific signal was obtained by using two purified IgG preparations raised against rabbit skeletal muscle RyR (Zorzato et al., 1990). In view of the weak or negative immunofluorescence results obtained with the second group Abs, their study was not pursued by the immunogold technique, which was therefore employed only with our first three Abs (against CS, $Ca^{2+}$ ATPase, and Bip).

## Conventional EM

The general ultrastructure of chicken Purkinje neurons (Fig. 2) closely resembles that of the rat (Palay and Chan-Palay, 1974; Satoh, 1990). In the cell body, large areas were occupied primarily by the rough-surfaced ER composed by numerous either parallel or irregularly distributed cisternae, often exhibiting direct lumenal continuity with smooth-surfaced elements: tubules and evenly spaced stacked cisternae (Rosenbluth, 1962; Satoh et al., 1990; Fig. 2 *A*). Compared to the rat, the latter structures were usually of smaller size: 2–6 instead of 2–12 piled cisternae. As in the rat (Satoh et al., 1990), the cisternae of these stacks appeared separated from each other by perpendicular, evenly spaced bridges, placed $\sim$25 nm from each other (center-to-center; Fig. 2 *A* and *inset*). Apparently discrete smooth elements of various size and shape, from small vesicles to larger vacuoles and tubules, were intermingled with the rough-surfaced cisternae and distributed also to the rest of the cytoplasm (Fig. 2 *B*), including the subplasmalemma region. At least

part of these structures are expected to correspond to the calciosomes identified by CS immunolabeling (see below). At the conventional EM level, however, a clear identification was impossible. The highly developed Golgi complex was equipped with numerous vesicles (coated and uncoated) and with a population of large ($\sim$0.5 $\mu$m in diameter), dense granules, sometimes engulfed with membrane profiles (Figs. 2 *B*; see 5 *A, inset*). Golgi cisternal lumena appeared clear except for the lateral tips where some moderately dense material was seen, similar to that contained within adjacent vesicles and small vacuoles (see Fig. 5 *A, inset*).

Smooth cisternal stacks were observed not only in the cell body but also in large dendrites. In addition, dendrites contained numerous longitudinal, smooth-surfaced elements (cisternae; convoluted and branched tubules), with some concentration near the attachment of or within the spines (the spine apparatus, not shown). Purkinje neuron's axons and axon terminals were not specifically investigated in the present work.

## CS Immunogold Labeling

As expected from immunofluorescence results, strong CS immunogold labeling was revealed in all regions of Purkinje neurons (Figs. 3–6), with no appreciable signal in the other cells and fibers of the cerebellar cortex. Of the organelles in the cell body, mitochondria, multivesicular bodies, and coated vesicles, were consistently unlabeled, and the same occurred with the nucleus. Consistent, but moderate, labeling was observed over the lumen of both rough-surfaced ER cisternae (recognized by their general architecture, distribution and, in many cases, also by ribosomal remnants at their surface) and smooth-surfaced cisternal stacks (Figs. 3, *A, B,* and *G*; see 5 *C*, 6, *A–C*; Table I). In these structures labeling was sometimes unevenly distributed. By far, however, the heaviest labeling was observed over a population of peculiar membrane-bound structures which will be indicated from hereon as calciosomes (overall labeling density, i.e., gold particles/$\mu m^2$ >10-fold the ER, Figs. 3, *A–G*; see 5, *A–C*, 6, *A* and *B*; Table I) accounting, on the average, for the 1.08% of the cell body cross-sectional area. Size and shape of calciosomes varied considerably, from vesicles to irregular or multilobated vacuoles (see Fig. 3, *C–F*). The electron density of their segregated content appeared often moderately higher than the cytosol and the content of ER cisternae. Calciosomes were widely distributed throughout the cytoplasm, adjacent to the plasma membrane (Fig. 3 *C*), mitochondria (Fig. 3, *A* and *E*), ER, and stacked cisternae (Figs. 3, *A, B,* and *F*; see 5 *C*, 6 *B*). Direct lumenal continuity with the latter organelles was never observed in the cell body. Of particular interest was the Golgi complex. Most cisternae were free of any CS immunolabeling (Figs. 3 *G*; see 5, *A–C*) except for the lateral tips, where moderately dense, CS-labeled material was often revealed (see Fig. 5, *A* and *B*). In the

*Figure 4.* CS immunolabeling of Purkinje neuron dendrites. High immunolabeling is observed over the heterogeneous population of calciosomes (marked in part by *solid arrows*) scattered around in the dendrite shaft, with some concentration near the entrance of the dendritic spines (*sp; A* and *E*). The organelles within the spines (the spine apparatus; *open arrows*) appear partially labeled (*A* and *D*) or unlabeled (*E*). The mostly longitudinal ER cisternae and cisternal stacks (*st; A–C*) appear variably but often moderately labeled. Mitochondria (*M; A–C*), the structures belonging to adjacent cells (in *A*, below the plasma membrane marked by *arrowheads*) and the impinging synapses (*A, D,* and *E*), are unlabeled. Bars, 0.5 $\mu$m.

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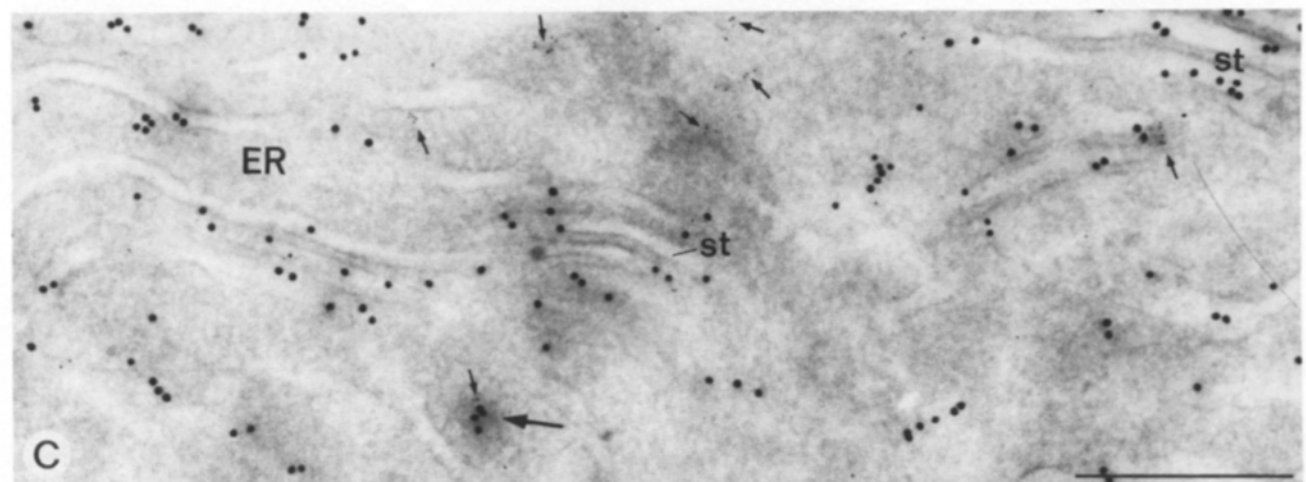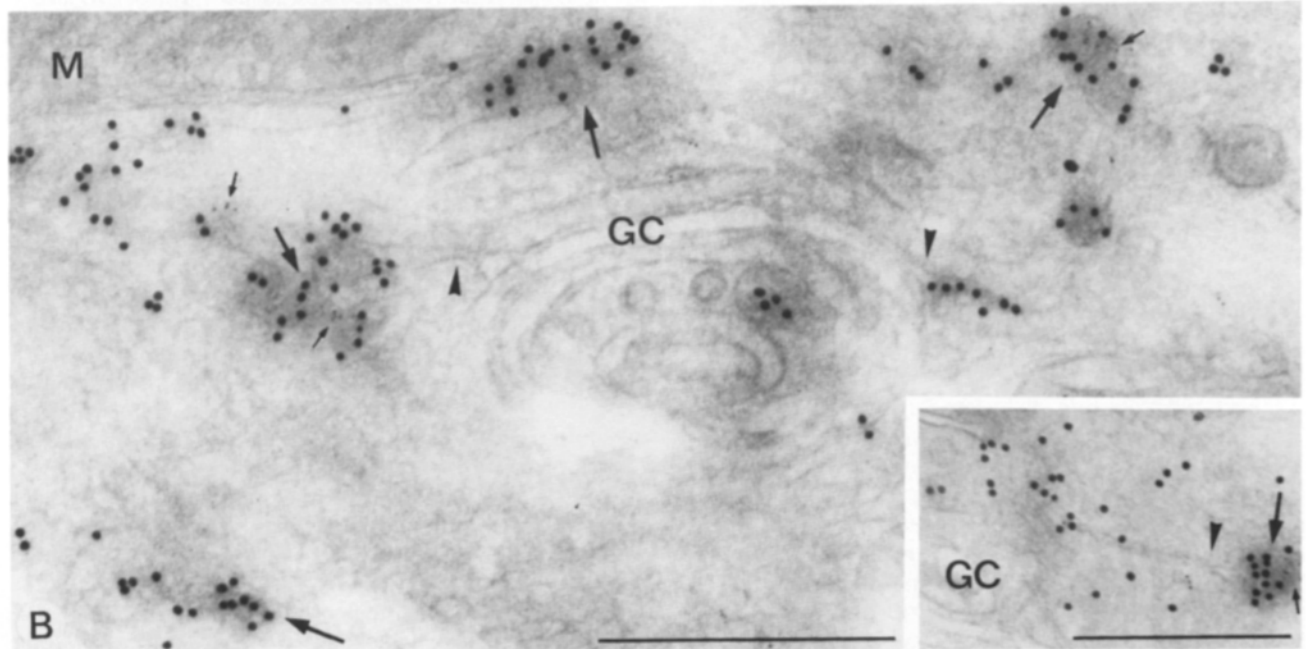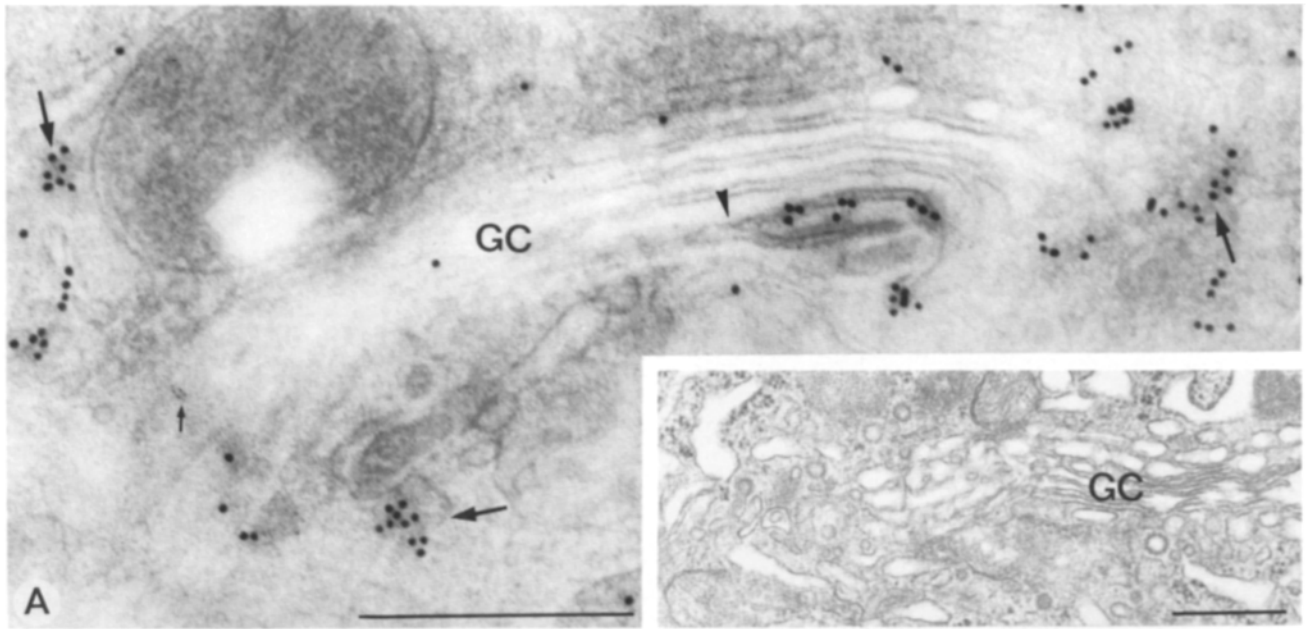
Golgi area, unlabeled vesicles and other structures were seen to coexist with numerous typical, highly labeled calciosomes (Figs. 3 G; see 5, A and B). Apparent direct continuity of a few of these juxta-Golgi calciosomes with Golgi cisternal tips was observed in favorable cryosections (see Fig. 5, A and B).

In dendrites (Fig. 4), a larger proportion of the intracellular membrane-bound structures ($\sim$2.6% of the cross-sectional area) appeared highly CS labeled. Compared to the cell body, the shape of these structures was more variable, with coexistence of moderately dense, apparently discrete calciosomes with larger structures (cisternae and tubules) less homogeneously (or very irregularly) labeled (Fig. 4, A and B), sometimes in apparent continuity with unlabeled tubules and vesicles. Concentration of highly labeled structures was seen especially at the entrance of spines (Fig. 4, A and E). Within the spines, however, labeling was low or absent (Fig. 4, A, D, and E). Similar to the cell body, ER and stacked cisternae of the dendrites were labeled, however, only to moderate extents (Fig. 4, A-C).

## Ca²⁺ ATPase and Bip Immunogold Labeling

Localization of these antigens was investigated primarily in relation to that of CS. Most of the experiments were thus carried out by dual labeling, with large gold addressed to CS and small gold to either one of the other proteins.

Compared to immunofluorescence, the immunogold signal obtained with the Ca²⁺ ATPase Ab was relatively weak (Fig. 5). However, specificity was clear because, at the Ab concentration employed, large areas (nuclei, mitochondria, cytosol) exhibited background labeling, while higher levels (8.2 and 9.6 times the background, respectively) were observed over individual ER cisternae (Fig. 5 C) and calciosomes, the latter recognized because of their intense large gold CS labeling (Fig. 5, A-C). Over these structures the small Ca²⁺ ATPase gold particles were not evenly distributed, but often arranged in small clusters, preferentially located over tangentially sectioned membranes. Golgi cisternae appeared unlabeled except for a few, labeled towards the tips (Fig. 5 A). In the ER stacked cisternae Ca²⁺ ATPase labeling was sometimes seen over the external, cytoplasmic face and the adjacent, lumenally continuous, rough-surfaced cisternae, but never over the stacks proper (Fig. 5 C).

Bip immunoreactivity (small particles in Fig. 6) was prominent in the ER (rough-surfaced cisternae as well as cisternal stacks). In this case, consistent with the recognized lumenal state of the protein (Bole et al., 1986; Pelham, 1989), labeling was located almost exclusively over the cisternal lumena. Nuclei, mitochondria, Golgi cisternae, trans-Golgi elements, and endosomal elements (peripheral coated vesicles and multivesicular bodies) were at the background level. Calciosomes were also negative (Fig. 6, A, B, and D), except for a few that exhibited either one or two small gold particles, located just beneath the limiting membrane. Countings were made in 12 randomly selected pictures. Large (CS) and small (Bip) gold labeling densities over 145 and 126 rough-surfaced and stacked ER cisternae were similar (78 ± 31 and 173 ± 67; 106 ± 60 and 174 ± 89 particles/$\mu$m², respectively). The large/small particle ratios over these two structures were thus also similar (0.45 and 0.61). In a population of 91 calciosomes only a few were labeled for Bip. The Bip labeling density of these structures (30 particles/$\mu$m²) was thus much lower, and the large/small particle ratio (36.5) much higher than in ER cisternae. The differential labeling of Bip and CS was observed not only in the cell body but also in the dendrites (Fig. 6 D). In the latter structures, however, precise countings have not been made yet.

## Discussion

As discussed extensively elsewhere (Meldolesi et al., 1990), any rapidly exchanging intracellular Ca²⁺ storage organelle needs to express at least three molecular components: (a) a Ca²⁺ uptake system, such as a Ca²⁺ ATPase; (b) a regulated Ca²⁺ channel, for rapid cation release; and (c) a low affinity-high capacity Ca²⁺ binding protein, to keep the free Ca²⁺ concentration within the store relatively low (in the mM range) and thus permit high calcium loading with no risk of excess formation (and precipitation) of calcium phosphates. In the storage organelles the relative concentration of these three components is expected to vary considerably. In spite of their high capacity (44–50 Ca²⁺ binding sites for CS and CR, Campbell, 1986 and Treves et al., 1990), storage proteins need in fact to be highly concentrated within the organelles, a condition favorable for immunocytochemical revelation. Indeed, with Abs against storage proteins, adequate signals have been reported by several groups, both in muscle fibers (SR terminal cisternae; Jorgensen et al., 1985) and in nonmuscle cells (calciosomes and/or ER cisternae, Volpe et al., 1988; Hashimoto et al., 1988; Henson et al., 1989; Koch et al., 1989; Treves et al., 1990). In contrast, the protein(s) responsible for Ca²⁺ uptake and, even more, the channels (each of which can transfer as many as 10⁷ ions/s) can be present in only a few copies in the limiting membrane of the stores, and thus remain inappreciable by immunocytochemistry. This has been found with the Ins-P₃R, a channel expressed (and functional) in many (maybe all) cells which so far could be mapped by immunogold labeling only in Purkinje neurons (Satoh et al., 1990; Otsu et al., 1990) where, for unknown reasons, it is largely overexpressed (Ross et al., 1989; Mignery et al., 1989). Overex-

Figure 5. Dual CS (large particles)-Ca²⁺ ATPase (small particles) immunogold labeling in the Purkinje cell body. A and B show two sections through the Golgi complex surrounded by numerous dense calciosomes (marked in part by arrows). Golgi cisternae are almost completely CS negative, except for their external tips in apparent direct continuity (arrowheads) with dense, CS positive, calciosome-like structures. Images reminiscent of the Golgi-calciosome interactions visible in the cryosections can be found by conventional microscopy (A, inset). The small gold particles of the Ca²⁺ ATPase immunolabeling are marked by small arrows. They often appear in clusters over tangentially sectioned membranes of calciosomes (B and C) and individual ER cisternae (C). Golgi cisternae (A and B) usually appear negative, except for some labeling at the tips (A, small arrow), and the same occurs with large, dense bodies (A). Also unlabeled are the evenly spaced membranes of ER stacks (C, st). Bars, 0.5 $\mu$m.
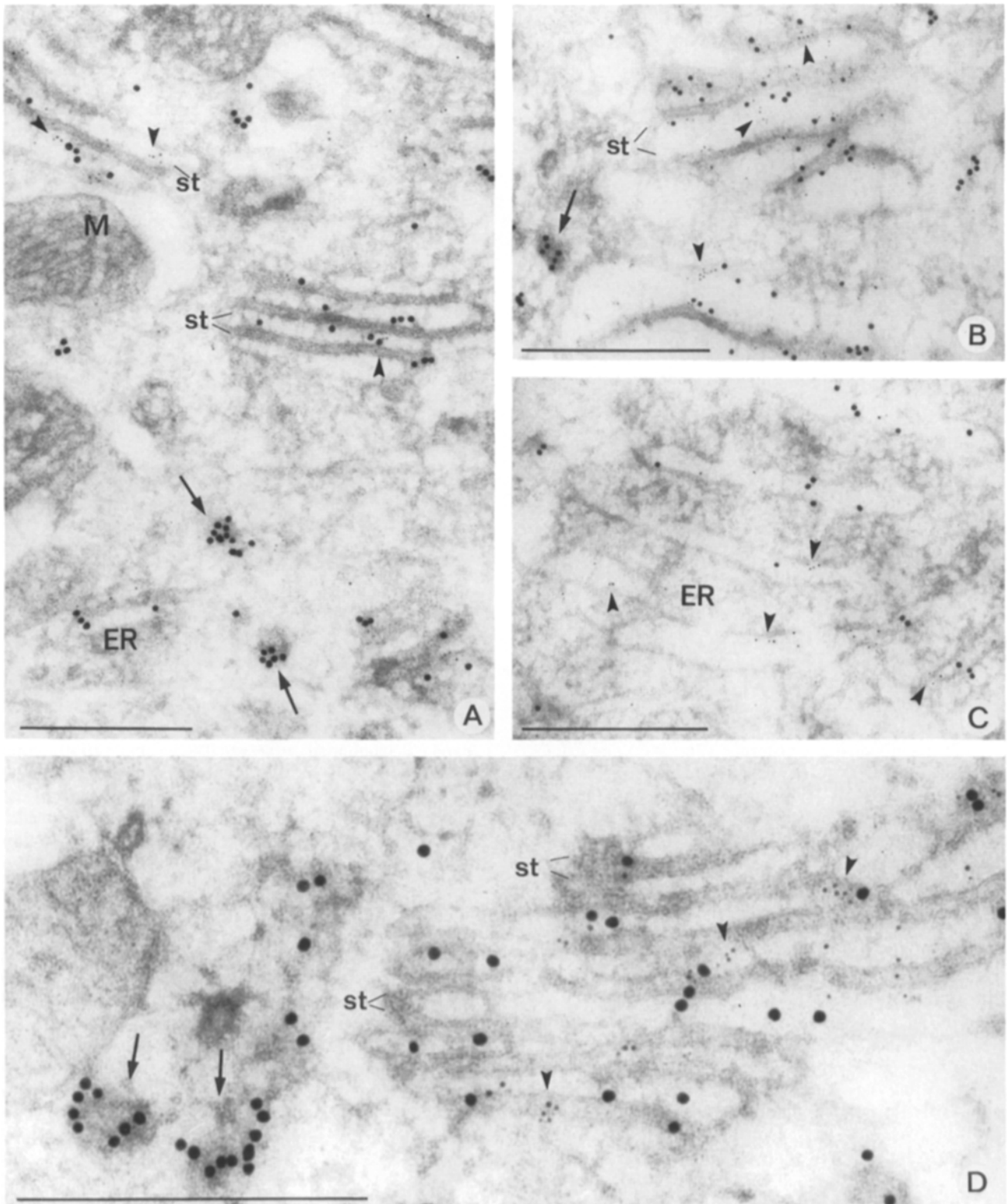
*Figure 6.* Dual CS (large particles)–Bip (small particles) immunogold labeling of the Purkinje neuron body (*A–C*) and dendrites (*D*). In both cell regions the distribution of CS reflects that of Figs. 3–5, with high concentrations in calciosomes (*arrows*) and low concentration in stacked (*st*; *A*, *B*, and *D*) and individual (*A* and *C*) ER cisternae. The small gold particles addressed to Bip (*arrowheads*) appear concentrated within the two types of ER cisternae. In contrast, no Bip labeling appears over CS-rich calciosomes. Bars, 0.5 μm.

pression in this cell type has been recently reported also for the other type of intracellular $Ca^{2+}$ channel, the RyR (Ellisman et al., 1990). Purkinje neurons can thus be considered as precious model cells for the immunocytochemical study of $Ca^{2+}$ stores. However, the main microsomal $Ca^{2+}$ storage protein expressed in the rat cerebellum is immunologically related to neither CS nor CR, and thus cannot be immunolocalized for the moment (see Satoh et al., 1990). This limitation has now been overcome in the chicken because of the expression of a protein indistinguishable from CS (see Volpe, 1990). Unfortunately, in that species both the anti-Ins-$P_3$R Abs we have available failed to yield any appreciable signal. A direct CS-Ins-$P_3$R dual labeling was thus precluded to us. However, because of the close ultrastructural similarity of Purkinje neurons of rats and chicken, we believe the distribution of the Ins-$P_3$R to be, if not the same, at least very similar in the two species. This assumption has been recently substantiated by Takei et al. (1990) by using a cross-species reactive anti-Ins-$P_3$R Ab. Therefore, we feel entitled to correlate our present results in the chicken with those on Ins-$P_3$R in the rat.

Dual labeling experiments could be carried out by the use of anti-CS with either anti-Bip or anti-$Ca^{2+}$ ATPase Abs, however, with different results. In fact, the immunogold signal was clear-cut with Bip while with $Ca^{2+}$ ATPase it was weaker, in spite of the strong immunofluorescence results obtained with the same Ab. This might be due to the fact that the major immunogenic determinant of the enzyme is located in the proximity of ATP binding, at a site difficult to reach by Abs (Mata et al., 1989) except when detergents are used, as it is the case with immunofluorescence. It should be emphasized, however, that in our experimental conditions, the $Ca^{2+}$ ATPase immunogold labeling of both calciosomes and individual ER cisternae was several fold above background. Therefore, the results with these structures, although not yet precisely quantizable, appear specific and worthy of attention.

The main finding of the present work is that, in the chicken Purkinje neurons, CS has a dual localization: within the ER (individual, mostly rough-surfaced cisternae and smooth-surfaced stacks) and (concentrated over 10-fold) within calciosomes. Both these structures might, thus, be active in the control of cell $Ca^{2+}$ homeostasis, however, with possibly different functional roles.

## ER Cisternae and Stacks

In the rat, these two, lumenally continuous ER subcompartments are known to differ markedly in their membrane concentration of Ins-$P_3$R, high in the stacks and ~10-fold lower in individual cisternae (Satoh et al., 1990; Otsu et al., 1990). A new membrane difference has now been revealed in the chicken, where individual cisternae exhibited $Ca^{2+}$ ATPase immunolabeling, whereas the stacked membranes did not. This last result was not entirely unexpected. In fact, the membranes of stacked cisternae appear to be joined together by parallel, perpendicular bridges, regularly spaced at a distance (~25 $\mu$m center-to-center) corresponding to the size of the Ins-$P_3$R (Maeda et al., 1990). This general structure resembles that of the junctional face of the muscle SR terminal cisternae, where the other type of intracellular $Ca^{2+}$ channel, the RyR, is clustered to form bridges (feet)

and $Ca^{2+}$ ATPase is excluded (Campbell, 1986; Franzini-Armstrong et al., 1987). From a functional point of view, however, the two endomembrane systems are probably quite different. In fact, terminal cisternae contain concentrated CS (Franzini-Armstrong et al., 1987) and the rest of the SR membranes, including the nonjunctional face of terminal cisternae, are very rich in $Ca^{2+}$ ATPase. $Ca^{2+}$ supply to the lumen, and ultimately to the RyR, is thus largely provided. In contrast, within their lumen, Purkinje neuron stacked cisternae are not specialized with respect to the rest of the ER, at least in terms of both CS and Bip (a typical ER lumen resident protein). As far as $Ca^{2+}$ storage is concerned, the stacks might thus work not independently, but coordinately with the adjacent lumenally continuous ER cisternae.

Individual ER cisternae, on the other hand, appear to possess the three basic components: $Ca^{2+}$ ATPase, Ins-$P_3$R, and CS, and could thus play the role of rapidly exchanging $Ca^{2+}$ stores, particularly important in the cell areas where calciosomes are absent or rare, for example, the dendritic spines. In this respect, it is interesting that the spines (in the rat) contain Ins-$P_3$R (Mignery et al., 1989; Satoh et al., 1990) and that evidence exists for changes of the calcium stored within those structures (Andrews et al., 1988). Because of their low CS concentration, the ER cisternae are, however, expected to accumulate only moderate amounts of calcium, and to be thus rapidly depleted on stimulation.

## CS-rich Structures: the Calciosomes

The population of relatively small, membrane-bound entities, quite heterogeneous in shape, characterized by high CS immunolabeling and moderate electron density of their content, together with expression of $Ca^{2+}$ ATPase in the membrane, meets the requirements previously defined in other nonmuscle cells for the calciosomes (Volpe et al., 1988; Hashimoto et al., 1988). These properties appear consistent with the ability of these structures to accumulate considerable amounts of calcium destined to sustain $[Ca^{2+}]_i$ transients of intracellular origin. However, in order to carry out this function, calciosomes need to express also a channel (Ins-$P_3$R and/or RyR), a property that, because of the reasons discussed above, could not be established yet. Formal assignment to calciosomes of specific $Ca^{2+}$ storage system(s) (sensitive to Ins-$P_3$, caffeine, or both) will thus be possible only when additional experimental evidence becomes available.

A new aspect of calciosomes revealed in this study is the almost complete exclusion of Bip from their content, at variance with ER cisternae. This represents a further criterion for calciosome identification. Based on this observation, the question can now be asked of whether Purkinje neuron structures exhibiting high concentrations of CS are all true calciosomes. In the cell body this appears to be largely the case. In the dendrites, on the other hand, images of CS clusters within the apparently empty lumen of ER-like elements have been observed. Although up to now coexistence of these clusters with high Bip immunoreactivity in the same lumena has never been observed, some caution, concerning at least CS-single labeled images, is probably appropriate.

The observed segregation of CS from Bip opens interesting clues on calciosome biogenesis. At the moment we do not know yet whether, in chicken Purkinje neurons, these

structures simply represent specialized portions of the ER or, alternatively, require involvement of the Golgi complex for assembly. In the first case, sorting of CS from the other molecules of the ER lumen could occur by simple aggregation of CS molecules, to each other or together with an additional protein. This latter possibility was proposed to account for CS concentration within muscle SR terminal cisternae (Franzini-Armstrong et al., 1987), while simple aggregation (of secretory zymogens) seems responsible for the assembly of ER intracisternal granules, sometimes occurring in pancreatic acinar cells (Tooze et al., 1989). Alternatively, sorting could take place via a trip through the Golgi complex, which is precluded to Bip and the other resident proteins expressing at the COOH terminus the retention sequence, KDEL (Pelham, 1989). In favor of this second possibility are the images we have obtained suggesting calciosome formation at the lateral tips of Golgi cisternae. Moreover, CS, at least in muscle, fails to express the KDEL sequence (Fliegel et al., 1987) and has been reported to travel, at least in part, along the Golgi complex, where its sugar chains are processed (Thomas et al., 1989). Whether this occurs also in Purkinje neurons, in particular whether (because of oligosaccharide chain processing within the Golgi) calciosome CS is molecularly different from the protein contained within ER cisternae, remains to be investigated.

## Conclusion

High resolution mapping of various proteins within chicken Purkinje neurons has yielded a number of interesting results that help to put into a new perspective the problem of the cytological nature of rapidly exchanging intracellular $Ca^{2+}$ pool(s). Clearly, the molecular characterization of the various structures (in particular, ER cisternae and stacks; calciosomes) is not yet complete and should be pursued by the use of additional, specific Abs. Moreover, these studies need to be ultimately complemented with direct measurements of calcium within the various structures, obtained by adequate techniques (such as electron probe microanalysis and electron spectroscopic imaging) in appropriately processed samples that were exposed to controlled treatments. The identification of the structures to be tested, and their cellular and molecular characterization, represent necessary steps of a comprehensive approach aimed to ultimately clarify the biology of $Ca^{2+}$ in nonmuscle cells.

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