Ryanodine and Inositol Trisphosphate Receptors Coexist in Avian Cerebellar Purkinje Neurons

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Abstract. Two intracellular calcium-release channel proteins, the inositol trisphosphate (InsP₃), and ryanodine receptors, have been identified in mammalian and avian cerebellar Purkinje neurons. In the present study, biochemical and immunological techniques were used to demonstrate that these proteins coexist in the same avian Purkinje neurons, where they have different intracellular distributions.

Western analyses demonstrate that antibodies produced against the InsP₃ and the ryanodine receptors do not cross-react.

Based on their relative rates of sedimentation in continuous sucrose gradients and SDS-PAGE, the avian cerebellar InsP₃ receptor has apparent native and subunit molecular weights of ∿1,000 and 260 kD, while those of the ryanodine receptors are ∿2,000 and 500 kD.

Specific [³H]InsP₃- and [³H]ryanodine-binding activities were localized in the sucrose gradient fractions enriched in the 260-kD and the ∿500-kD polypeptides, respectively.

Under equilibrium conditions, cerebellar microsomes bound [³H]InsP₃ with a K_d of 16.8 nM and B_{max} of 3.8 pmol/mg protein; whereas, [³H]ryanodine was bound with a K_d of 1.5 nM and a capacity of 0.08 pmol/mg protein.

Immunolocalization techniques, applied at both the light and electron microscopic levels, revealed that the InsP₃ and ryanodine receptors have overlapping, yet distinctive intracellular distributions in avian Purkinje neurons. Most notably the InsP₃ receptor is localized in endomembranes of the dendritic tree, in both the shafts and spines. In contrast, the ryanodine receptor is observed in dendritic shafts, but not in the spines. Both receptors appear to be more abundant at main branch points of the dendritic arbor.

In Purkinje neuron cell bodies, both the InsP₃ and ryanodine receptors are present in smooth and rough ER, subsurface membrane cisternae and to a lesser extent in the nuclear envelope. In some cases the receptors coexist in the same membranes. Neither protein is observed at the plasma membrane, Golgi complex or mitochondrial membranes.

Both the InsP₃ and ryanodine receptors are associated with intracellular membrane systems in axonal processes, although they are less abundant there than in dendrites.

These data demonstrate that InsP₃ and ryanodine receptors exist as unique proteins in the same Purkinje neuron. These calcium-release channels appear to coexist in ER membranes in most regions of the Purkinje neurons, but importantly they are differentially distributed in dendritic processes, with the dendritic spines containing only InsP₃ receptors.

THE results of pharmacological and physiological studies suggest that several distinct intracellular calcium release events exist in many neuronal cell types, including sympathetic neurons (12, 18), dorsal root ganglia (37), adrenal chromaffin cells (5, 27), and certain neuroblastoma cell lines (38). Neurotransmitter stimulated increases in intraneuronal inositol triphosphate (InsP₃)[1] result in the release of calcium from sites associated with the ER (21, 25, 29, 30). The InsP₃ receptor present in mammalian brain, has been identified as a homotetrameric protein composed of a 260-kD polypeptide subunit (34). This protein has been purified and demonstrated to embody a calcium channel (9), and recently cloned and sequenced (10, 25, 26).

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1. *Abbreviation used in this paper:* InsP₃, inositol 1,4,5,trisphosphate.

A second, intracellular calcium-release event has been demonstrated to be insensitive to InsP₃, but stimulated by caffeine and inhibited by ryanodine (22, 37). The physiological trigger for release of calcium from this store is unknown, but by analogy with striated muscle, it may involve either transient increases in cytoplasmic calcium (calcium-induced release), or depolarization of the plasma membrane (depolarization-induced release). The calcium release channel protein associated with this store has yet to be unequivocally identified; but recently, ryanodine receptors have been identified in both avian and mammalian neurons (8, 23), that are biochemically similar to those in avian skeletal muscle (1).

The identification of InsP₃ and ryanodine receptors is consistent with the existence of two distinct intracellular calcium-release processes in Purkinje neurons, however, this possibility remained to be confirmed by the demonstration that both receptor proteins coexist within the same cell rather than in two subsets of these cells. In addition, a knowledge of the intracellular distributions of each protein is required to understand how the calcium-release event mediated by each protein contributes to neuronal function. The latter issue is of particular relevance to Purkinje neurons, which receive interactive inputs via parallel fibers, thought to increase neuronal InsP₃ levels, and climbing fibers, thought to evoke calcium entry into neurons (19, 20, 32, 36). Moreover, oscillatory changes in intracellular calcium have been observed in the dendritic processes of Purkinje neurons (36) and one model for the generation of such variations in calcium involves interplay between two calcium-release systems (2).

In the present studies we have established that the ryanodine and the InsP₃ receptors coexist within avian cerebellar Purkinje neurons. In dendritic processes, these proteins have distinctive distributions, with the ryanodine receptor existing only in dendritic shafts and the InsP₃ receptor in both dendritic shafts and spines. In contrast, in Purkinje neuron cell bodies, these proteins have overlapping distributions; in some cases, existing within the same membranes.

Materials and Methods

Materials

Fertilized White Leghorn chicken eggs were purchased from Weber Egg Co. (Rio Linda, CA). Leupeptin, PMSF, CHAPS, L-alpha-phosphatidylcholine, polyethylenimine, and n-propyl gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid from Pierce Chemical Co. (Rockford, IL), SDS-PAGE molecular weight standards from Bio-Rad Laboratories (Richmond, CA), InsP₃, and [³H]InsP₃ were purchased from New England Nuclear (Boston, MA). Alkaline phosphatase-conjugated goat anti–mouse IgG was from Tago Inc. (Burlingame, CA) and Fisher Scientific (Santa Clara, CA). OCT compound from Miles Laboratories, Inc. (Elkhart, IN), FITC and Texas red-conjugated goat anti–mouse antibodies, normal goat, and normal mouse sera were from Organon Technika (Durham, NC). 5- and 10-nM gold conjugated antibodies were from Janssen Pharmaceutica (Beerse, Belgium) and LR White was obtained from the London Resin Co. (London, England).

Microsomal Membrane Preparation

Two 3-wk-old chicks were sacrificed by decapitation, the cerebellums excised, and immediately frozen in liquid N₂. Frozen tissue was subsequently weighed and homogenized at 0–4°C in 5 ml/gm of solution A containing 0.3 M sucrose, 10 mM imidazole, pH 7.4, 5 mM DTT, 0.23 mM PMSF, 1.1 μM leupeptin, using 3 × 30-s bursts of a Polytron at setting of

four. The homogenate was centrifuged at 10,000 g for 30 min and microsomes were collected from the resulting supernatant by centrifugation at 130,000 g for 2 h (33). The microsomal pellet was resuspended in solution A at a protein concentration of 10–20 mg/ml, rapidly frozen in liquid N₂ and stored at −90°C. Membrane protein was quantitated using BCA (31) and BSA as a standard.

Solubilization and Partial Purification of Ryanodine and InsP₃ Receptors

Cerebellar microsomes were thawed at 37°C, diluted 1:1 with ice-cold solution B, containing 0.5 M KCl, 20 mM Tris, pH 7.4, 5 mM DTT, 0.23 mM PMSF, and 1.1 μM leupeptin, and centrifuged at 130,000 g for 1 h. The membranes were resuspended at a protein concentration of 9 mg/ml, mixed with solution B containing 5% CHAPS and 2.5% phosphatidylcholine to give final protein, CHAPS and phosphatidylcholine concentrations of 6 mg/ml, 2 and 1%, respectively, and incubated on ice for 30 min. Solubilized and nonsolubilized material were separated by centrifugation at 100,000 g for 30 min. Either 2.5- or 5-ml aliquots of solubilized protein were layered onto 3–30% continuous sucrose gradients prepared in solution B with 1% CHAPS and 0.5% phosphatidylcholine. The gradients were centrifuged at 23,000 rpm in a SW 27 rotor (Beckman Instruments, Palo Alto, CA) for 14–16 h and fractionated into 12 × 3 ml samples. The protein profile of each sample was analyzed by SDS-PAGE as described below. Solubilized protein was quantitated using the method of Kaplan and Pedersen (15) and BSA as a standard. Gradient fractions were either used immediately, or rapidly frozen in liquid N₂ and stored at −90°C.

Antibodies

A mouse mAb (34C) raised against avian skeletal muscle ryanodine-binding proteins (1) which recognizes both ryanodine receptor isoforms in avian cerebellar Purkinje neurons (8) and a rabbit polyclonal sera produced against a synthetic peptide containing the carboxy-terminal 19 amino acids of the InsP₃ receptor (25) were used in these studies.

SDS-PAGE and Western Blot Analyses

Microsomal samples for SDS-PAGE (16) were adjusted to a protein concentration of 5 mg/ml and mixed 1:1 with 2× concentrated load buffer to give a final SDS concentration of 2%. Samples of solubilized proteins from the sucrose gradient fractions were prepared in a 4× concentrated load buffer. Aliquots of 40–50 μl were loaded onto continuous 4–20% linear polyacrylamide gradient SDS minigels and run under a constant voltage of 130 V at 0–4°C. Gels were stained for protein with 0.2% Coomassie brilliant blue.

Proteins were blotted onto nitrocellulose using a Transphor TE 50 (Hoeffer Scientific Instruments, San Francisco, CA) at an initial setting of 100 V for the first 2 h and then at 40 V overnight in 10 mM 2[N-cyclohexylamino]ethane-sulfonic acid, pH 9.6; 10% ethanol (41). Proteins transferred to the nitrocellulose were detected by staining with 0.5% Ponceau S in 5% acetic acid and destaining in water. Blots were probed with the monoclonal, anti-avian skeletal muscle ryanodine receptor antibody, 34C, and a peptide-specific anti–InsP₃ receptor polyclonal sera. Blotted proteins identified by these antibodies were visualized using either goat anti-mouse, or goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase.

[³H]ligand Binding

[³H]ryanodine and [³H]InsP₃ binding to both cerebellar microsomal membranes and solubilized ryanodine and InsP₃ receptors was measured under equilibrium conditions.

[³H]ryanodine binding to cerebellar microsomes was measured in a final volume of 0.2 ml containing 1.0 M KCl, 20 mM Tris, pH 7.4, 5 mM DTT, 200 μM CaCl₂, 0.23 mM PMSF, 1.1 μM leupeptin (solution C), 0.5-mg membrane protein, varying concentrations of [³H]ryanodine (sp act = 53.2 Ci/mmol; 35) and 50 μM unlabeled ryanodine for measurement of nonspecific binding for 2 h at 37°C. The binding reaction was terminated by filtration through GF/B glass fiber filters and washed with 4 × 4-ml aliquots of an ice-cold solution containing 1.0 M KCl, 20 mM Tris, pH 7.4, and 200 μM CaCl₂. Ligand binding to solubilized receptor in the sucrose gradient fractions was determined in a 0.2-ml volume of solution C, which also contained 1% CHAPS, 0.5% phosphatidylcholine, 50-nM [³H]ryanodine, and 0.166 ml of the gradient fraction. The binding reaction was per-

mitted to proceed at room temperature for 1 h, terminated by filtration through GF/B glass fiber filters treated with 3% polyethylenimine (4), and washed with 7 × 4-ml aliquots of the above wash solution.

[^{3}H]InsP$_3$ binding to cerebellar microsomes was measured as described by Chadwick et al. (6). Briefly, 50 μg of membrane protein was incubated in 0.1 ml of a solution containing 50 mM Tris, pH 8.3; 1.0 mM EDTA, 1.0 mM DTT (solution D), and variable concentrations of [^{3}H]InsP$_3$ (sp act = 17 Ci/mmol) and 5 μM unlabeled InsP$_3$ for measurement of nonspecific binding for 10 min at 0–4°C. The membranes were subsequently collected by centrifugation at 65,000 rpm in a TL-100 (Beckman Instruments), the pellet washed with ice-cold solution D, resuspended in 0.2 ml of water, and bound radioactivity determined by liquid scintillation counting. Ligand binding to solubilized receptor was determined using the conditions described by Suppattone et al. (34). Sucrose gradient fraction samples (167.5 μl) were incubated in a final volume of 0.2 ml of solution D containing 12.5-nM [^{3}H]InsP$_3$ for 10 min at 0–4°C. Binding reactions were terminated by centrifugation (5 min, 1,000 g) through prechilled 1 ml Sephadex G50-80 spin columns (28) and the radioactivity present in the void volume was measured by liquid scintillation counting.

Determinations of the affinities and capacities of cerebellar microsomal membranes for [^{3}H]ryanodine and [^{3}H]InsP$_3$ involved analyses of saturation isotherms by the method of Scatchard. Estimates of the relative levels of ligand binding to solubilized receptors present in sucrose gradient fractions involved the application of the single ligand concentrations indicated above. Levels of nonspecific binding usually did not exceed 10% of the total binding measured. All binding reactions were conducted in duplicate.

Confocal Laser Immunofluorescence

Chicks (3–7-d old) were anesthetized and perfused transcardially with Ringer's solution followed by 2% paraformaldehyde in 0.1 M PBS (pH 7.4) at 35°C for 2 min. Whole brain was removed, bisected sagittally, and fixed for an additional hour at 4°C. 50 μm-thick sections were obtained with a vibratome (Lancer, St. Louis, MO) and placed in 0.1 M PBS with 0.05 M glycine at 4°C for 10 min. Sections were blocked with 1% normal goat serum, 0.5% BSA, and 0.5% gelatin in PBS-glycine for 20 min at 4°C. Sections were incubated in either anti-ryanodine receptor antibody (34C), anti-InsP$_3$ receptor antibody, or a combination of both antibodies for 12 h at 4°C. After repeated washes for 1 h, the sections were incubated in either goat anti–mouse FITC, goat anti–rabbit Texas red, or a combination of both secondary antibodies for 1 h at 4°C. After washing for 1 h the sections were mounted on clean, untreated glass slides and covered with antifade media consisting of 4% n-propyl gallate in a glycerol/PBS mixture. The secondary antibodies used in these studies were tested to insure that there was no species cross reactivity.

Optical sections and digitally generated stero pairs were obtained using a confocal laser scanning microscope (series MRC-600; BioRad Laboratories). Digital images stored on optical disc were photographed with an LFR Plus camera (Lasergraphics, Irvine, CA) using Kodak Ektachrome 100 film (Ektachrome 100; Eastman Kodak Co., Rochester, NY).

Immunoelectron Microscopy

Chick cerebellum was fixed with 2% paraformaldehyde, cryoprotected in sucrose, and rapidly frozen (39). Immunoelectron microscopy was carried out as previously described (8). Briefly, ultrathin cryosections were obtained with an ultramicrotome (Ultracut E; Reichert-Jung, S. A., Paris) equipped with an FC-4E cryo attachment (Cambridge Instruments Inc., Monsey, NY). The sections were mounted on formvar-coated gold grids and blocked with 1% normal goat serum, 1% BSA, and 1% gelatin in PBS-glycine for 20 min before incubation in either anti-ryanodine receptor antibody, anti-InsP$_3$ antibody, or for double labeling a combination of both antibodies for 1 h at 37°C. After washing in PBS glycine, the sections were incubated in either goat anti–mouse IgG conjugated with either 5- or 10-nm gold particles, or goat anti–rabbit IgG 10-nm gold conjugates for 30 min. For double labeling studies, a combination of goat anti–mouse IgG and goat anti–rabbit IgG labeled with 5- and 10-nm gold particles, respectively, were used. The sections were washed for 30 min in PBS, postfixed in freshly mixed 1% osmium tetroxide, 1% gluteraldehyde in PBS for 5 min, rinsed with double distilled water, counterstained with uranyl acetate, dehydrated in ethanol, and embedded in a thin layer of LR White.

Cryosections were examined at 100–200 keV using either a JEOL 100CX or 2000FX II electron microscope (10CX or 200FX II; JEOL USA, Electron Optics, Peabody, MA).

Results

Distinct InsP$_3$ and Ryanodine Receptors Are Present in the Avian Cerebellum

The objectives of the present study were to determine whether ryanodine and InsP$_3$ receptors coexist in avian cerebellum and to establish the inter- and intracellular distributions of these two calcium release channels in this tissue.

Both receptors could be identified biochemically and pharmacologically in avian cerebellar microsomes. Intact membranes were found to bind [^{3}H]ryanodine with K$_d$ and B$_{max}$ values of 1.5 nM and 79.3 fmol/mg protein, while these parameters were found to be 16.8 nM and 3.8 pmol/mg protein for [^{3}H]InsP$_3^2$. In both cases these values are similar to those reported for mammalian brain (23, 34).

Ryanodine receptors have been demonstrated previously to be present in avian cerebellar Purkinje neurons (8). These proteins are physically similar to those in striated muscle in both native and subunit molecular weights (1). The native proteins solubilized with CHAPS in the presence of phosphatidylcholine sediment as 30 s particles in continuous 3–30% sucrose gradients and are comprised of subunits that migrate with an apparent molecular weight of ~500,000 in SDS-PAGE (Fig. 1 A). These proteins copurify with [^{3}H]ryanodine binding (Fig. 1 F), and are precipitated by the antibody against the skeletal muscle ryanodine receptors (Fig. 1 B). This antibody also precipitates specifically bound [^{3}H]ryanodine (Fig. 1 C). In the presence of the anti-ryanodine receptor antibody (Fig. 1 C, lane "+") 177.1 fmol of [^{3}H]ryanodine were precipitated, whereas no specifically bound ryanodine was detected in the absence of primary antibody (lane "−"). The InsP$_3$ receptor is also present in the avian cerebellum. This protein appears to be physically similar to that identified in rat and mouse cerebellum (10, 25, 26, 34) in that it sediments in a continuous 3–30% sucrose gradient as a native protein of ~1,000-kD comprised of a 260-kD polypeptide subunit (Fig. 1 D). This polypeptide is recognized in Western analysis by an anti–rat brain InsP$_3$ receptor antibody (Fig. 1 E) and copurifies with specifically bound [^{3}H]InsP$_3$ (Fig. 1 F).

As would be anticipated from previous studies, the avian cerebellar ryanodine and InsP$_3$ receptors are distinct proteins. This is indicated by the results presented in Fig. 1. The two receptors (indicated by arrows in Fig. 1, A and D) have quite different native molecular masses as evidenced by their different rates of sedimentation in identical continuous 3–30% sucrose gradients. The ryanodine receptors were localized in gradient fractions 4 and 5 (numbered from the bottom of the gradient), while the InsP$_3$ receptor was found primarily in gradient fraction 7. Based on the ligand-binding capacities of cerebellar microsomes noted above, and the relative staining intensities of the 500- and 260-kD bands indicated by the arrows in Fig. 1, the InsP$_3$ receptor appears to be ~50-fold more abundant than the ryanodine receptors in the cerebellum. Because of this difference in abundance it was necessary to load twice the quantity of solubilized protein onto the sucrose gradient to clearly detect the ryanodine receptor (Fig. 1 A). Under these conditions, the InsP$_3$ receptor is overloaded and appears smeared across several gradient fractions. The actual distribution of this protein can be ap-
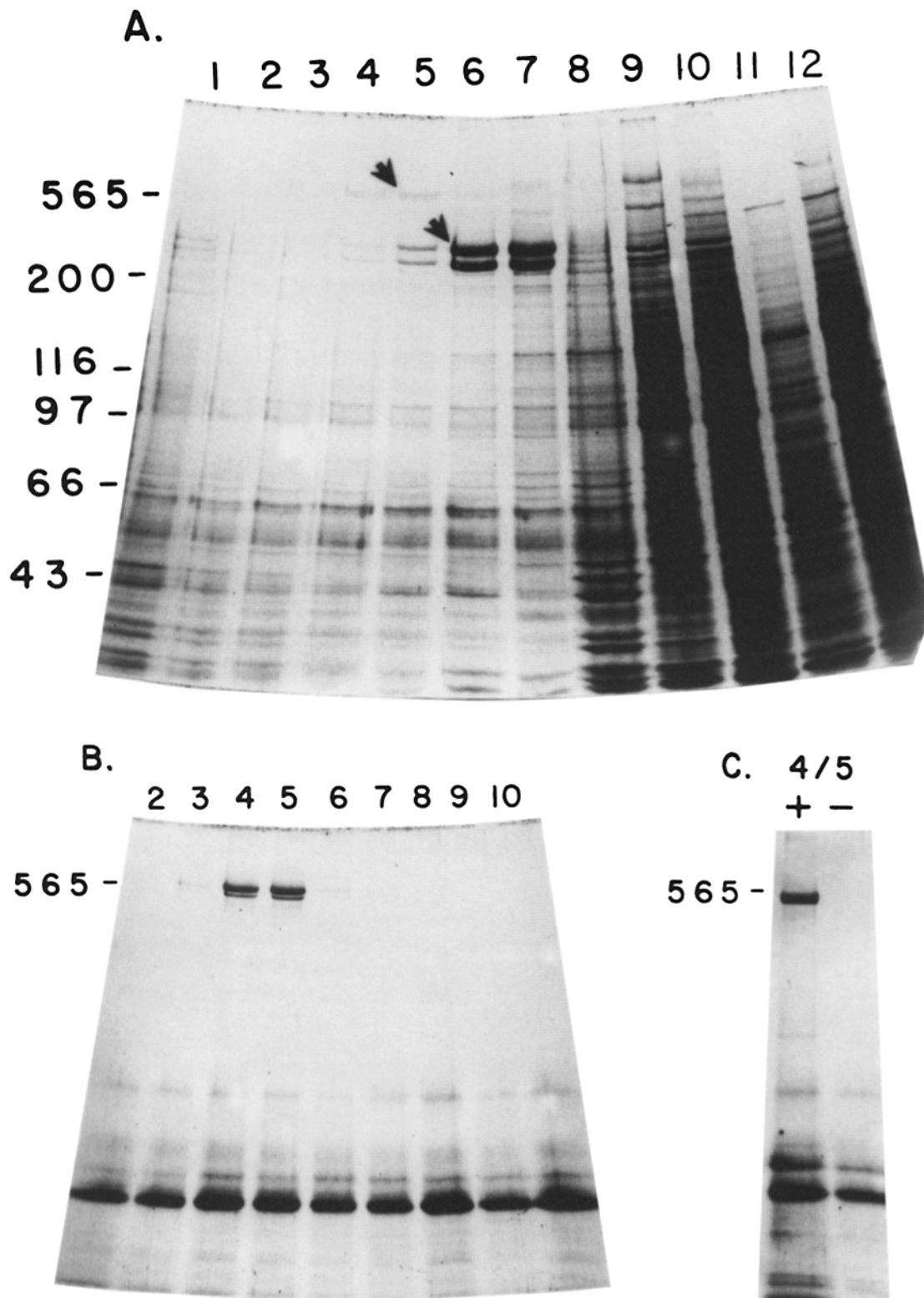
Figure 1. Distinct ryanodine and InsP₃ receptors exist in avian cerebellum. Cerebellar microsomal proteins, solubilized with CHAPS, were sedimented through continuous 3–30% sucrose gradients. The gradients were divided into 12 × 3-ml fractions (numbered *1–12* from the bottom of the gradient) and the protein profile of these fractions were visualized in continuous 4–20% polyacrylamide gradient SDS gels stained with Coomassie brilliant blue (*A* and *D*). 5 ml of solubilized protein was applied to the gradient shown in *A*, while 2.5 ml was loaded onto that shown in *D.* The ryanodine and InsP₃ receptors are indicated by the arrows at ∼500 and 260 kD, respectively. Gradient fractions 2–10 were precipitated with the anti-ryanodine receptor antibody, 34C (*B*) or analyzed after Western blotting using the anti-InsP₃ receptor antisera (*E*). Sucrose gradient fractions 4 and 5 were pooled, permitted to bind [³H]ryanodine, and then precipitated in the presence (lane +) and absence (lane −) of ryanodine receptor antibody, 34C (*C*). 177.1 and 0 fmol of specifically bound

D.

E.

F.
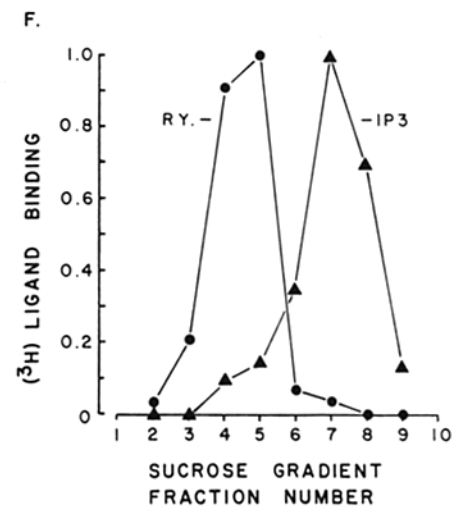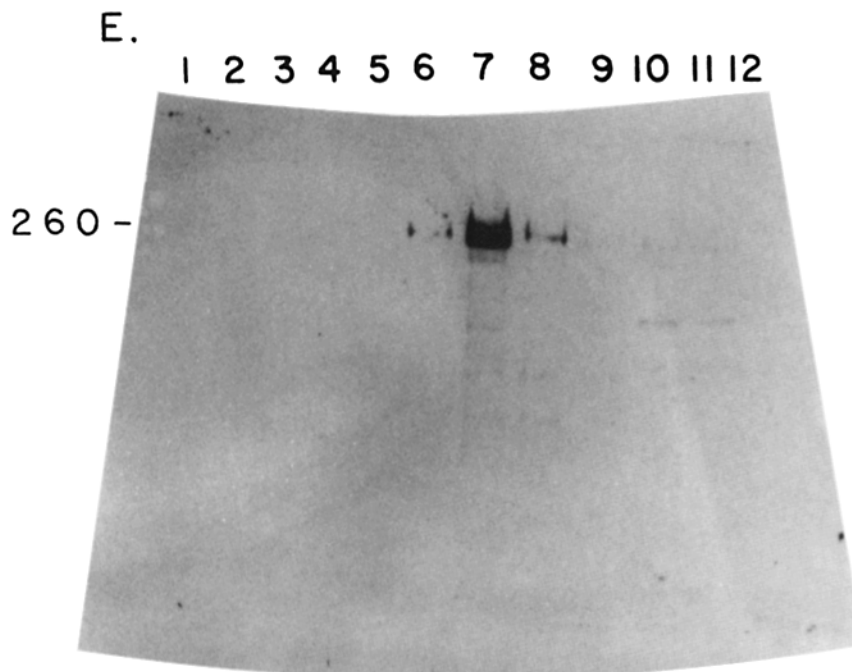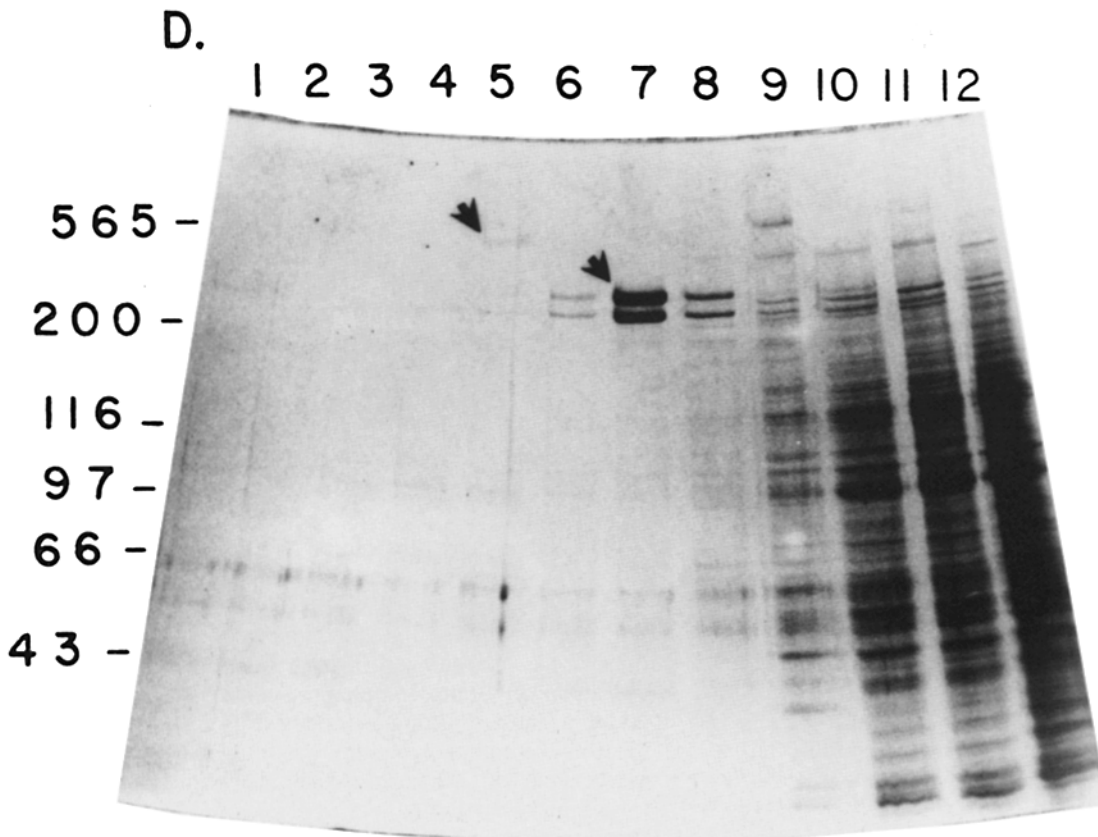
[³H]ryanodine were present in the samples shown in lanes + and −, respectively. The gradient fractions shown in *A* and *D* were assayed for specific [³H]ryanodine and [³H]InsP₃ binding (*F*). In these experiments equal volumes (rather than equal protein) of each fraction were assayed to demonstrate the relative levels of ligand binding in each fraction. Maximal [³H]ryanodine and [³H]InsP₃-binding levels were 41.7 and 785.6 fmol.
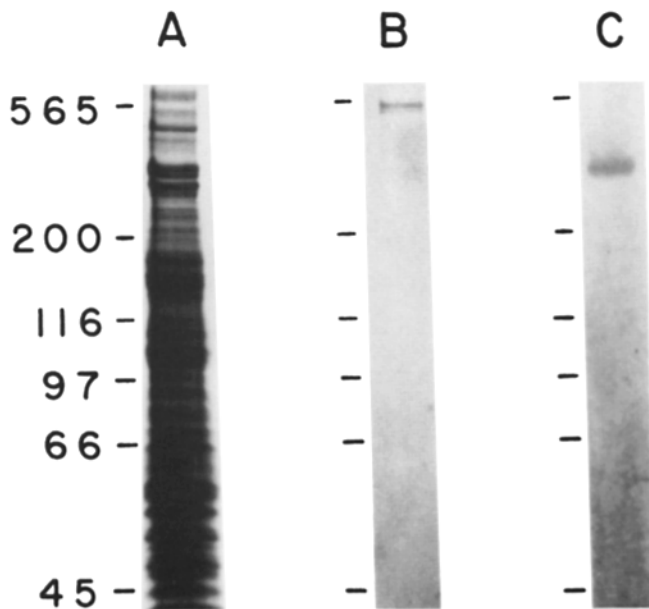
Figure 2. Avian cerebellum ryanodine and InsP₃ receptors are immunologically distinct proteins. Three identical samples of cerebellum microsomal protein from 3-wk-old chickens were resolved in a continuous 4–20% polyacrylamide gradient SDS gel. One lane of the gel was stained for protein with Coomassie brilliant blue (A). The other two lanes were Western blotted and probed with either an anti-chicken skeletal muscle ryanodine receptor antibody (34C) (B), or rat brain InsP₃ receptor antisera (C).

preciated more accurately in Fig. 1 D, where a smaller sample of solubilized protein was applied to the gradient.

Because of sequence and general structural homology between the InsP₃ and ryanodine receptors (6, 10, 24, 25, 26), it was necessary to determine whether the antibodies to be used in the immunocytochemical studies described below reacted with both receptors. As shown in Fig. 1, the anti-ryanodine receptor antibody, 34C recognizes only the two polypeptides of ~500 kD associated with the ryanodine receptors (Fig. 1 B; 8), while the InsP₃ receptor anti-sera identifies only a 260-kD polypeptide (Fig. 1 E). The specificity of these antibodies was confirmed further by Western analysis of total microsomal protein (Fig. 2).

Intracellular Distribution of the Ryanodine and InsP₃ Receptors in Avian Cerebellar Purkinje Neurons

Ryanodine receptors have been identified in dendritic and axonal processes, as well as in the cell bodies of chick cerebellar Purkinje neurons (8), while the InsP₃ receptor has been observed in the cell body and dendritic processes of mammalian cerebellar Purkinje neurons (21, 25, 29, 30). In view of the pharmacological and biochemical evidence that both receptors are present in chick cerebellum described above, we investigated whether these proteins coexist in the same neuron, or if there are neuronal subpopulations that contain only one receptor.

InsP₃ and ryanodine receptors present in sections of chick cerebellum were visualized simultaneously using confocal immunofluorescence microscopy and appropriate secondary antibodies conjugated with FITC and Texas red, respec-

tively, (Fig. 3). The results of these studies demonstrate that first, within the cerebellum these proteins are primarily, if not exclusively in Purkinje neurons; and second, that both proteins coexist in the same neurons with overlapping distributions.

When viewed in stereo, the InsP₃ and ryanodine receptors in the cell body appear to be arranged as a three dimensional network. Immunoreactivity associated with both receptors is observed to the distal regions of the Purkinje neuron dendritic processes, with regions of relatively high abundance evident at main branch points (Figs. 3 and 4). As observed for mammalian cerebellum (21, 25, 29, 30), the InsP₃ receptors are present in both dendritic shafts and spines (Fig. 4, A and C); whereas, consistent with our previous findings (8), the dendritic labeling for ryanodine receptors is limited to the dendritic shaft and was not observed within the head or neck regions of the dendritic spines (Fig. 4, B and D).

The subcellular distribution of the neuronal ryanodine receptor was investigated further using immunogold labeling of ultrathin cryosections. The results obtained by EM are consistent with the immunofluorescence observations just described, and add information regarding the density of internal membrane components containing ryanodine receptors. In the Purkinje cell body (Fig. 5 A), ryanodine receptor-like immunoreactivity was frequently observed to be associated with subsurface cisternae. Immunolabeling was also found on both the rough and smooth ER, as well as the nuclear envelope. Staining was not observed on the Golgi apparatus, mitochondria, or on the plasma membrane. In the proximal portion of the dendrite (Fig. 5 B) and axon (Fig. 5 C), staining is restricted to a subset of membrane cisternae in the dendritic and axonal shafts.

Similarities and differences in the intracellular distribution of the ryanodine and InsP₃ receptors was investigated directly in double-labeling studies. Secondary antibodies conjugated with either 5- or 10-nm gold particles were used to differentiate and localize both proteins in the same sections (Fig. 6). The electron micrographs of Purkinje cell bodies shown in Fig. 6, A and B, demonstrate that both receptors are found in several types of membranes and are occasionally colocalized to the same membrane bounded structure. Elements of both rough and smooth ER contain both proteins, although the distribution of the ryanodine receptor appears less continuous than that of the InsP₃ receptor. In addition, the InsP₃ and ryanodine receptors are evident in subsurface cisternae and the nuclear envelope, although the labeling of the latter membranes was relatively low for both proteins. Neither receptor was observed to be associated with the Golgi apparatus, mitochondria, or the plasma membrane. Although differences in avidity, clonal nature, and fixation sensitivity of the antibodies used in these studies preclude quantitative comparison, the relative densities of the two receptors demonstrated by immunogold staining is consistent with the biochemical and pharmacological data noted above, and indicate that the InsP₃ receptor is more abundant than the ryanodine receptors.

In view of the different distributions observed for the InsP₃ and ryanodine receptors in the dendritic spines by immunofluorescence, the localization of these proteins in the distal regions of the dendritic tree was investigated in greater detail in immunolabeled cryosections (Fig. 7). The results
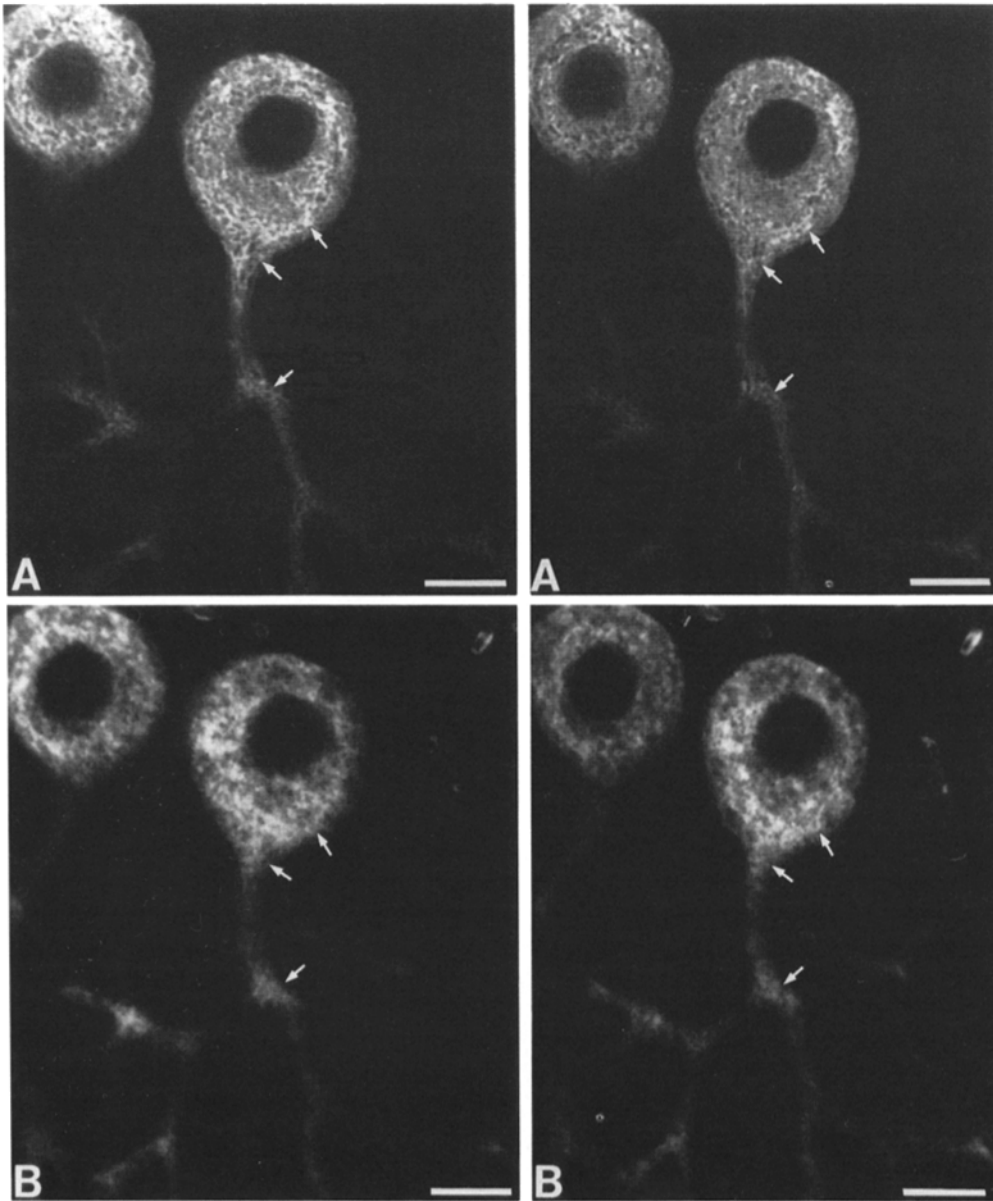
Figure 3. Paired stereo images of cerebellar Purkinje neurons labeled immunofluorescently for both the InsP₃ (*A*, Texas red) and ryanodine (*B*, FITC) receptors obtained with confocal microscopy. The arrows indicate regions of colocalization in the cell body and the dendritic processes. Bars, 10 μm.

obtained by EM support the observations obtained by immunofluorescence. The InsP₃ receptor was visible on a cisternal membrane network in both the dendritic shaft and spine (Fig. 7 *A*), whereas the ryanodine receptor is localized to a similar membrane system in the shaft, but is not detected in the head or neck regions of the spines (Fig. 7 *B*).

Discussion

The results presented provide evidence for the existence of two biochemically and immunologically distinct intracellular calcium-release channel proteins in the same cerebellar Purkinje neuron. These findings are consistent with previous pharmacological studies which demonstrated multiple calcium release events in several cell types other than Purkinje neurons (12, 18, 37, 38). The present observation of two

calcium-release channels in Purkinje neurons supports the idea that intracellular calcium serves as a mediator of neuronal function and that the levels of this cation are regulated in a complex manner. Moreover, the observation that both the ryanodine and InsP₃ receptors are expressed at much greater levels in Purkinje cells than in other neurons in the cerebellum indicates that the calcium-release events mediated by these proteins participate in cellular processes which are emphasized in this neuron type.

Purkinje neurons provide the only pathway for neuronal output from the cerebellar cortex (13) and this output is modulated by excitatory inputs received via parallel and climbing fibers. Consequently, Purkinje neurons serve as a site for the processing and selective transfer of information. A clue to the possible functional roles of the ryanodine and InsP₃ receptors comes from the differential distribution of
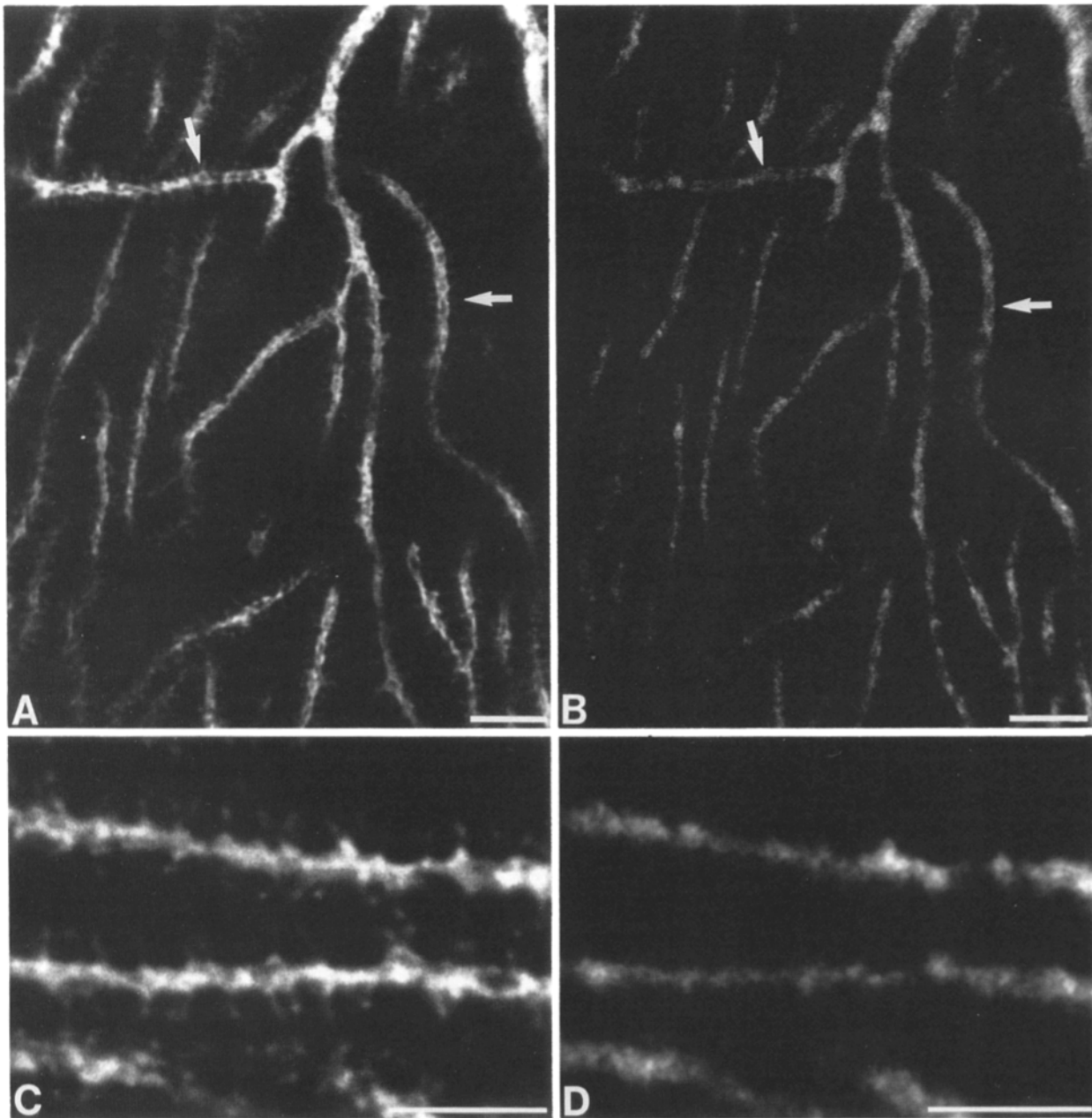
Figure 4. Confocal images of Purkinje neuron dendritic processes stained for both the InsP$_3$ (*A* and *C*, Texas Red) and ryanodine (*B* and *D*, FITC) receptors obtained at two different magnifications. The arrows indicate the staining of the dendritic spines in *A*, and the absence of staining of these structures in *B*. Similar patterns of staining are obtained for both receptors in dendritic shafts. Bars, 10 μm.

these proteins in dendritic processes. The dendritic spines, which contain only the InsP$_3$ receptor receive inputs from parallel fibers coming from cerebellar granule cells. Parallel fiber synapses appear to involve postsynaptic glutamate receptors, and to result in postsynaptic increases in InsP$_3$ (3). A second input to the Purkinje neuron, provided by climbing fibers from cell bodies in the brain stem, is received via synapses on the dendritic shafts and soma which contain the ryanodine receptors, as well as the InsP$_3$ receptors. This latter input results in large postsynaptic potentials that are associated with calcium influx, which could activate ryanodine-sensitive ion channels in either a calcium- or a

depolarization-induced manner. Inputs from parallel and climbing fibers interactively modify the output of Purkinje neurons through phenomena known as long-term depression and long-term potentiation. These latter two events appear to involve changes in intraneuronal calcium, as both phenomena are affected by injections of the calcium chelator, EGTA (14). Whether this modulation is achieved through alterations in InsP$_3$ and ryanodine-sensitive calcium–release events occurring in the spines, and dendritic shafts, respectively, remains to be determined; as do the mechanism by which such changes in intraneuronal calcium levels could have opposite effects on Purkinje neuron output and the
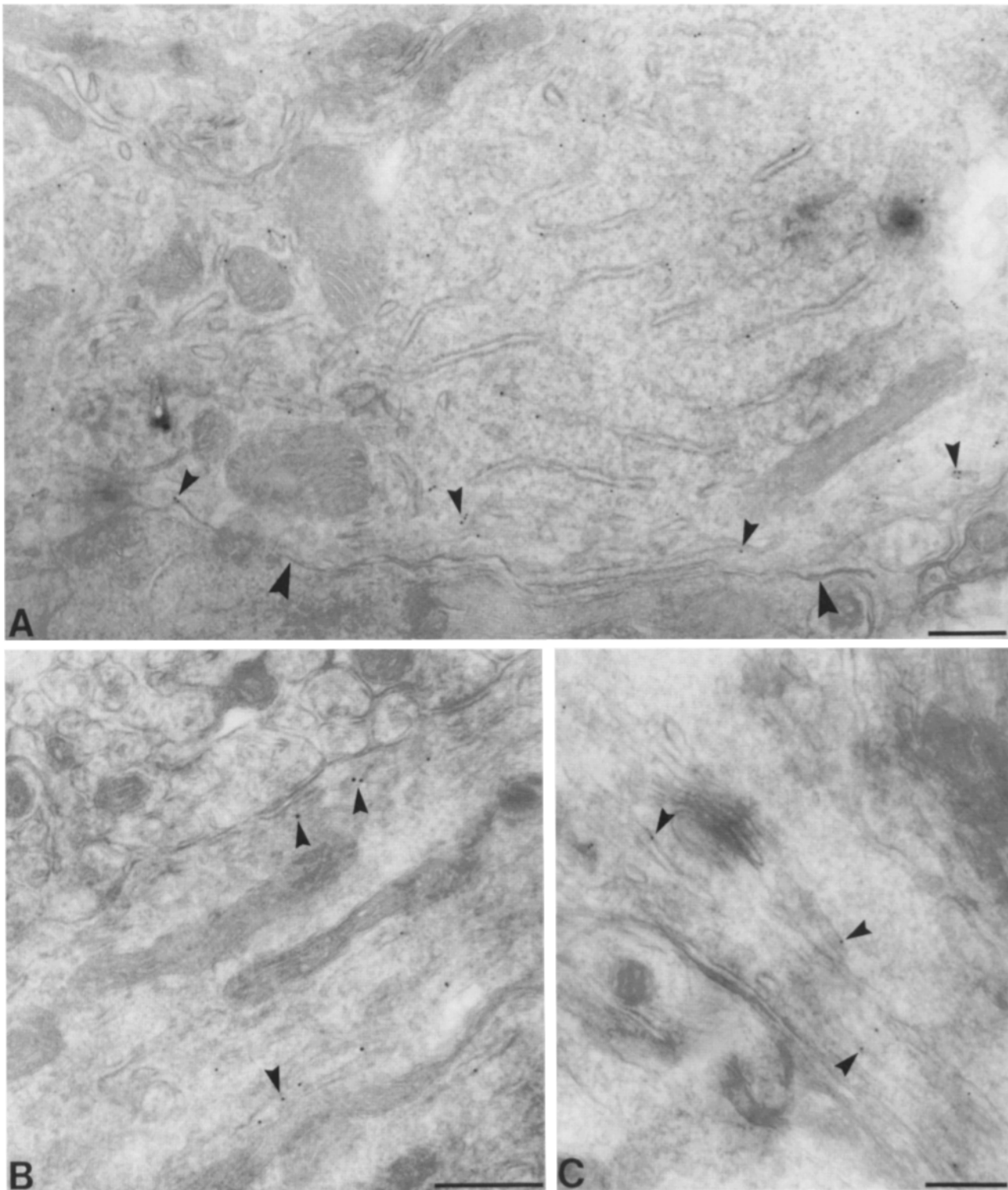
Figure 5. Localization of ryanodine receptors in chick cerebellar Purkinje neurons using 10 nm gold particles. (*A*) In the cell body, labeling is seen in the ER and in small membraneous cisternae (*small arrowheads*), while no labeling of the plasma membrane (*large arrowheads*) is evident. Magnification, 26,400×. Bars, 0.5 μm. (*B*) In the proximal dendrite, membranous tubules present in the shaft are labeled. (*C*) In the proximal axon, labeling is observed in membranous cisternae. Bars, 0.5 μm.

significance of the relative enrichment of both proteins at the branch points of the dendritic tree. The ryanodine (and presumably caffeine) sensitivity of one of putative calcium-release channels in this series of events should provide the means for testing this possibility.

In avian cerebellum, the InsP₃ receptor appears to be more abundant than the ryanodine receptor. The importance of differences in the abundance of these proteins to their possible functional contributions is difficult to assess. For example, the reticulated pattern of distribution of the ryanodine
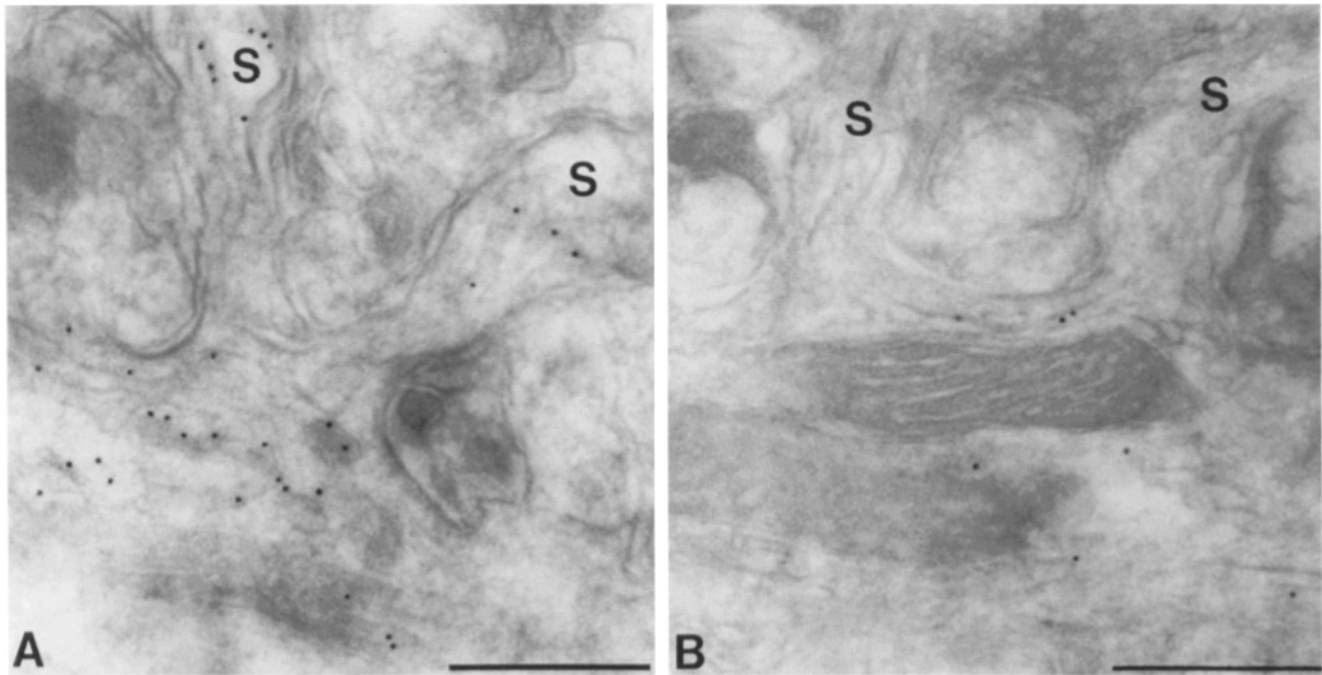
N

A

B
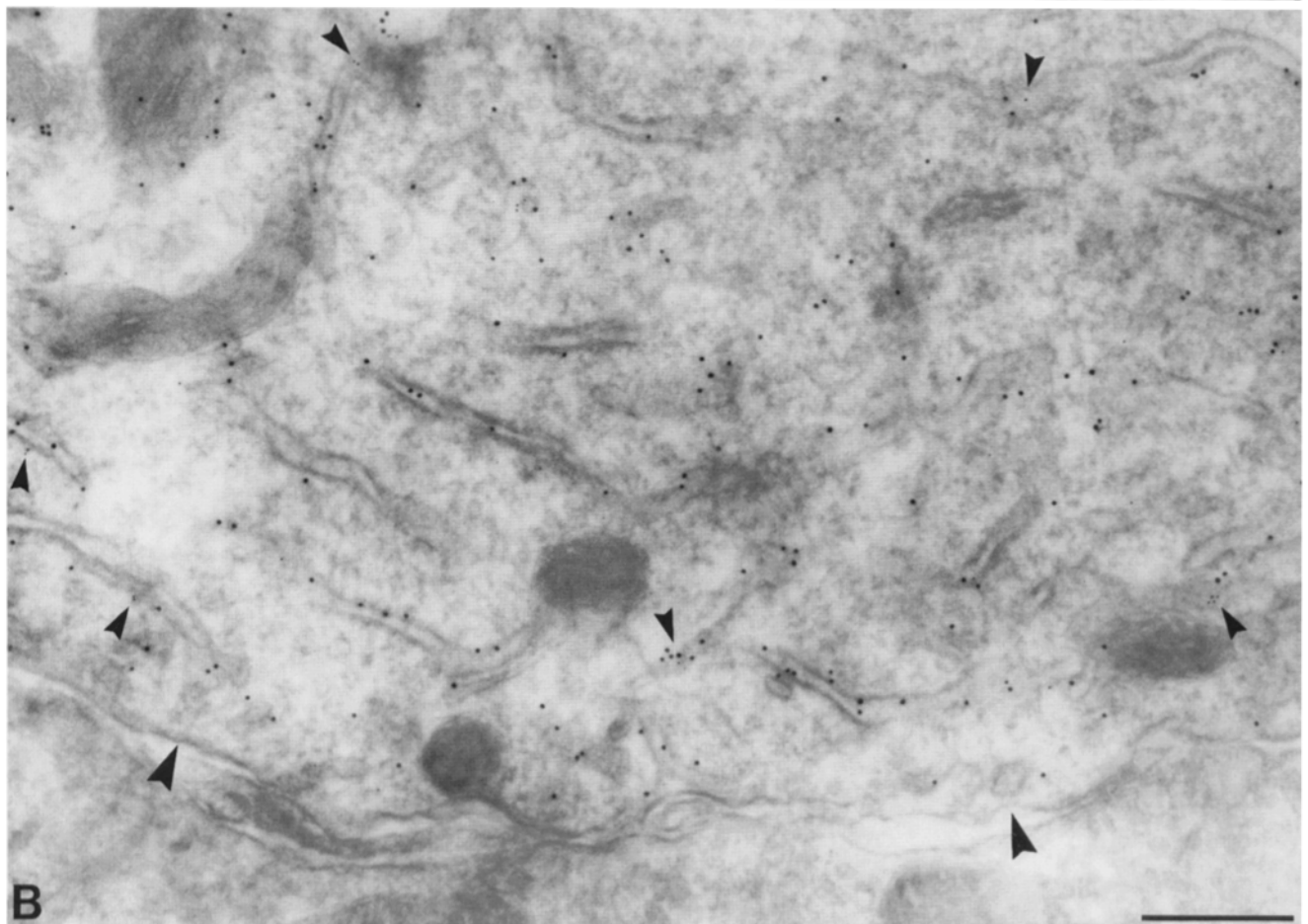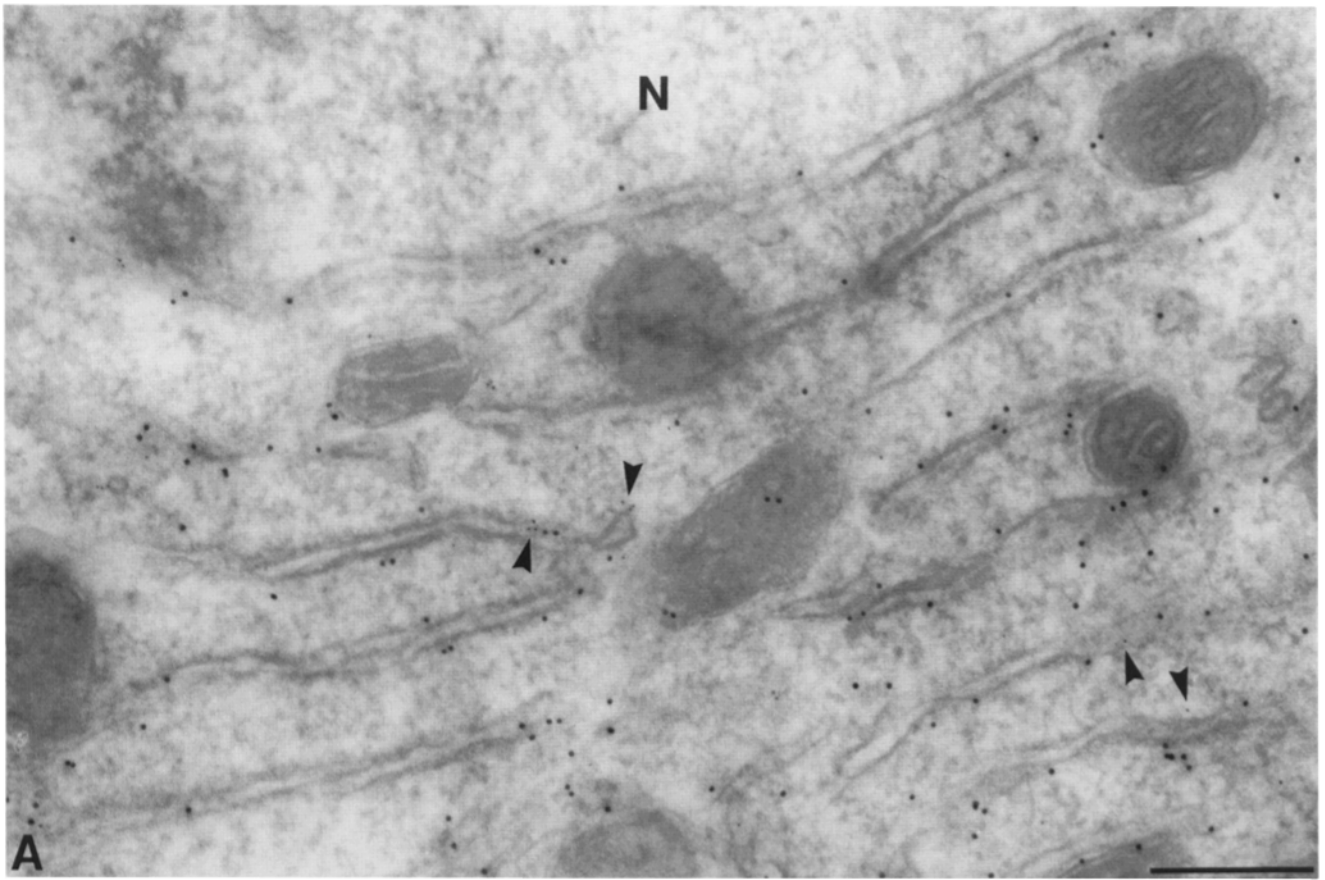
Figure 7. Immunogold labeling of InsP₃ and ryanodine receptors in ultrathin cryosections of the distal region of Purkinje neuron dendrites. (*A*) Labeling of the InsP₃ receptor with 10-nm gold in membranous elements in both the dendritic shaft and spines (*S*). (*B*) Labeling of the ryanodine receptor with 10-nm gold in membranous elements in the dendritic shaft, but not in the spines (*S*). Bars, 0.5 μm.

receptor calcium channel could produce a sphere of influence in the three-dimensional volume of the neuron equal to that of a more abundant calcium-release channel having a more spatially restricted distribution. Moreover, the kinetic characteristics of the calcium release and the size of the calcium store associated with each receptor will also determine the relative influence exerted by each calcium-release event.

In view of the presence of the ryanodine and InsP₃ receptors in the same subsets of membranes and in some cases the same membranes in the Purkinje neuron cell body, the calcium-release events mediated by each protein may interact in a complex manner and perhaps be specialized for different aspects of intracellular calcium homeostasis. For example, a caffeine-sensitive calcium store identified in bovine adrenal chromaffin cells has been suggested to serve as a calcium buffer, rather than directly in stimulus-secretion coupling (7). In addition, this colocalization of calcium-release channels could provide the machinery necessary for the generation of oscillations in intraneuronal calcium as suggested by Berridge and Galione (2). It should be noted that it remains to be determined whether all of the proteins identified by the antibodies used in the present studies are functional calcium channels. Polypeptide subunits being synthesized and assembled, or proteins that are in transit to the site of their functional activity, but which are not yet func-

tional as calcium release channels, could be recognized by the antibodies.

If the ryanodine and InsP₃ receptors which coexist in the same membranes in Purkinje neurons are verified to be functional, then their differential distribution observed in distal dendritic processes, would indicate that there are regional differences in the localization of the retention signals for both proteins. The identification of these signals in the dendritic shaft may be particularly interesting, since in some micrographs the ryanodine receptor appears to reside in membranes within the shaft that may be continuous with those in the spine (Fig. 5 *B*). A more rigorous three-dimensional reconstruction of the membrane systems present in the dendritic processes is necessary to establish whether this is the case. We have identified two isoforms of the ryanodine receptor in avian skeletal muscle (1) and cerebellum (8). Recently, both isoforms have been localized in the same Purkinje neurons (Ouyang, Y., P. D. Walton, J. A. Airey, J. L. Sutko, C. F. Beck, T. J. Deerink, and M. H. Ellisman, manuscript in preparation), and both isoforms are recognized by the anti-ryanodine receptor antibody used in the present studies; therefore, differences in the two isoforms could contribute to regional differences in the distribution of these proteins. We are currently using ryanodine receptor isoform–specific antibodies to investigate this possibility.

Figure 6. Co-localization of ryanodine and InsP₃ receptor in chick cerebelluar Purkinje neurons using 5- and 10-nm gold particles, respectively. (*A*) Region of the cell body near the nucleus (*N*) demonstrating the co-localization of the InsP₃ and ryanodine receptors in smooth and rough ER and to a lesser extent in the nuclear envelope. The arrowheads indicate ryanodine receptors labeled with 5-nm gold particles. (*B*) The cell body near the plasma membrane (*large arrowheads*) showing co-localization of the InsP₃ and ryanodine receptors on ER and on subsurface cisternae. The small arrowheads indicate ryanodine receptors. Bars, 0.5 μm.

The distribution of the InsP₃ receptor observed in avian Purkinje neurons in the present study are in general agreement with that reported for mammalian Purkinje neurons (21, 22, 29, 30). The localization of the ryanodine and InsP₃ receptors to subsurface cisternae is consistent with earlier observations of morphological similarities between these structures and skeletal muscle triad junctions and suggestions of their involvement in neuronal calcium release (11). The present findings and those by others cited above indicate that intracellular membranes in many cell types contain multiple systems capable of regulating cytoplasmic calcium levels. These calcium uptake/release systems are generally attributed to ER membranes. In addition, a specialized organelle, termed the calciosome, has also been described (40). It would appear likely though that once the components of each system, their spatial distribution in the cell, the extent of their interactions, and the physiological significance of their contributions to cell function are appreciated that a more specific taxonomy will be developed for the involved membranes.

In summary, the major findings of the present studies are that in chicken cerebellum: (a) ryanodine and InsP₃ receptors exist as biochemically and immunologically distinct entities with the InsP₃ receptor being more abundant; (b) these receptors exist primarily in Purkinje neurons and are found in the same neurons with overlapping distributions in dendritic processes and the cell body, where the ryanodine receptor has a less continuous distribution than the InsP₃ receptor; (c) in dendritic processes, relatively high concentrations of both proteins are present at branch points and the InsP₃ receptor is present in both dendritic shafts and spines, whereas the ryanodine receptor is found only in dendritic shafts; and (d) in the cell body the InsP₃ and ryanodine receptors reside in a variety of membranes, including rough and smooth ER, the subsurface cisternae, and to a lesser extent the nuclear envelope; moreover, in some instances both receptors coexist in the same membrane.

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