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Immunocytochemical localization of the plasma membrane calcium pump, calbindin-D_{28k}, and parvalbumin in Purkinje cells of avian and mammalian cerebellum

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ABSTRACT **A monoclonal antibody produced against the human erythrocyte plasma membrane calcium pump (PMCA) was shown to react immunohistochemically with an epitope of the PMCA in avian and mammalian cerebellum. Western blot analysis of purified synaptosomes and homogenates from avian cerebellum revealed major immunoreactive proteins with molecular masses (130 kDa and 138 kDa) similar to those of purified erythrocyte PMCA. Dual-imaging confocal immunofluorescence microscopy of avian cerebellum showed that the PMCA antibody stained the periphery of the soma whereas calbindin-D_{28k} was located in the cytosol. PMCA heavily stained the more distal dendrites of the Purkinje cells and, within the resolution of the fluorescence procedure, colocalized with calbindin-D_{28k}. By using alkaline phosphatase-conjugated second antibody, PMCA was again localized to the peripheral soma, to a segmental pattern in dendrites, and to presumed spiny elements. The soma periphery and dendrites of Purkinje cells of the rat cerebellum were also prominently stained with anti-PMCA antibody and compared to parvalbumin localization. Dendritic depolarization and dendritic spiking behavior are significant Ca^{2+}-dependent events of Purkinje cells. The rapid decline of intracellular free Ca^{2+} after the rapid rise time of Ca^{2+} transients is considered to be due to sequestration by Ca^{2+} buffers, uptake by intracellular stores, and Ca^{2+} extrusion mechanisms, the latter a function of PMCA now shown immunohistochemically to be a prominent feature of Purkinje cell dendrites.**

The only output from the cerebellar cortex arises from Purkinje cells whose GABAergic axons terminate in deep cerebellar nuclei and other regions of the central nervous system (1) (GABA is γ-aminobutyric acid). The Purkinje cells receive two types of excitatory input from the periphery by way of the granule-cell-derived parallel fibers that invest the more distal dendrites and from climbing fibers that make contact with the Purkinje cell soma and proximal dendrites. GABAergic inhibitory inputs to these cells originate from the interneurons, the basket and stellate cells, resident in the cerebellar molecular layer.

The excitatory neurotransmitters from parallel fibers and climbing fibers are considered to be glutamate and aspartate, respectively, and activate both ionotropic and metabotropic receptors on the Purkinje-cell surface (1–4). Activation of Purkinje-cell dendrites is characterized initially by a slow Ca^{2+}-dependent depolarization and, when of sufficient magnitude, all-or-none Ca^{2+}-dependent spikes are generated (5, 6). The Ca^{2+} contributing to the Ca^{2+}-dependent spiking might be both from the influx of Ca^{2+} from the extracellular fluid through activation of voltage- and agonist-dependent Ca^{2+} channels and from the release of Ca^{2+} from the intracellular stores shown to contain ryanodine receptors (7).

Real-time imaging of Purkinje cells, using a fluorescent intracellular Ca^{2+} probe showed that the Ca^{2+} transients are characterized by a rapid rise time followed by a rapid return to baseline concentrations (6, 8). The rapid decrease in intracellular Ca^{2+} concentration is probably due to the operation of several processes, including the sequestration by the endoplasmic reticulum shown to contain a Ca^{2+} pump (9–11) and calsequestrin (11, 12) and by Ca^{2+} binding to the high-affinity calcium-binding proteins, calbindin-D_{28k} (CaBP-28) and parvalbumin (PV), previously identified in Purkinje cells (13, 14). Another mechanism that could participate meaningfully in rapidly reducing intracellular Ca^{2+} concentrations is the energy-dependent extrusion of Ca^{2+} from dendrites into the extracellular space. The presence of an ATP-dependent Ca^{2+} pump in isolated brain synaptosomes has been shown biochemically (15, 16). Three isoforms of the plasma membrane calcium pump (PMCA) mRNA (PMCA1, PMCA2, and PMCA3) were identified in rat brain by Greeb and Shull (17), and Stahl *et al.* (18) showed, by *in situ* hybridization, that the Purkinje cells of rat cerebellum contained each of the three PMCA isoforms. Quantitative analysis indicated that the concentration of PMCA2 mRNA was ≈10-fold greater than that of the PMCA1 and PMCA3 mRNAs.

A monoclonal antibody produced against the human erythrocyte PMCA was shown to cross-react immunohistochemically and by Western blot analysis with the PMCA of rat (19) and chicken (20) intestine, human (21, 22) and rat (23) kidney, placenta (24), and mammalian choroid plexus (25). In the present study, this antibody, 5F10, is shown to cross-react with an epitope of the PMCA of the soma and dendritic tree of avian and mammalian Purkinje cells. The localization of the PMCA was compared with established markers of the Purkinje cell, CaBP-28 (13) and PV (14).

MATERIALS AND METHODS

Four-week-old White Leghorn chickens or adult rats were anesthetized with Nembutal (50 mg/kg) and perfused through the cardiac ventricle with 60 ml of phosphate-buffered saline (0.01 M phosphate, pH 7.4; PBS), followed by 300 ml of a fixative containing 4% (wt/vol) paraformaldehyde or 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.4). The brains were placed in the same

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Abbreviations: PMCA, plasma membrane calcium pump; CaBP-28, calbindin-D_{28k}; PV, parvalbumin.

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fixative for 24 h at 4°C and then immersed in 0.1 M sodium phosphate, pH 7.4/0.15 M NaCl with 0.03% sodium azide. Cerebella were also directly fixed in 10% (vol/vol) phosphate-buffered formalin. Paraffin sections (4 μm) were used for immunohistochemistry.

Antisera. The polyclonal anti-CaBP-28 antibody was produced in the rabbits from chicken intestinal CaBP-28. The anti-PMCA monoclonal antibody was produced against the purified human erythrocyte PMCA and described by Borke *et al.* (23). The polyclonal chicken muscle anti-PV antisera was a generous gift from J.-F. Pechere (Montpellier, France). Monoclonal anti-carp muscle PV antibody was purchased from Sigma.

Synaptosomal Preparation and Western Blot Analysis. Chickens were killed by decapitation and the cerebellum was rapidly removed. The synaptosomes were prepared by the procedure of Booth and Clark (26). Synaptosomal proteins were separated by SDS/polyacrylamide gel electrophoresis by the method of Laemmli (27), and the proteins were transferred to nitrocellulose sheets by the procedure of Towbin *et al.* (28). The presence of PMCA in the synaptosomes was done by Western blot analysis as described (20).

Protein analysis was by the Lowry procedure (29).

Fluorescence Immunohistochemistry. The localization of the Ca^{2+}-binding proteins and PMCA was visualized by indirect immunofluorescent staining. The dewaxed rehydrated paraffin sections, after preincubation in 5% (vol/vol) normal rabbit and 5% (vol/vol) normal goat sera, were incubated sequentially with anti-CaBP-28 (1:500 dilution) and anti-PMCA (1:2000 dilution) antibodies. After rinsing, the section was incubated with a 1:50 dilution of rhodamine-conjugated anti-rabbit IgG (heavy and light chains) (Boehringer Mannheim) and a 1:50 dilution of fluorescein-conjugated anti-mouse IgG (whole molecule) (Sigma). Control sections were incubated with BALB/c ascites fluid and normal rabbit serum in place of their respective first antibodies.

The dual localization of the PMCA/PV or CaBP-28/PV pairs was carried out using a similar technique.

Confocal Microscopy. Laser-scanning confocal microscopy was carried out with a Zeiss Axiovert 10 and Bio-Rad MRC-600 equipped with a krypton–argon ion laser (Ion Laser Technology, Salt Lake City). Fluorescein and rhodamine emission were simultaneously measured using the K1 and K2 filters provided with the instrument. Excitation was at 488 and 568 nm, and emission was measured through 522DF35 and 585LP filters. An achroplan ×40 objective was used during acquisition. Software merging of images was carried out using the COMOS software. Images were recorded using a Screenstar film recorder (Presentation Technologies, Sunnyvale, CA).

Immunocytochemistry with Alkaline Phosphatase-Conjugated Second Antibody. Dewaxed rehydrated paraffin sections of avian cerebellum were preincubated with 10% (vol/vol) normal rabbit serum for 15 min at room temperature. Incubation with the primary antibody (anti-PMCA, 5F10; 1:2000 dilution) was at 37°C for 2 h and, after rinsing with PBS, followed by incubation with the biotinylated second antibody for 15 min. After rinsing, the streptavidin-alkaline phosphatase conjugate was added for 5 min and this step followed by rinsing. Color development was by the use of the AP-Blue kit from Zymed, incubating the tissue with chromagen at 37°C for 30 min.

RESULTS

The immunoblot analysis of chicken cerebellar synaptosomal membranes and homogenate and purified erythrocyte PMCA is shown in Fig. 1. Major polypeptides reacting with the 5F10 antibody produced against the erythrocyte PMCA are in the

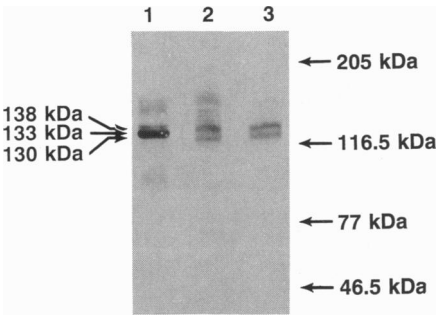

FIG. 1. Western blot analysis of the reactivity of the anti-PMCA antibody 5F10 with cerebellar proteins. The proteins of cerebellar homogenate (lane 2, 83 μg of protein) and cerebellar synaptosomes (lane 3, 15 μg of protein) and the purified erythrocyte PMCA proteins (lane 1, 1 μg of protein) were separated by SDS/PAGE and transferred electrophoretically to a nitrocellulose membrane. The primary antibody was the monoclonal antibody produced against the erythrocyte PMCA and the second antibody was anti-mouse IgG conjugated with horseradish peroxidase.

molecular mass range of 130–138 kDa, which is in the expected range for PMCA, as recorded by Carafoli (30). These sharp bands in each pattern presumably represent the monomeric Ca^{2+} pump polypeptides. The diffuse bands and minor bands are probably aggregation products and/or proteolytic degradation products of the pump, as suggested for the human erythrocyte Ca^{2+} pump by Niggli *et al.* (31). Monomers of PMCA have been shown to dimerize (32, 33), with the intermolecular bonding region suggested to be associated with the calmodulin-binding sites (33). Western blots of various tissues, including kidney (21, 22) and intestine (19, 20), have also shown multiple bands with the 5F10 antibody.

The immunofluorescent images of CaBP-28 and PV localization in chicken cerebellum showed, as did others (13, 14), heavy staining of the soma and dendrites of Purkinje cells (Fig. 2 *A* and *B*). PV was also present in basket and/or stellate cells of the molecular layer of the cerebellum (14). The superimposed video images accentuate the colocalization of proteins as shown by the resultant yellowish color (Fig. 2*C*).

The PMCA epitope in cerebellum reacting with the 5F10 antibody was visualized by the immunofluorescence method on the periphery of the soma and the dendrites of the Purkinje cell (Fig. 2*D*). In the same section, the intracellular localization of CaBP-28 (Fig. 2*E*) in Purkinje cells is shown. Distinctly different patterns of localization of the PMCA as compared to CaBP-28 are apparent in the soma and primary dendrites, with calbindin present within the cytosol and the Ca^{2+} pump present on the periphery of the soma and presumably the plasma membrane of the primary dendrites. Within the resolution of the procedure, there is an apparent colocalization of the two proteins in the finer more distal dendrites, emphasized by the superimposed images (Fig. 2*E*). The periphery of cells in the granular layer stained with 5F10 antibody, and other structures seemed to have both PMCA and CaBP-28-like immunoreactivity.

Immunohistochemical staining with the 5F10 anti-PMCA antibody and the alkaline phosphatase-conjugated second antibody (Fig. 3) revealed more detail than the above fluorescent series. The association of the Ca^{2+} pump with the periphery of the soma and the primary dendrites is again readily apparent. The occasionally labeled spikes and segmental pattern of labeling throughout the molecular layer might signal the presence of PMCA units on dendritic spiny elements and possibly at sites of neuronal/neuronal interactions.

The localization of PV and PMCA in rat cerebellum is depicted in Fig. 4 *A* and *B*, respectively. The soma and

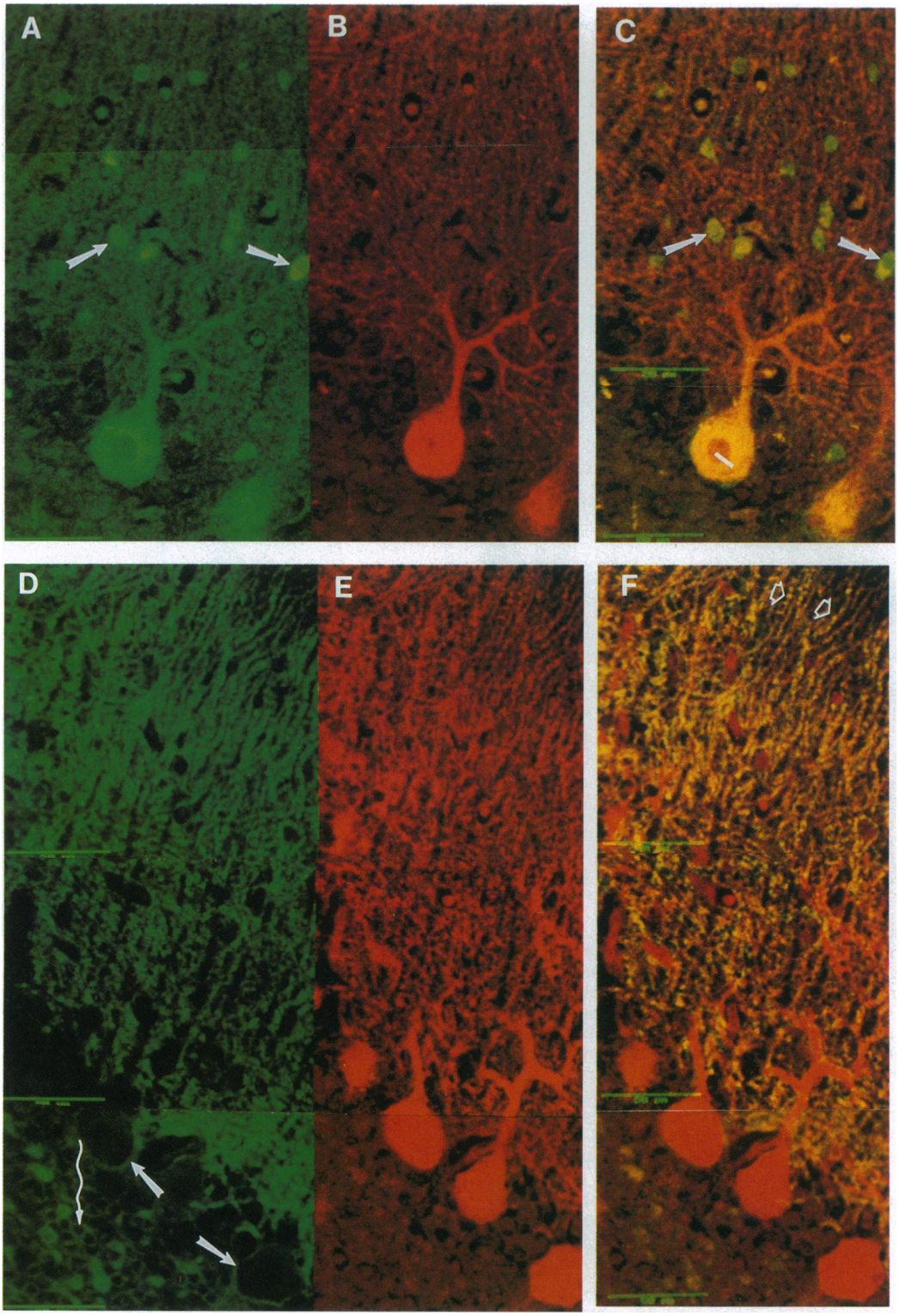

FIG. 2. Localization of CaBP-28, PV, and the PMCA in the Purkinje cells of avian cerebellum by confocal fluorescence microscopy. The antigens on cerebellar tissue sections were visualized by a double antibody technique and the secondary antibody was conjugated to either fluorescein (*A* and *D*) or rhodamine (*B* and *E*). Fluorescence localization was determined by laser confocal microscopy, and the figures represent a montage of two photographs taken at sequential depths of the Purkinje and molecular layers. (*A*) PV localization. Note the presence of antigen within the soma, primary and more distal dendrites, and stellate/basket cells (*A* and *C*, arrows). (*B*) CaBP-28 localization. Similar to PV but note the absence in stellate/basket cells. (*C*) Superimposed images of PV and CaBP-28, accentuating colocalization in soma and dendrites, and specific presence of PV in the stellate/basket cells. (*D*) PMCA localization. Note presence of antigen on the periphery of the Purkinje cell soma (*D*, straight arrows) and prominent staining of the dendrites in the molecular layer. Staining of the periphery of a cell in the granule layer is designated with a wavy arrow. (*E*) CaBP-28 localization. Similar to *B* above. (*F*) Superimposed PMCA and CaBP-28 images, emphasizing the differential localization in the Purkinje cell layer and colocalization in the more distal dendrites. Note "hot spots" stained with both PMCA and CaBP-28 (*F*, open arrows). (Bar = 50 μm.)

dendrites of the Purkinje cells are heavily stained with the anti-PV antisera. The anti-PMCA antibody stained the periphery of the soma whereas the interior of the soma and the primary dendrites did not stain, again as expected for a

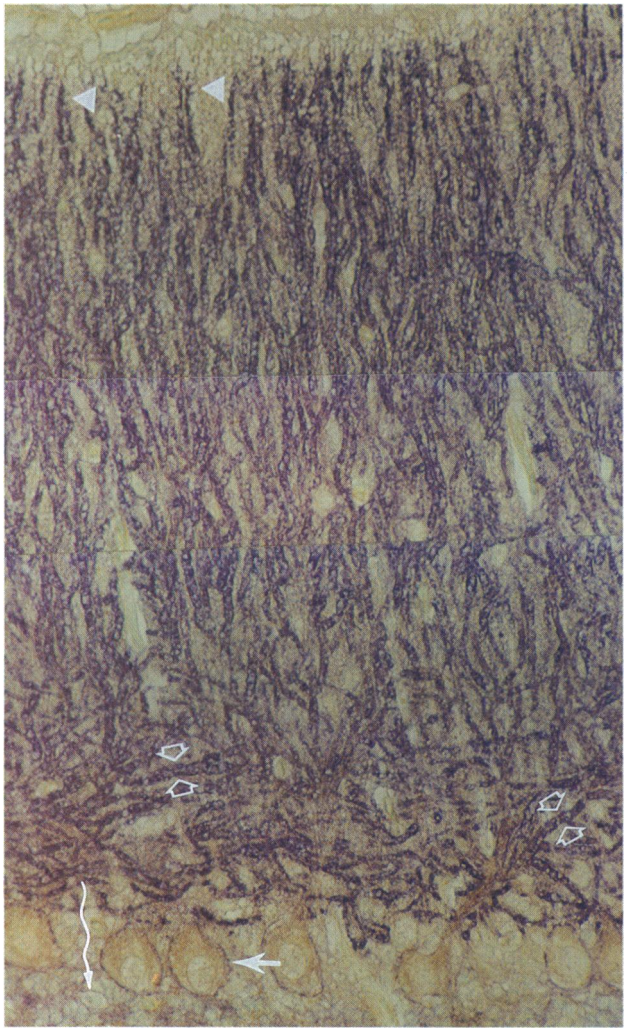

FIG. 3. Visualization of the localization of the PMCA in the Purkinje cell and molecular layers of the avian cerebellum by conventional immunohistochemistry. The tissue section of cerebellum was incubated in sequence with the anti-PMCA monoclonal antibody, a biotinylated second antibody, streptavidin–alkaline phosphatase conjugate, and chromagen. The figure is a montage of three photomicrographs taken at sequential depths of the Purkinje cell and molecular layers and shows staining of the periphery of the soma (straight arrow) and of the dendrites (open arrows). The labeled spikes (arrowhead) and segmental pattern of labeling might reflect sites of spiny elements and/or neuronal–neuronal interactions. The periphery of cells in the granular layer was also stained (wavy arrow). (×270.)

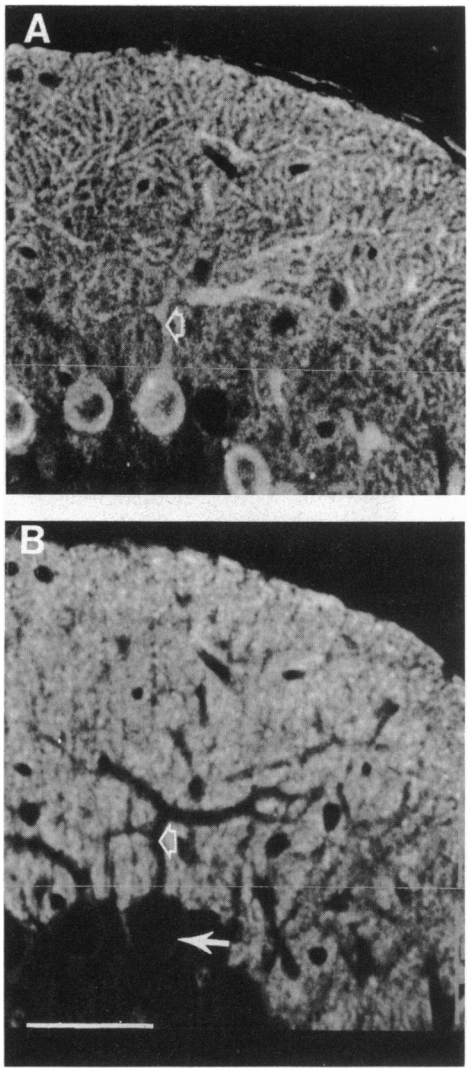

FIG. 4. Localization of the PMCA and PV in Purkinje cells of the rat cerebellum by confocal fluorescence microscopy. The cerebellar tissue section was incubated with rabbit anti-PV antibody and mouse anti-PMCA monoclonal antibody, followed by their respective second antibodies, anti-rabbit IgG conjugated with rhodamine and anti-mouse IgG conjugated with fluorescein. (*A* and *B*) Immunoreactivity with the anti-PV and anti-PMCA antibodies, respectively. Each figure is a montage of two photomicrographs taken at sequential depths of the Purkinje cell and molecular layers. The PMCA antibody stained the periphery of the Purkinje cell soma (*B*, solid arrow). Note the absence of anti-PMCA antibody staining of the internal aspect of the primary Purkinje cell dendrites (*B*, open arrow), which are heavily stained by anti-PV antibody (*A*, open arrow). Both antibodies heavily stained the more distal dendrites. (Bar = 50 μm.)

plasma membrane-associated protein. It is apparent, however, that the tertiary finer dendrites are heavily stained by both anti-PMCA (5F10) and anti-PV antibodies.

DISCUSSION

The present studies demonstrate that the 5F10 antibody produced against the PMCA of the erythrocyte plasma membrane cross-reacts with PMCA epitopes in chicken and rat cerebellum. The reactivity of 5F10 antibody with neuronal elements of the avian and mammalian central nervous system was not previously shown, although the localization of PMCA in the mammalian choroid plexus was reported (25). By Western blot analysis, the molecular mass of the prominent 5F10-reactive proteins in chicken cerebellar homogenates and isolated synaptosomes was within the molecular mass range expected of PMCAs (30).

The localization of the 5F10 antibody on the periphery of the soma of the Purkinje cells and lack of intracellular staining in the primary dendrites are consistent with a protein that is resident to the plasma membrane (Figs. 2 and 4). The laser confocal fluorescent images clearly show colocalization of PMCA with the relatively specific Purkinje cell markers, CaBP-28 and PV, in the more distal dendrites. The higher resolution of the immunochemically stained section (Fig. 3) gave more detail of the localization pattern of the PMCA epitope than the fluorescence images; a segmental pattern of staining of the dendrites and staining of presumed dendritic spiny elements are seen.

The simultaneous presence of relatively high concentrations of the PMCA and CaBP-28 in Purkinje cells is reminiscent of a similar colocalization in Ca^{2+}-transporting epithelia (20, 22, 34, 35). CaBP-28 in these polarized epithelia has been considered to increase the rate of intracellular diffusion of

calcium (36) and might serve a similar function in Purkinje cells. There is also evidence that the calbindins directly stimulate the activity of the PMCA of intestinal (37, 38) and erythrocyte (39) membranes, and modulate the activity of voltage-dependent Ca^{2+} channels (43). Thus, CaBP-28 might have multiple functions in Purkinje cells, serving as an intracellular Ca^{2+} buffer (44), as an intracellular Ca^{2+} transporter, and as a modulator of the activity of PMCA and other Ca-dependent enzymes and proteins. Iacopino *et al.* (40) further suggested a role of calbindin in the maturation and maintenance of Purkinje cells. PV, distinct from CaBP-28, binds Mg^{2+} at basal levels of intracellular Ca^{2+} and, as cytosolic intracellular Ca^{2+} concentration rises, the bound Mg^{2+} is displaced by Ca^{2+} (41). In addition to buffering intracellular Ca^{2+}, PV also represents a potential source of the Mg^{2+}.

As emphasized in a recent review (42) and noted above, the Ca^{2+} is a significant factor in the physiological behavior of the Purkinje cell. Some of the molecular and biochemical entities involved in the Ca^{2+}-related mechanisms of these neuronal cells have been mentioned. As reported herein, the dendrites of the Purkinje cells contain a relatively high density of PMCA, suggestive of an important role of this protein in the homeostatic regulation of intracellular Ca^{2+} concentration. When consideration is given to the relative uniqueness of Ca^{2+}-dependent inward currents in the functioning of Purkinje cells, the Ca^{2+} extrusion process assumes particular significance. It is evident that, over time, the rate of influx of Ca^{2+} upon dendritic activation must be balanced by the rate of extrusion by PMCA and other Ca^{2+} extruding mechanisms, otherwise the total Ca^{2+} concentration would continue to rise, potentially leading to Ca^{2+}-induced neurotoxicity.

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