RESEARCH ARTICLE

Developmental Distribution of Plasma Membrane Ca^{2+}-ATPase Isoforms in Chick Cerebellum

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The plasma membrane Ca^{2+}-ATPase (PMCA) is highly expressed in the nervous system, but little information is available about its implication in neuronal development. We have analyzed the expression and localization of different isoforms of PMCA in membrane vesicles and sections of chick cerebellum from embryonic day 10 to hatching. We found that the relative amount of each PMCA isoform and their spatiotemporal distribution in the cerebellum are directly linked to precise cellular types during the cerebellar maturation, even in a non-neural tissue as choroid plexus. Purkinje cells contain the highest diversity of PMCA isoforms of the cerebellar cortex since the moment of its morphogenesis. From embryonic day 15, the PMCA2 was highly expressed in the whole Purkinje cell, while PMCAs 1 and 3 had a more restricted distribution in the soma and dendritic branches, and these distributions were evolving according with cell maturation. Other cellular types seem to contain a specific combination of isoforms, but with a well-defined distribution pattern at late moments of development. Thus, PMCAs 1 and 3 were located in the soma of molecular layer interneurons, and only the PMCA2 was observed in granule cells at hatching. Furthermore, PMCA isoforms are also expressed in cellular compartments characterized by a high amount of synapses, suggesting a key role of these proteins in synaptogenesis and in the maturation of neuronal electrophysiological properties. ***Developmental Dynamics 236:1227–1236, 2007.*** **© 2007 Wiley-Liss, Inc.**

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INTRODUCTION

The plasma membrane Ca^{2+}-ATPase (PMCA) transports Ca^{2+} from the cytoplasm to the extracellular space at expenses of ATP hydrolysis. The protein plays a crucial role in the regulation of cytosolic Ca^{2+} concentration in the cell, specifically in the nervous system (Mata and Sepulveda, 2005). There are four different genes that generate four isoforms (PMCA1, PMCA2, PMCA3, and PMCA4), which by alternative splicing in two regions of primary transcripts, generate more than 22 variants with a broad distribution in the majority of eukaryotic cells (Strehler and Zacharias, 2001). These isoforms share the basic function of Ca^{2+} transport, although they show some differences in kinetic properties and regulation mechanisms (reviewed in Strehler and Treiman, 2004). The high diversity of variants in the PMCA family could be due to functional specialization of each isoform, which could be directly related to its specific cellular and subcellular localization.

ABBREVIATIONS DAPI 4′,6-diamidino-2-phenylindole **MV** membrane vesicles **PMCA** plasma membrane Ca^{2+}-ATPase

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Calcium ions play a key role in neuronal development (Spitzer et al., 1994; Mandeville and Maxfield, 1997; Komuro and Rakic, 1998a,b; Wong and Ghosh, 2002; Moya and Valdeolmillos, 2004), being involved in the complex mechanisms of specification and differentiation of neural identity. In cerebellum, as in other regions of the nervous system, these mechanisms take place through a sequence of events that includes neurogenesis, cell migration, axon extension, and synaptogenesis (Hatten et al., 1997; Sotelo, 2004). The cerebellum is an anatomic and physiologically conserved structure of the vertebrate central nervous system, involved in motor coordination. The layered organization of the cerebellum and the well-characterized development of individual cell types and their interconnections (Ramón y Cajal, 1911; Sotelo, 2004) made this region an excellent model for studying the role of proteins involved in Ca^{2+} regulation during the histogenesis of developing central nervous system. In a previous study, we have reported the expression of PMCA family during chick cerebellum ontogeny (Sepulveda et al., 2005), without discriminating among isoforms. In this work, we have analyzed the expression levels and the localization of the different PMCA isoforms during neuronal maturation in chick cerebellum using membrane fractions and tissue sections, to better understand the specific task of these proteins in critic moments of development.

RESULTS

Distribution of PMCA Isoforms in Membrane Fractions From Developing Chick Cerebellum

The presence and expression levels of PMCA isoforms were analyzed in chick cerebellum at different stages of development, according to Sepulveda et al. (2005). Cerebellar membrane vesicles (MV) were used from chick embryos and hatchlings (P0) and the proteins were identified by Western blot with specific antibodies (Fig. 1). We have, first, analyzed the distribution of PMCA using the a-cb polyclonal antibody (Fig. 1A), raised in our laboratory against the synaptosomal PMCA purified from pig cerebellum, which is a mixture of isoforms (as shown in Sepulveda et al., 2004). Therefore, this antibody does not discriminate among PMCA isoforms. From embryonic day 10 (E10) to hatching (P0) the a-cb antibody labeled several proteins with different intensities and molecular weights among 126–145 kDa, which is the expected range for PMCA isoforms. The immunostaining for the homogeneously expressed β-tubulin was used as a protein loading control to confirm that differences in band intensities were due to changes in their relative protein expressions at different developmental stages.

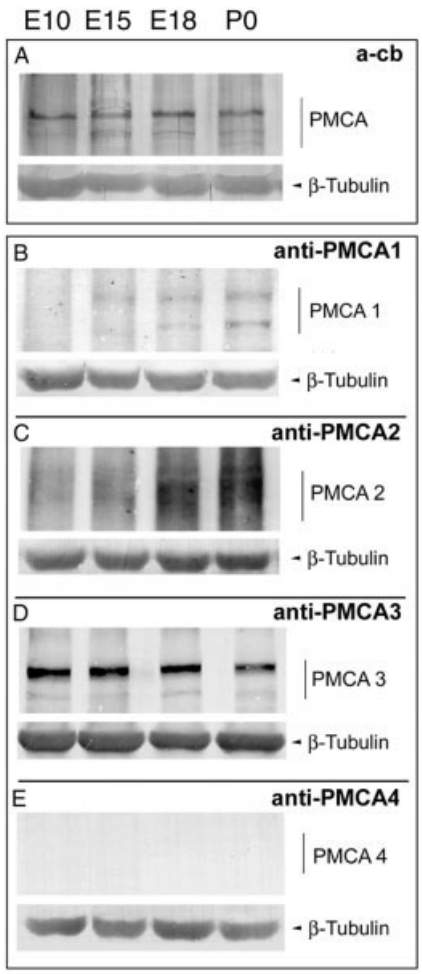

Fig. 1. Immunodetection of plasma membrane Ca^{2+}-ATPase (PMCA) isoforms in cerebellar membrane fractions during development. **A–E:** Thirty micrograms of protein from membranes vesicles of the indicated developmental stages were electrophoresed in a 6.5% sodium dodecyl sulfate-polyacrylamide gel, blotted onto PVDF membranes, and incubated with the a-cb antibody (A) and with the indicated PMCA isoform-specific antibodies (B–E). An antibody against β-tubulin was used to confirm equal protein loading in all lanes.

To investigate the specific expression of the different isoforms of PMCA pump during cerebellar development, Western blot assays were performed with specific anti-isoform antibodies (Fig. 1B–E). The anti-PMCA1 antibody stained two bands of PMCA protein, from the E15 stage (Fig. 1B) of molecular mass around 134 and 130 kDa, which corresponded to variants 1b and 1a, respectively (according to Filoteo et al., 1997). The expression of PMCA1b was constant, whereas that of PMCA1a increased with development. The anti-PMCA2 antibody labeled a broad spectrum of bands around 126–152 kDa (Fig. 1C), whose expression levels clearly increased from E10 to P0. With respect to PMCA3 (Fig. 1D), the specific antibody immunolabeled a strong 133-kDa band and a weak 127-kDa band, which could correspond to PMCA3b and 3a, respectively (according to Filoteo et al., 1997). These PMCA3 isoforms were expressed from the first embryonic stage analyzed, and their intensities did not change significantly during the analyzed stages. The monoclonal anti-PMCA4 antibody did not show any reaction neither in these membrane fractions (Fig. 1E) nor in sections of chick cerebellum. However, this antibody recognizes the chick PMCA4 protein in brain, although very weakly (not shown).

Localization of PMCA Isoforms in Sections of Developing Chick Cerebellum

Distribution of PMCA isoforms in the developing cerebellar cortex.

The specific localization of PMCA proteins was analyzed on parasagittal sections of chick cerebellum from the E10 stage to hatching using the PMCA antibodies described above. During this period, the cerebellar development involves a transformation from a curved structure (at E10) to a structure with a final folial pattern (at P0), very similar to the organization and cellular maturation of an adult, as

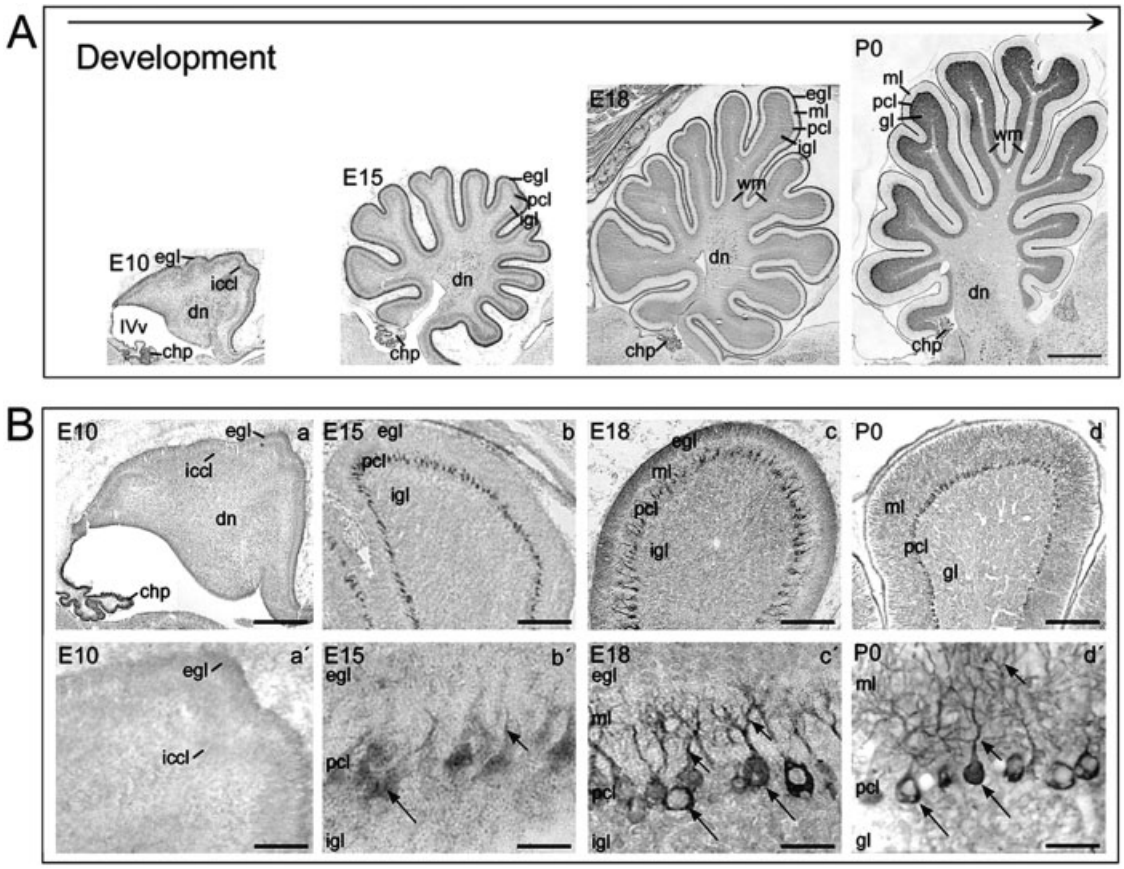

Fig. 2. Cresyl violet staining and a-cb immunolabeling on cryostat sections of developing chick cerebellum. **A:** The development of chick cerebellum from embryonic day (E) 10 to hatching (P0) is shown in parasagittal sections stained with cresyl violet. **B:** Immunohistochemistry with the a-cb antibody at low and high magnifications. At stage E10 (a,a′), a very weak immunoreaction was observed in the inner cortical cell layer (iccl), external granular layer (egl), and in the deep nuclei (dn) neurons, while a strong a-cb staining was present in the choroid plexus (chp). From E15 to P0 (b–d′), staining was observed in the soma and primary dendritic trunks (large arrows and short arrows, respectively, in b′, c′, and d′) of Purkinje cells. igl, internal granular layer; lVv, fourth ventricle; ml, molecular layer; pcl, Purkinje cells layer; wm, white matter. Scale bars = 480 μm in a, 96 μm in a′, 525 μm in b, 50 μm in b′, 600 μm in c, 58 μm in c′, 620 μm in d, 62 μm in d′.

shown in Figure 2A. The immunostaining with the a-cb antibody (Fig. 2B) showed very weak or no-detected reactions at E10 in the inner cortical cell layer, external granular layer, and deep nuclei (Fig. 2Ba,a′). However, an intense a-cb labeling was detected in the choroid plexus of the fourth ventricle (Fig. 2Ba). At the E15 stage, the Purkinje cell precursors, which begin to be organized in a monolayer, showed a clear a-cb staining in the soma and incipient dendritic trunks (Fig. 2Bb,b′). At E18 and P0 stages (Fig. 2Bc–d′), PMCA proteins were detected in the soma and dendritic trees of Purkinje cells. The axon and smaller dendrites of these cells were always devoid of a-cb immunoreaction. The interneurons of the molecular layer did not show immunoreaction at any analyzed stage.

Figure 3 shows the specific distribution of PMCA1, PMCA2, and PMCA3 isoforms during development in serial sections. At the E10 stage (Fig. 3A–C), the antibodies gave weak or no reactions in the inner cortical cell layer, external granular layer, and deep nuclei. Conversely, a strong staining for the three PMCA isoforms was detected in the choroid plexus (Fig. 3A–C), similar to that observed with the a-cb antibody at this stage (Fig. 2Ba). In the cerebellar cortex at E15, Purkinje cells showed clear PMCA1 and PMCA3 expressions in the soma (Fig. 3D,F), whereas PMCA2 labeling was found in the cell membrane (Fig. 3E). At the E18 stage, the three analyzed isoforms were localized in the soma of Purkinje cells (Fig. 3G–I), being the PMCA2 isoform mainly expressed in the cell membrane (Fig. 3H). It is interesting to note a specific distribution of these isoforms in the dendritic trees of the Purkinje cells: the PMCA1 isoform was weakly detected in the dendritic trunk (Fig. 3G), the PMCA3 expression was extend further in the dendritic tree (Fig. 3I), and the PMCA2 isoform was present in the whole dendritic tree, including the spiny branchlets (Fig. 3H). At hatching, the distribution of PMCA isoforms in Purkinje cells was similar to that observed at the E18 stage, according to the increment of cerebellar cortex maturation (Fig. 3J–L). Then, the immunoreaction pattern observed in the Purkinje cell arborization with the a-cb antibody corresponded mainly with PMCA1 and 3 because the PMCA2 was largely expressed in the spiny branchlets of Purkinje cell. In addition, PMCA1 and PMCA3 were also detected in interneurons of the molecular layer, specifically in the soma of basket and stellate cells (Fig.

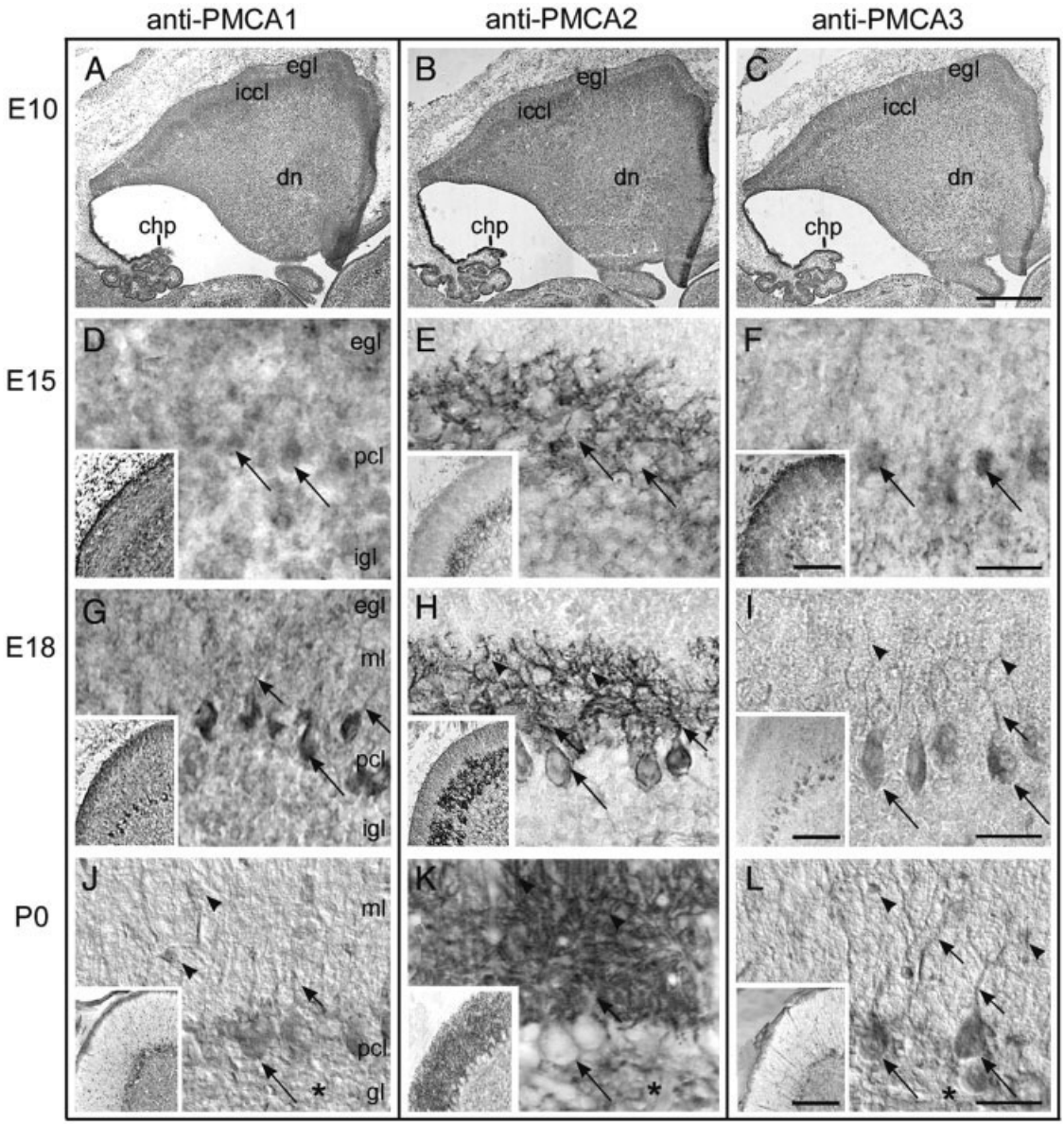

Fig. 3. Plasma membrane Ca^{2+}-ATPase (PMCA) 1, PMCA2, and PMCA3 specific immunoreactions on cryostat sections of developing chick cerebellar cortex. **A–C:** At embryonic day (E) 10 stage, the inner cortical cell layer (iccl), the external granular layer (egl), and deep nuclei (dn) were weakly or not immunolabeled with the specific anti-PMCA1 (A), anti-PMCA2 (B), and anti-PMCA3 (C) antibodies, whereas the choroid plexus (chp) showed a more evident immunostaining with these antibodies. **D–F:** At E15, the labeling of PMCA1 and PMCA3 was located in the Purkinje cell soma (arrows in D and F), while the PMCA2 protein was found in the cell membrane (arrows in E). **G–L:** At E18 and hatching (P0), PMCA isoforms were selectively distributed in the soma of Purkinje cells (large arrows). The dendritic trunks of Purkinje cells expressed the three PMCA isoforms (short arrows in G and I), while the main dendritic branchlets were PMCA2- and PMCA3-positive (short arrows in H, I, K, and L), and the spiny branchlets were exclusively PMCA2-positive (arrowheads in H and K). PMCA1 and PMCA3 immunostaining was also observed in the basket/stellate soma in the molecular layer (ml; arrowheads in J and L). All isoforms were expressed in the granular layer (gl; asterisk in J–L). The inserts in D–L show low magnifications of the developing cerebellar cortex. igl, internal granular layer. Scale bars = 500 μm in C, 50 μm in F, 175 μm in insert in F, 58 μm in I, 218 μm in insert in I, 62 μm in L, 235 μm in insert in L.

3J,L). The three PMCA isoforms were strongly immunodetected in the granular layer at this stage (Fig. 3J–L).

The extremely reduced cytoplasm of the soma of the granule cell around its nucleus and the disposition of the cerebellar glomeruli between groups of granule cells allows us to straightforwardly locate the PMCA protein in these structures of granular cell layer. A detailed examination of the PMCA-specific labeling in the granular layer was performed at P0 by immunofluorescence assays and 2 μM 4′,6-diamidino-2-phenylindole (DAPI) staining (to visualize cellular nuclei) to determine the specific distribution of these proteins in this layer (Fig. 4). The a-cb and the anti-PMCA1, anti-PMCA2, and anti-PMCA3 antibodies stained the cerebellar glomeruli, placed among groups of granule cells, with different intensity. The highest labeling in these structures corresponded to PMCA2, being the only isoform detected in the cell membrane of granule cells soma (Fig. 4E,F).

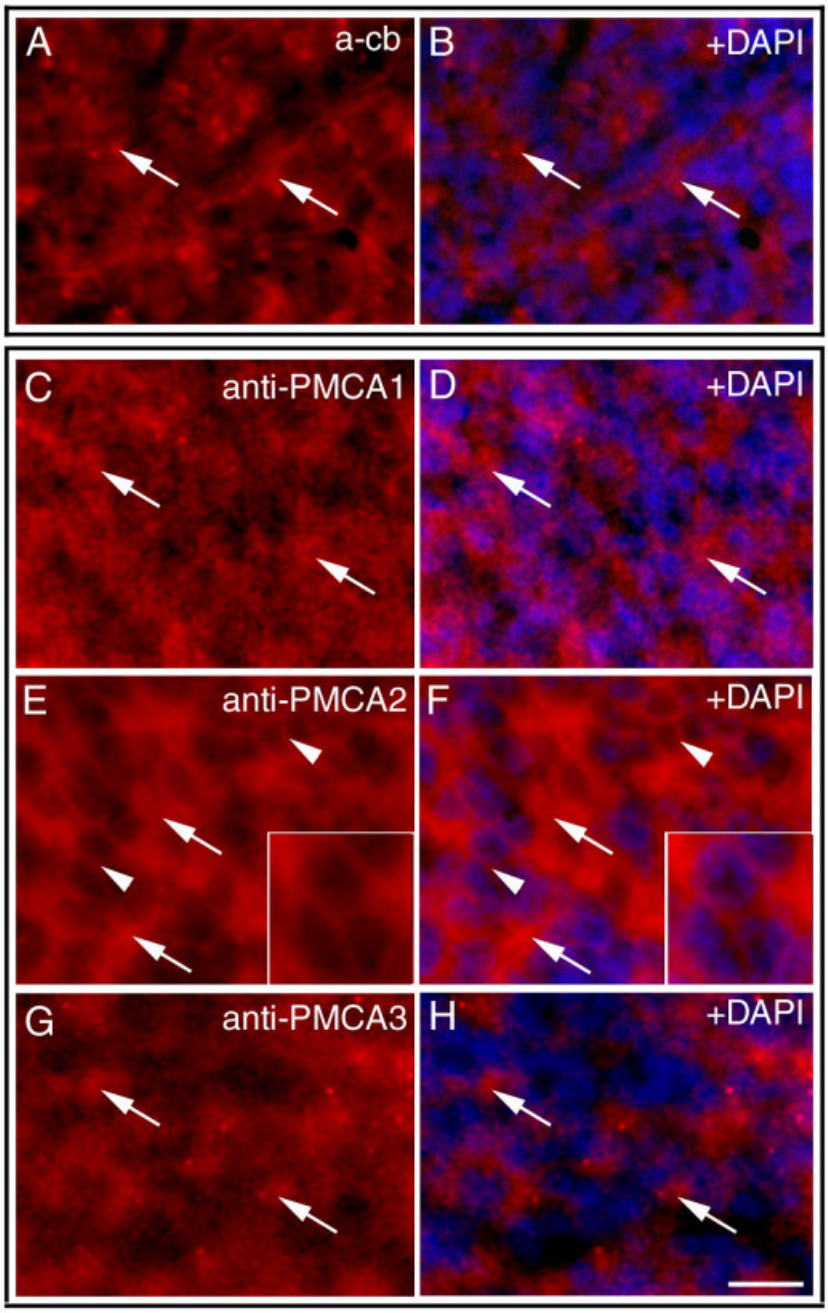

Fig. 4. Immunofluorescence with plasma membrane Ca^{2+}-ATPase (PMCA) antibodies in the granular layer at hatching (P0). **A,C,E,G:** The a-cb (A), and the specific anti-PMCA1 (C), anti-PMCA2 (E), and anti-PMCA3 (G) antibodies stained the cerebellar glomeruli (arrows). The PMCA2 was also expressed in the cell membrane of the soma of granule cells (arrowheads in E,F). **B,D,F,H:** All sections were counterstaining with 2 μM 4′,6-diamidino-2-phenylindole (DAPI) to locate granule cell nuclei (blue). Scale bar = 12 μm.

Localization of PMCA isoforms in cerebellar deep nuclei during development.

The expression of PMCA proteins in deep nuclei was observed from the earliest analyzed stages (Figs. 2Ba, 3A–C), to further stages (Fig. 5). At E15, the a-cb antibody labeled the soma of precursors of deep nucleus neurons (Fig. 5A), while at P0, it stained processes around these cells (Fig. 5B). The PMCA1 and PMCA3 proteins were detected in the soma of the neurons (Fig. 5C,D,G,H), and the PMCA3 was the only isoform located in the dendritic trunks (Fig. 5G–H). In addition, a general reduction of PMCA3 expression was observed at P0 (Fig. 5H). The PMCA2 immunoreaction was always observed in the cell membrane of these neurons and in several processes among them (Fig. 5E,F).

Localization of PMCA isoforms in the choroid plexus during development.

The choroid plexus of the fourth ventricle was strongly stained by all antibodies in a similar way from the first stage analyzed (Figs. 2Ba, 3A–C). A detailed analysis (Fig. 6) showed the expression of PMCA at E10 in the epithelial cells of choroid plexus (Fig. 6A,C,E,G). However, the expression of these proteins decreased and was more restricted in the apical side of these cells as the development proceeds (Fig. 6B,D,F,H).

DISCUSSION

We have analyzed the expression of different PMCA isoforms during development of chick cerebellum in membrane fractions and tissue sections. The PMCA pumps are functional proteins in the developing chick cerebellum from embryonic stage E10, showing a significant ATPase activity and Ca^{2+} transport that increase during development (Sepulveda et al., 2005). Membrane fractions showed a high content of PMCA isoforms and variants, with different patterns of expression during development (Fig. 1). The low abundance of PMCA1 with respect to PMCA2 and 3 in chick cerebellum has also been reported in adult rat cerebellum (Stahl et al., 1992; Filoteo et al., 1997; Burette et al., 2003). In addition, variants 1a and 1b showed a different pattern of expression during neural development that can be compared with that reported in developing rat brain (Brandt and Neve, 1992; Strehler and Zacharias, 2001; Kip et al., 2006). It has been suggested that PMCA1a expression begins with synaptic maturation, which starts at around the E12 stage in developing chick cerebellum (Bertossi et al., 1986), while PMCA1b would be associated to undifferentiated neurons. With respect to PMCA2

expression, the increase observed from E10 to P0 in chick cerebellum was similar to the up-regulation reported from P2 to P30 in developing rat cerebellum (Brandt and Neve, 1992), which has a postnatal development (Armengol and Sotelo, 1991). The high amount of PMCA2 variants observed in the last stages of development is similar to that reported in adult rat cerebellum (Stahl et al., 1992; Filoteo et al., 1997; Stauffer et al., 1997). By contrast, the content of variants PMCA3a and 3b was quite different at each stage, although their expression levels did not change during development. Thus, PMCA3 could be the first isoform involved in the different events that proceed during cerebellar development. On the other hand, that the anti-PMCA4 antibody did not react in chick cerebellum neither in Western blot nor in immunohistochemistry, does not confirm the lack of this isoform in chick cerebellum. The predicted sequence for chicken PMCA4 (Medline XM_418055) does contain a 25-amino acid region that differs from the epitope of anti-PMCA4 (which has been mapped to amino acids 51–75 of the human PMCA4; Caride et al., 1996) in four amino acids. These amino acid residues may play a critical role in antibody binding, being responsible for the non–cross-reaction of the antibody in chick cerebellum. The antibody does recognize the isoform in the cerebellum of other species, such as rat (Filoteo et al., 1997; Burette et al., 2003) and pig (Sepulveda et al., 2006).

The analysis of PMCA isoforms in tissue sections gave a detailed distribution of the isoforms expression associated to specific cell types and processes (summarized in Fig. 7). In cerebellar cortex, a precise expression of PMCA was not observed during the first events of neurogenesis and cell migration, suggesting that these proteins do not play an important role until a more definite stage of development. In fact, the expression of PMCA isoforms appeared in Purkinje cells, the only output of information from cerebellum, from its morphogenesis and neurite extension (E15 stage). These cells are also enriched in several other proteins involved in the regulation of intracellular Ca^{2+} as calbindin and parvalbumin (Bastianelli, 2003) or the SERCA2 calcium pump (Plessers et al., 1991; Sepulveda et al., 2004, 2005). The restricted distribution of PMCA1 and 3 and the broad expression of PMCA2 in the Purkinje cells could be related to the establishment of different synapse types on the Purkinje cell. Indeed, distal dendritic branches (in which PMCA2 is located) receive input from parallel fibers of granule cells and from stellate cell axons, whereas the more proximal branches and the soma (containing the three isoforms) are innervated by multiple synapses from a single climb-

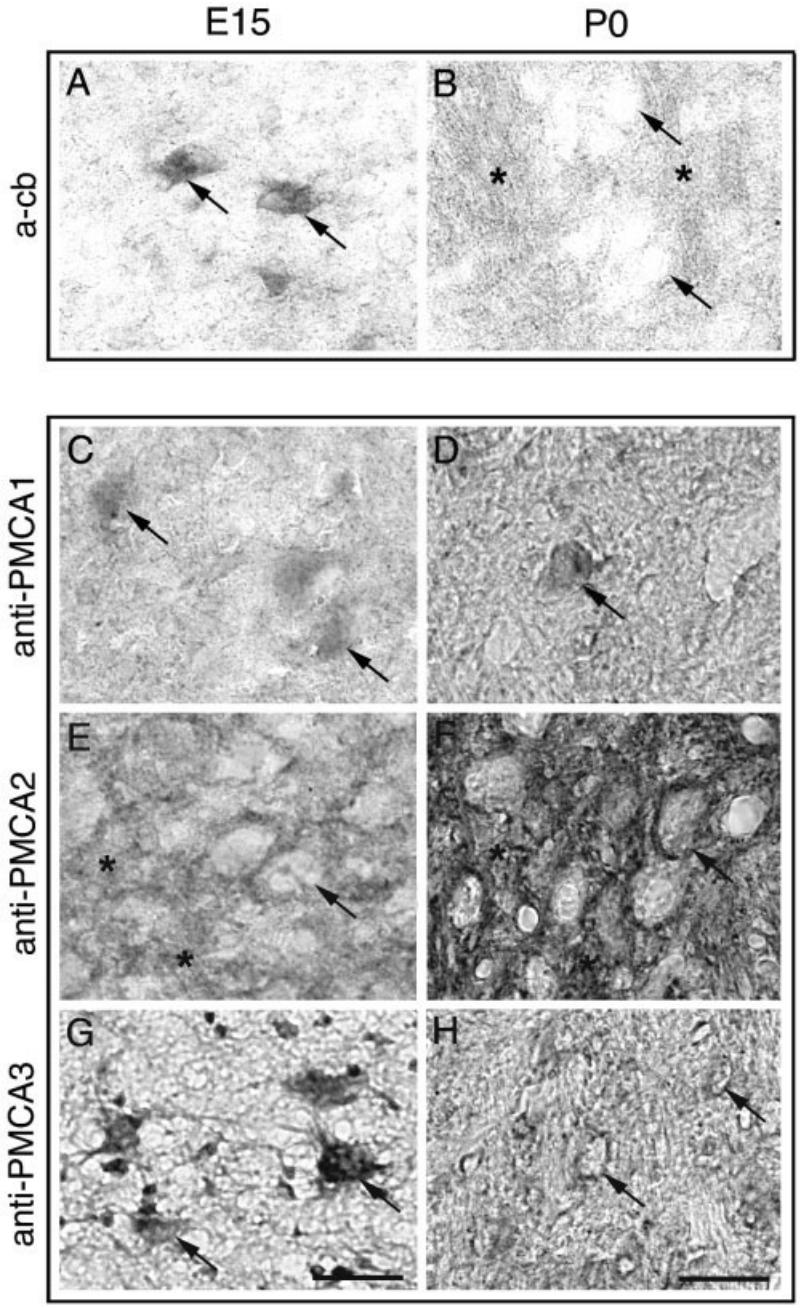

Fig. 5. Immunolocalization of plasma membrane Ca^{2+}-ATPase (PMCA) proteins in deep nucleus during development. **A,B,C,D,G,H:** The a-cb, anti-PMCA1, and anti-PMCA3 antibodies stained the soma of deep nucleus neurons from embryonic day (E) 15 (arrows in A,C,G) to hatching (PO, arrows in D,H), changing only the a-cb immunoreaction to label processes among neurons at hatching (P0, asterisks in B). **E,F:** The anti-PMCA2 stained the cell membrane of the soma of deep nucleus neurons (arrows) and processes among them (asterisk). Scale bars = 38 μm in G, 42 μm in H.

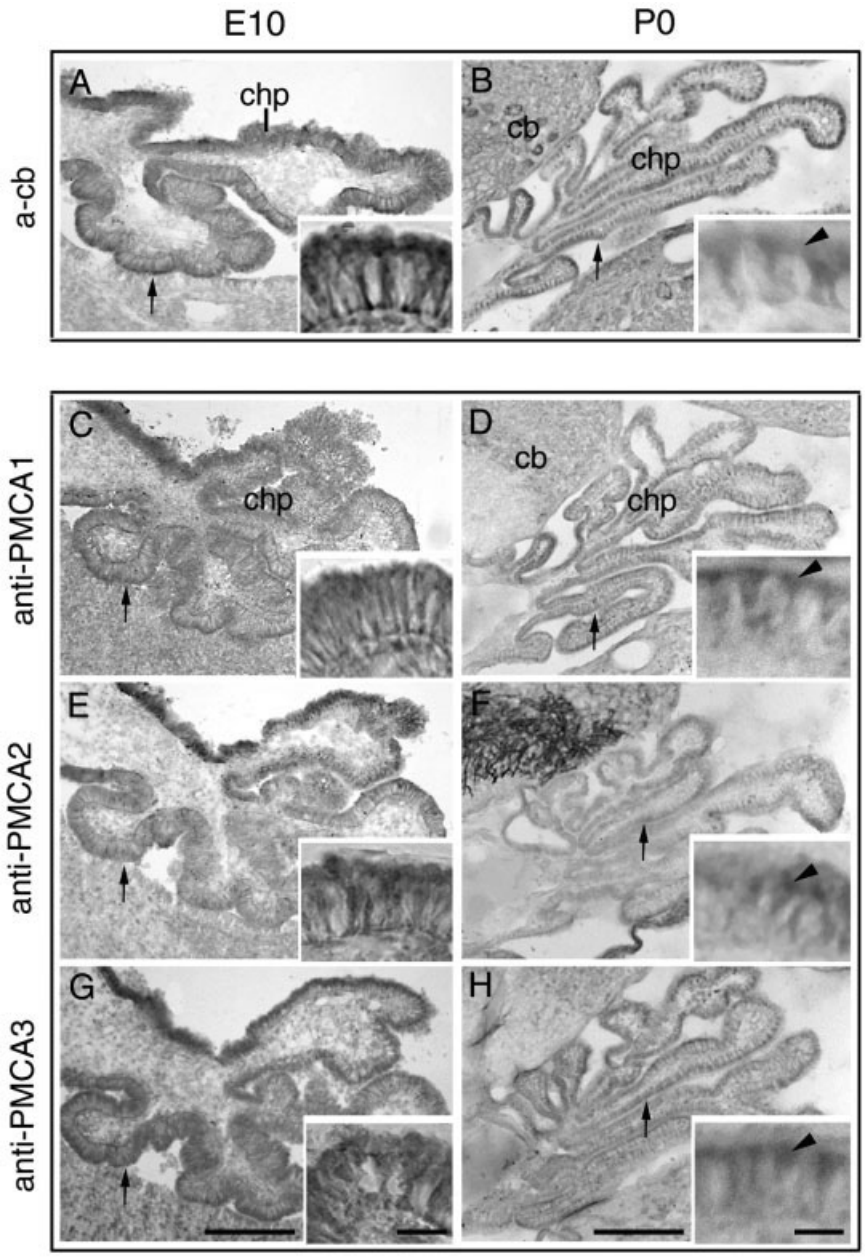

Fig. 6. Immunodetection of plasma membrane Ca^{2+}-ATPase (PMCA) proteins in the choroid plexus during development. **A–H:** The a-cb and the specifics anti-PMCA1, anti-PMCA2, and anti-PMCA3 antibodies stained epithelial cells of the choroid plexus (chp) from embryonic day (E) 10 (arrows in A,C,E,G; see also inserts) to hatching (P0, arrows in B,D,F,H). The labeling was lower at hatching and more concentrated in the apical side of these epithelial cells (arrowheads in inserts of B,D,F,H). cb, cerebellum. Scale bar = 110 μm in G, 11 μm in insert in G, 280 μm in H, 28 μm in insert in H.

ing fiber and from basket cell axons, respectively (Foelix and Oppenheim, 1974; Palay and Chan-Palay, 1974). In addition, the PMCA2 was the only isoform located in dendritic spines of these neurons, in which Ca^{2+} plays a key role in the generation and transport of excitatory and inhibitory signals from spines to soma and in synaptic plasticity (Berridge, 1993). Besides, the presence of the three PMCA isoforms in other regions very enriched in synapses such as cerebellar glomeruli suggest their involvement in the regulation of the magnitude and duration of Ca^{2+} spikes in these areas.

The expression of PMCA1 and PMCA3, but not PMCA2, in the bodies of stellate and basket cells was only detected at hatching, when the organization of chick cerebellum is complete, and is similar to the distribution found in adult rat for PMCA1 (Stauffer et al., 1997) and PMCA3 (Burette et al., 2003). These interneurons establish inhibitory synapses with different regions of Purkinje cells (Palay and Chan-Palay, 1974). On the contrary, PMCA2 was the only isoform identified in mature granule cells of chick cerebellum, although the presence of PMCA1 and 3, and even PMCA4, have been reported in granule cell of adult rat cerebellum (Stahl et al., 1992; Stauffer et al., 1997; Burette et al., 2003).

The choroid plexus, a non-neural tissue that contains epithelial cells involved in the production and secretion of cerebrospinal fluid also has a high content of PMCAs 1, 2, and 3 in chick. However, in adult rat cerebellum, the PMCA3 seems to be more abundant than the other isoforms (Stahl et al., 1992; Stauffer et al., 1997, Burette et al., 2003). Interestingly, the labeling was reallocated and decreased with development as observed also with the a-cb antibody, and with the 5F10 antibody that recognize all PMCA isoforms (Sepulveda et al., 2005). A down-regulation in the expression levels of PMCAs during development has been also reported in some cells of gerbil cochlea (Crouch and Schulte, 1995) and in rat mammary tissue (Reinhardt et al., 2000), being related to different requirements of Ca^{2+} at definite stages of development.

This distribution of isoforms in different cellular types and processes may be related to their specific functional properties, such as basal activity and calmodulin stimulation (Penniston and Enyedi, 1998; Padanyi et al., 2003), or their sensibility to modulators located in different subcellular compartments. Furthermore, the finding of the three isoforms simultaneously in some cellular regions does not exclude a different distribution in the plasma membrane. In this sense, we have recently showed a differential association of PMCA isoforms in plasma membrane lipid rafts from pig cerebellum (Sepulveda et al., 2006), which could be related to a interaction of certain PMCA isoforms with specific proteins and molecules involved in a more specific function.

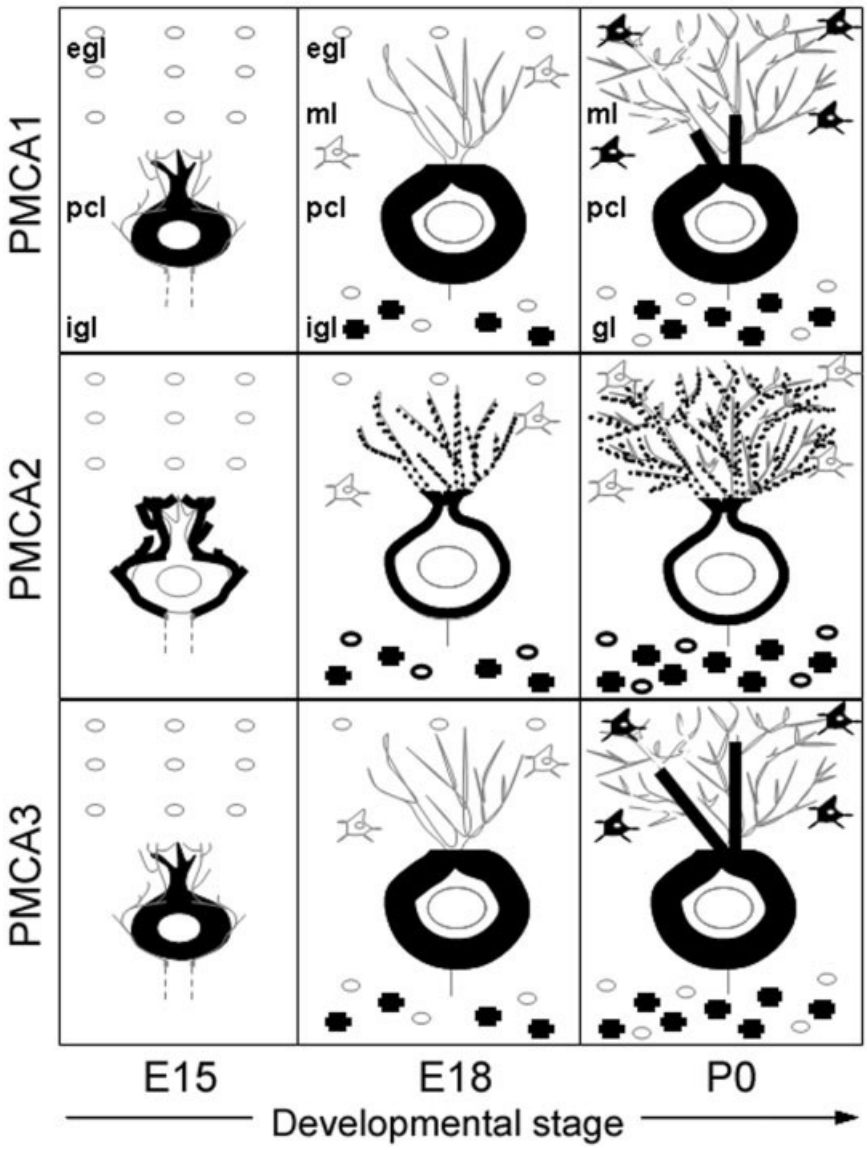

Fig. 7. Schematic representation of the temporal and spatial expression patterns of plasma membrane Ca^{2+}-ATPase (PMCA) isoforms (in black) during development of chick cerebellar cortex. egl, external granular layer; igl, inner granular layer; gl, granular layer; ml, molecular layer; pcl, Purkinje cells layer; circles, granule cells; plus signs, cerebellar glomeruli.

EXPERIMENTAL PROCEDURES

Preparation of Membrane Vesicles

Fertilized White Leghorn chick eggs were incubated until E10, E15, E18, and P0 in a humidified atmosphere at 37°C. Chicks were anaesthetized with chloroform, decapitated, and dissected, and the cerebella were immediately homogenized in a Potter with a buffer that contained 10 mM Hepes/KOH (pH 7.4), 0.32 M sucrose, 0.5 mM $MgSO_4$, 0.1 mM phenylmethyl sulfonyl fluoride, 2 mM 2-mercaptoethanol, and protease inhibitor cocktail (Roche). Then, the homogenates were centrifuged for 10 min at 1,500 × *g*, the pellets were discarded, and the supernatants were centrifuged for 45 min at 100,000 × *g*. The final pellets were resuspended in 10 mM Hepes/KOH (pH 7.4) and 0.32 M sucrose and stored at −80°C until use. The protein content was evaluated by the Bradford method (1976).

Electrophoresis and Immunoblotting

Electrophoresis was performed in 6.5% (w/v) polyacrylamide gels according to the method of Laemmli (1970). Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot SD semidry system (Bio-Rad). After blocking in Tris-buffered saline (TBS) containing 2% (w/v) of nonfat dry milk (TBS–milk) for 30 min, immunostaining reactions were performed by incubating the membranes for 3 hr at room temperature with the following primary antibodies at dilution 1:500 in TBS-0.05% (v/v) Tween 20: the polyclonal a-cb antibody (raised in our laboratory against synaptosomal PMCA from pig cerebellum), the polyclonal antibodies anti-PMCA1 (raised against the synthetic peptide: A(5) N N S V A Y S G V K N S I K E A N(22), which corresponds to amino acid residues 5–22 from rat PMCA1), anti-PMCA2 (raised against the synthetic peptide: T(5) N S D F Y S K N Q R N E S S(19), which corresponds to amino acid residues 5–19 from human PMCA2), and anti-PMCA3 (raised against the synthetic peptide: A(5) N S S I E F H P K P Q Q Q R E V P(22), which corresponds to amino acid residues 5–22 from rat PMCA3), and the monoclonal antibody anti-PMCA4 (raised against the purified human erythrocyte PMCA; the epitope for this antibody has been mapped to amino acids 51–75 of human PMCA4). These antibodies were purchased from Affinity Bioreagents. Afterward, membranes were incubated for 1 hr at room temperature with peroxidase-conjugated secondary antibodies (1:3,000, Bio-Rad) and stained with 4-methoxy-1-naphtol. The membranes were washed extensively between steps with TBS–milk. The monoclonal anti–β-tubulin antibody (Sigma) at dilution 1:1,000 was used as control of protein loading.

Tissue Preparation for Immunohistochemistry

Embryos from stages E10, E15, E18, and P0 were anesthetized with chloroform and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) solution by transcardiac perfusion. After dissection, brains

were post-fixed by immersion in the same fixer for 24 hr at 4°C. After several washes with PBS, brains were cryoprotected in 10% (w/v) sucrose in PBS for 2 days and then embedded in 10% (w/v) gelatin, 10% (w/v) sucrose in PBS. The blocks were frozen for 2 min in isopentane cooled to −70°C by dry ice, and stored at −80°C. Serial parasagittal sections of 20 μm were collected using a cryostat Leica CM1900 on Super-Frost Plus slides.

Immunohistochemistry

Sections in slides were immersed in PBS-0.05% (v/v) Triton X-100 (PBS-T) for 15 min, and then endogenous peroxidase activity was quenched with PBS-0.5% (v/v) H_2O_2 for 45 min. Before the incubation of the sections with the primary antibody, the tissue was treated with a solution containing 0.2% (w/v) gelatin, 0.25% (v/v) Triton X-100 in PBS (PBS-G-T), and blocked with 0.1 M lysine in PBS-G-T for 1 hr. Immunodetection was performed with the primary antibodies described above incubated overnight at room temperature in a humidified chamber, and diluted in PBS-G-T at 1:200 for a-cb and at 1:50 for specific PMCA isoform antibodies, and subsequently incubated with biotinylated goat anti-mouse or anti-rabbit antibodies (1:200 dilution, Sigma) and then with ExtrAvidin-peroxidase (1:200, Sigma). The immunodetection of the peroxidase activity was carried out using 0.03% (w/v) 3,3′-diaminobenzidine tetrahydrochloride. The sections were dehydrated and mounted with Eukitt for their observation in the microscope. Several cryostat sections of developing analyzed stages were stained with a solution containing 0.01% cresyl violet, 0.01% tionin, 0.6% acetic acid, and 1.25% sodium acetate during 5 min at 37°C and differentiated with 80% ethanol and 2 drops of acetic acid. Alternatively, immunofluorescence assays were performed with the primary antibody indicated in each case and the secondary Alexa 594 goat anti-rabbit antibody (Molecular Probes) at dilution 1:500 in PBS-G-T. Then, sections were treated with DAPI after immunofluorescence assays. Afterward, the sections were covered with FluorSave mounting medium and analyzed using a Nikon E600 microscope. Negative controls were performed for each set of experiments by omitting the primary antibody.

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