ELSEVIER

Brain Research 748 (1997) 21–29

BRAIN RESEARCH

Research report

Immunolocalization of the plasma membrane Ca^{2+} pump isoforms in the rat brain

Thomas P. Stauffer [a], Danilo Guerini [a], Marco R. Celio [b], Ernesto Carafoli [a,*]

[a] *Laboratory for Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zurich, Switzerland*
[b] *Institute of Histology and General Embryology, University of Fribourg, Pérolles, 1705 Fribourg, Switzerland*

Accepted 15 October 1996

Abstract

Ca^{2+} homeostasis in nerve cells is dependent on at least three mechanisms: Ca^{2+} channels, calcium-binding proteins and Ca^{2+} exchangers/pumps. Only limited information is available on the regional/cellular distribution of these Ca^{2+}-regulating systems in the brain. The distribution of three of the isoforms of one of the systems, plasma membrane Ca^{2+}-ATPase (PMCA), was analyzed in this study. Using antibodies against epitopes specific for each isoform, a map of the distribution of the pump in the whole brain was produced. The pump was mainly expressed in neurons and was apparently absent from glia cells. Isoform 1 was ubiquitous and occurred in varying, but always significant, concentrations in almost all nerve cells. Isoform 2 was abundant in cerebellar Purkinje cells but less concentrated in other brain regions. Isoform 3 had a predominantly extra neuronal location, e.g. it was abundant in the choroid plexuses. The three isoforms were found to be distributed in a highly characteristic manner, suggesting that nerve cells have different requirements for the preservation of their intracellular calcium homeostasis.

Keywords: Intracellular calcium homeostasis; Plasma membrane; Calcium channel; Calcium-binding protein; Immunolocalization; Intracellular signaling

1. Introduction

Calcium ions (Ca^{2+}) are the key to the process of intracellular signaling in all cells, but in the nervous system their role is more complex; in addition to intracellular signaling, they are also involved in neurotransmission, in the control of excitability and in axonal transport. Therefore, in the nervous system the precise regulation of the free intracellular calcium is particularly critical. Four different systems control the intracellular Ca^{2+} concentration: Ca^{2+} channels, intracellular calcium-binding proteins, exchangers and pumps. Only the first two have been fairly well characterized in the nervous system.

The plasma membrane Ca^{2+}-ATPase (PMCA) is thought to play a major role in the export of intracellular calcium and in the fine tuning of its resting level. The enzyme was first described in the plasma membrane of erythrocytes [22] and later found to be present in almost all cells. cDNA cloning resulted in the finding of four distinct gene (PMCA1–4) codings for the pump [12–14,25,30,32]. This diversity is further increased by alternative splicing of the primary transcript at three distinct sites [7]. The isoforms (gene products) and splice forms of the enzyme differ mainly in their affinity for Ca^{2+} and for the prominent regulator, calmodulin [11,14]. Testing the tissue distribution of the four gene products by Northern blot [12,25], RT-PCR [18,28] or Western blot analysis [29] has shown that two housekeeping isoforms (PMCA1 and PMCA4) are present in virtually all cells, whereas the other two only occur in significant amounts in nerve tissue (PMCA2 [3]). In addition, nearly all splice forms were found in nerve tissue, frequently in unusually high amounts. A prominent role for the plasma membrane Ca^{2+} pump in Ca^{2+} homeostasis in nerve tissue can thus be safely predicted. In earlier studies the regional distribution of the transcripts of the PMCA isoforms in brain was analyzed by RT-PCR [33] and in situ hybridization [26,27]. However, only partial information is so far available on the presence, the

Abbreviations: DMSO = dimethylsulfoxide; DTT = dithiothreitol; nt = nucleotide; PMCA = plasma membrane Ca^{2+}-ATPase; PMSF = phenylmethylsulfonyl fluoride; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; PVDF = polyvinylidene difluoride; RT = reverse transcription; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate

* Corresponding author.

PII S0006-8993(96)01282-6

distribution and abundance of the expressed proteins in brain [3,9]. In this study the localization of the three rat PMCA isoforms (rat PMCA1–3) was thus determined in brain by immunohistochemistry and for rat PMCA2 also by immunoelectron microscopy using antibodies specific for the three rat PMCA isoforms.

2. Material and methods

2.1. Generation of isoform-specific antibodies

Polyclonal antibodies were raised against the N-terminal regions of the human PMCA isoforms (isoform 1: amino acids 1–88: isoform 2: amino acids 1–96: isoform 3: amino acids 1–83) as described in ref. [29]. The sera were purified by affinity chromatography [29]. The specificity of the antibodies was demonstrated by Western blot analysis using human PMCA pump isoforms expressed in Sf9 cells [29].

2.2. Microsome preparation from rat brain

The brains of adult white Wistar rats were removed after decapitation and frozen in liquid nitrogen and kept at −80°C until use.

Two grams of tissue were homogenized with a Polytron in buffer A (0.1 M KCl, 0.05 M HEPES NaOH, pH 7.0, 0.4 mM PMSF, 0.5 mM DTT) and the homogenate was centrifuged at $800 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $8000 \times g$ for 10 min at 4°C and the fraction containing the microsomes was sedimented for 1 h at $100\,000 \times g$ (4°C). The pellet was resuspended in a small volume of buffer A and the protein concentration determined according to Bradford [4].

2.3. SDS-polyacrylamide gel electrophoresis and Western blot analysis

The microsome proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [19] and then transferred to PVDF membranes (Millipore, USA) [31]. The membranes were blocked overnight with 1% BSA and incubated with the primary antibody (1:500 diluted in TBS-T: 10 mM Tris-HCl pH 7.0; 500 mM NaCl; 0.05% Tween-20; 0.1% BSA) for 1 h. Incubation with the secondary antibody (alkaline phosphatase coupled to an anti-rabbit antibody; Promega, Madison, WI, USA) was followed by the staining reaction, carried out according to the manufacturer's protocol (ProtoBlot AP; Promega Biotec, Madison, WI, USA). As a control the antibodies were preincubated overnight with a two-fold molar excess of the peptides used for the immunization and processed as described above.

2.4. Immunohistochemical procedure

Male and female rats (100–200 g) were anesthetized with 1 ml/100 g body weight of a 4% solution of chloral hydrate in PBS (0.05 M NaH_2PO_4, pH 8; 0.5 M NaCl) and transcardially perfused with 4% paraformaldehyde in 0.1 phosphate buffer, pH 7.3. The brain was dissected, sliced, stored in 18% sucrose for 2 days and then frozen in pulverized dry ice. Cryostat sections, 40 μm thick (coronal and sagittal), were incubated by floating with the primary antibodies, diluted (1:500, isoform 1 and 2; 1:100 isoform 3) in 0.1 M Tris-buffered saline, pH 7.3, and further processed by the avidin-biotin method [15] using the Vectastain kit (Vector Laboratories, Burlingame, CA, USA). As a control the antibodies were preincubated overnight with a two-fold molar excess of the peptides used for the immunization and processed as described above.

2.5. Immunoelectron microscopy

The brains were fixed as previously described. Forty μm thick vibratome sections were cut pendicular to the surface of the cerebellum. Free floating sections were incubated for 36 h at 4°C with the primary antibody diluted 1:500. The primary antibody was detected by the avidin-biotin method using the Vectastain kit (Vector Laboratories, Burlingame, CA, USA). After reaction with diaminobenzidine tetrahydrochloride (DAB), the sections were osmicated in 1% OsO_4 in PB with 4% sucrose for 1 h. This was followed by dehydration by using graded concentrations of ethanol and propylenoxide before embedding flat in Epon.

Under low-power stereomiroscopic control small pieces of the cerebellum were cut out. They were re-embedded in Epon in Beem capsules to allow for ultrathin sectioning. The ultrathin sections were mounted on grids, without counterstaining, and examined in a Zeiss TEM 10 electron microscope operated at 80 kV.

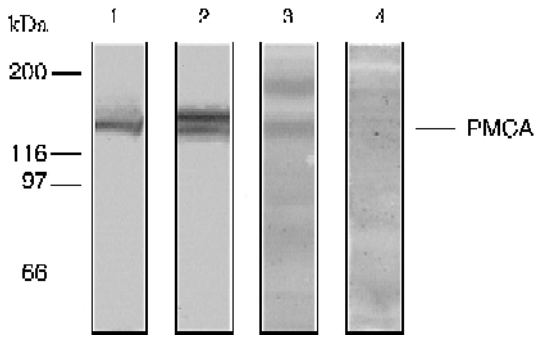

Fig. 1. Western blot analysis of rat brain membrane microsomes. Membrane proteins (60 μg) of rat brain were tested for the presence of the three isoforms of the pump. Lane 1: antibody against isoform 1 (dilution: 1/500); lane 2: antibody against isoform 2 (dilution: 1/500); lane 3: antibody against isoform 3 (dilution: 1/200); lane 4: antibody against isoform 3 (dilution: 1/200) preabsorbed with a two-fold molar excess of the peptide against which it was raised.

Table 1
Distribution of the three PMCA isoforms in rat brain

Isoform	rPMCA1		rPMCA2		rPMCA3	
	Cell body	Neuropil	Cell body	Neuropil	Cell body	Neuropil
Telencephalon						
Olfactory bulb						
Periglomerular cells	xx	–	–	–	–	–
Mitral cells	xx	–	–	–	–	–
Cerebral cortex						
Laminae I–IV	–	xx	–	xx	–	–
Lamina V	xx	x	–	x	–	–
Lamina VI	–	x	–	xx	–	–
Hippocampus	xx	–	–		(x)	–
Stratum oriens	xx		–	xx	(x)	–
Stratum pyramidale	xx		–	xx	(x)	–
Stratum radiatum	–	–	–	x	–	–
Stratum laculosum molec.	–	–	–	x		
Caudate-putamen	–	x	–	x	–	–
Dentate nucleus	(x)	(x)	–	x	–	–
Diencephalon						
Thalamus	–	(x)	–	(x)	–	–
Hypothalamus						
Paraventricular nucleus	xxx	–	–	–	–	–
Subependymal layer	xx	–	–	–	–	–
Supraoptic nucleus	xxx	–	–	–	x	–
Corpus mammillare	–	–	–	–	x	–
Mesencephalon (midbrain)						
Basal ganglia	–	x	–	x	–	–
Bed nucl. stria terminalis	–	xx	–	–	–	–
Caudal linear nucleus raphe	–	xxx	–	–	–	–
Interpeduncular nucleus	–	xxx	–	–	–	–
Magnocellular preoptic nucl.	x	–	–	–	–	–
Oculomotoric nucleus	xxx	x	–	–	–	–
Retrorubral field	–	–	–	x	–	–
Septum	–	xxx	–	–	–	–
Substantia nigra	x	xx	–	x	–	–
Metencephalon						
Cerebellum						
Molecular layer	(x)	(x)	–	xxx	–	–
Purkinje cell layer	xx	–	xx	–	x	–
Granular layer		x	x		(x)	–
Deep cerebellar nuclei	x	–	–	–	x	–
Myelencephalon (medulla oblongata)						
Colliculus superior	x	xx	–	x		
Colliculus inferior	–	–	–	x		
Gigantocellular nucleus	–	–	–	–	xx	–
Locus ceruleus	xxx	xxx	–	–	–	–
Nucleus facialis	xx	xx	–	x	–	–
Nucleus reticularis	–	–	–	–	(x)	–
Nucleus solitary tract	–	xx	–	–	–	xx
Nucleus subceruleus	xxx	xx	–	x	–	–
Superior olive	–	–	–	xx	–	–
Vestibular nuclei	xx	xx	–	x	–	–
Choroid plexus	–	–	–	–	xx	–

Summary of the presence of PMCA1–3 in rat brain, detected using isoform specific antibodies. – = not detectable; (x) = weak; x = present; xx = abundant; xxx = very abundant.

3. Results

3.1. Characterization of the antibodies

Antibodies against the human plasma membrane Ca^{2+}-ATPase isoforms were raised using the N-terminal sequence of the pumps [29]. The antibodies against isoforms 1–3 recognized the human and rat isoforms, whereas the antibody against isoform 4 failed to do so [29].

The antibodies were characterized on protein extracts of rat brain microsomes by immunoblots, separated by SDS-PAGE and transferred to PVDF membranes. Fig. 1 shows that only signals at the molecular mass range of the PMCA pump (130–135 kDa) were detected (Fig. 1, lanes 1–3). An additional band of about 160 kDa was revealed by the antibody against isoform 3 (Fig. 1, lane 3), which was very likely the result of the formation of an aggregate or a complex with calmodulin. Under different conditions a band with similar molecular mass could be observed also for PMCA2 (not shown, see ref. [8]). Preincubation of the antibodies with the peptide against which they were generated, abolished completely all signals in the immunoblots (results not shown).

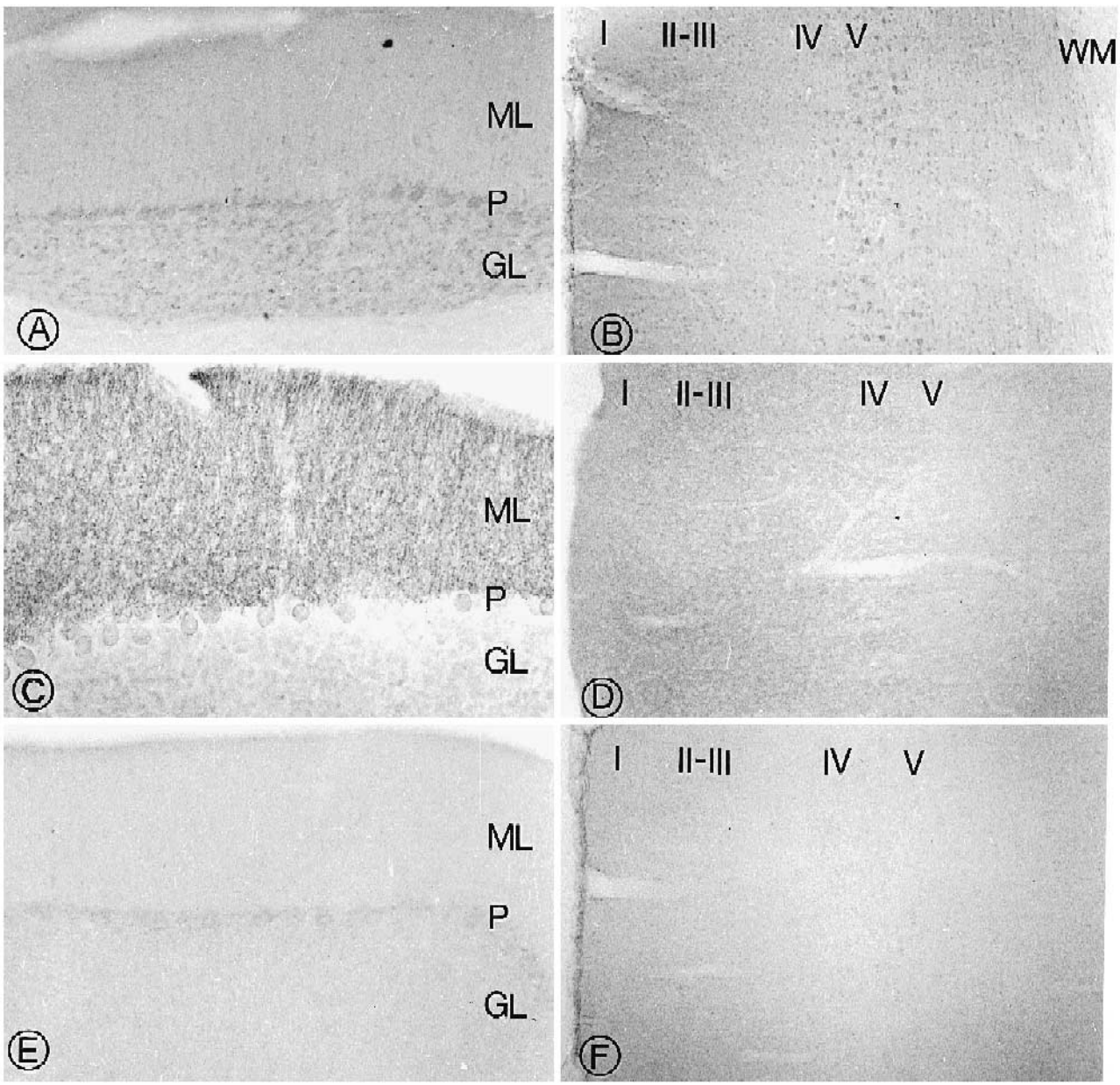

Fig. 2. Immunohistochemical localization of the PMCA isoforms in cerebellum and cerebral cortex. Micrographs were obtained using bright-field optics. Sagittal sections of cerebellum (left) and cerebral cortex (right) were stained with the antibodies against PMCA1 (A, B), PMCA2 (C, D), PMCA3 (E, F). GCL, granular cell layer; ML, molecular layer; P, Purkinje cells; I–V, layers of the cerebral cortex. ×120.

3.2. Localization of the plasma membrane Ca^{2+} pump in rat brain

The localization of the ATPase isoforms was studied in both sagittal and coronal brain sections of adult rat brain by immunohistochemistry using the specific antibodies. Preincubation of the diluted antibodies with their respective antigens completely abolished the immunolabeling (results not shown). With all antibodies the staining was clearly neuronal. Oligodendrocytes showed no reaction with any of the antibodies. However, PMCA positive astrocytes were detected in the optical nerve using the antibody against PMCA1 (Fig. 3F).

3.3. The rat PMCA1 pump

The staining pattern of brain sections incubated with the antibody specific for isoform 1 revealed that the isoform was present in nearly all brain regions (Table 1). Depend-

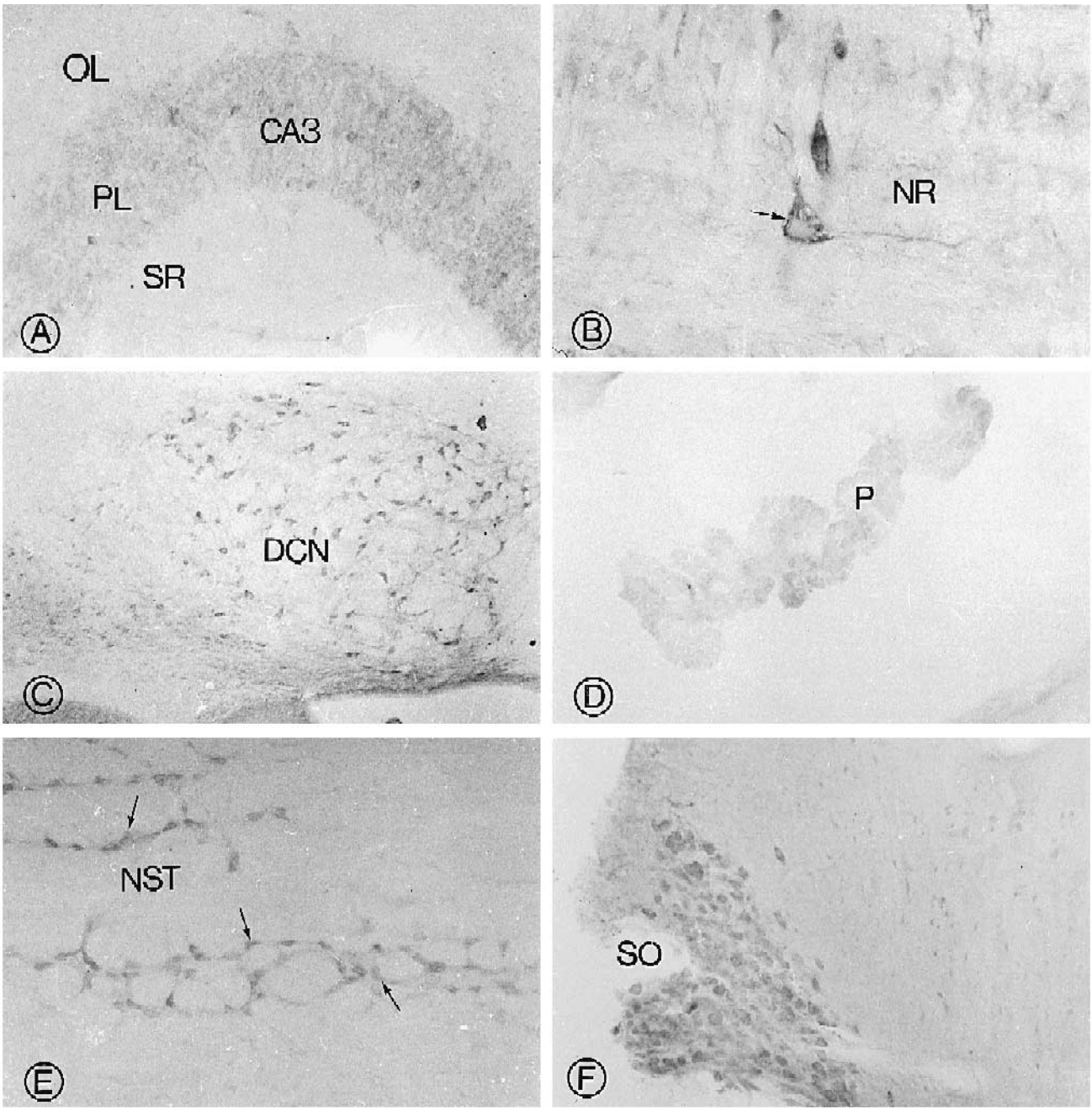

Fig. 3. Immunohistochemical localization of the PMCA isoforms in defined brain regions. Sagittal sections of hippocampus (A), reticular nucleus (B), deep cerebellar nuclei (C) and supraoptic nucleus (F) stained with the antibody against isoform 1. Choroid plexus of the fourth ventricle (D) and solitary tract nucleus (E) stained with the antibody against isoform 3. CA3, CA3 of Ammon's horn; DCN, deep cerebellar nuclei; OL, oriens layer; PL, pyramidal layer; SR, stratum radiatum; ON, optic nerve; STN, solitary tract nucleus. The arrows in B indicate the unhomogenous coloration of the soma. ×150.

ing on the region, either the cell body or neuropil (axons, dendrites synapses), or both, were PMCA1 positive. Moderate to high levels of PMCA1 were detected in the cerebral cortex, hypothalamus, hippocampus, olfactory bulb and in some midbrain and hindbrain nuclei, whereas only low levels were detected in the cerebellum, thalamus, dentate nucleus and in some midbrain regions (Table 1). The immunoreactivity in the cerebral cortex was very unhomogenous. The cell bodies of the pyramidal cells of layer V showed strong staining, whereas the pyramidal neurons in other layers were stained only weakly. The neuropil was stained in all layers (Fig. 2B). In the hippocampal CA1–CA4 cells, in the neuropil of the pyramidal layer and also in some cells of the oriens layer PMCA1 reactivity was found (Fig. 3A). The cell body of mitral and periglomerular cells in the bulbus olfactorius showed moderate immunoreactivity, whereas very strong labeling was found in some regions of the hypothalamus and of the mid- and hindbrain (Fig. 3B). Different parts of the cells (soma and axons) of the nucleus reticularis were PMCA1 positive. The staining of the cell bodies of these cells was often unhomogenous; stained and unstained regions alternated in patches (Fig. 3B). In the cerebellum low immunoreactivity for PMCA1 was found in the cell bodies of neurons of the molecular layer and in the Purkinje cells. The dendrites of the latter were stained very weakly, the axons showing no immunoreactivity at all (Fig. 2A). In addition, the cell body and the dendrites of the cells of the deep cerebellar nuclei were clearly stained (Fig. 3C). Stronger staining was detected in the neuropil of the granular layer.

3.4. The rat PMCA2 pump

Isoform 2 was detected in large amounts in the cerebellum and in moderate amounts in the cerebral cortex and the hippocampus. Minor amounts of the isoform were also detected in some midbrain and hindbrain regions (see Table 1). The cell bodies, dendrites and spines of Purkinje cells were clearly marked by the labeling (Fig. 2C). At the electron microscopical level a clear submembranous staining (Sharat et al. [23,24]) along the intracellular surface of

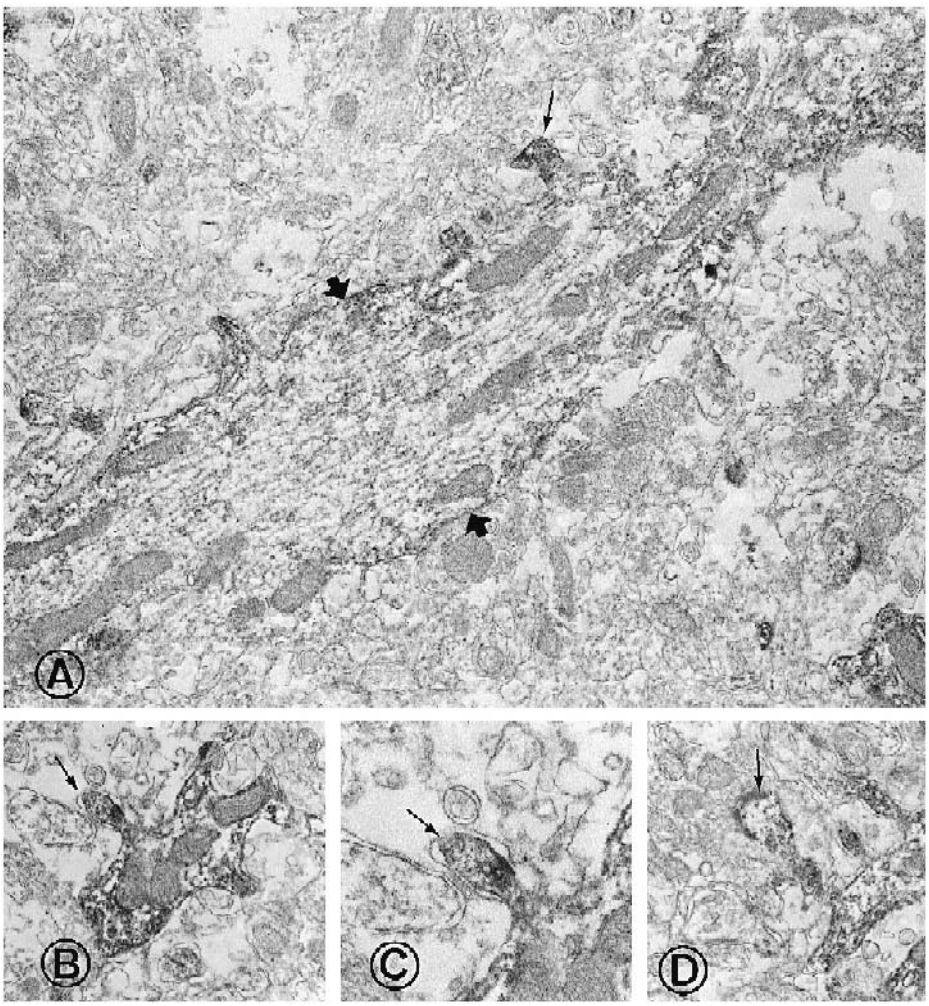

Fig. 4. Electron microscopical localization of the PMCA2 isoform in the molecular layer of the cerebellum. Longitudinal section through a Purkinje cell dendrite (A). PMCA2 positive dendritic spines (arrows in B, C and D). ×8000. Insets: ×12 000.

plasma membranes, and more diffuse towards the cytoplasm, was observed (Fig. 4A). Strong staining was also found in the dendritic spines (Fig. 4B,C). In the cerebral cortex a diffuse staining of the neuropil was observed. Layers I–IV became stained moderately, layers V and VI more weakly (Fig. 2D). In the hippocampus neuropil labeling was observed in the oriens and pyramidal cell layers, the other structures in the hippocampus showing only weak staining or no staining at all.

3.5. The rat PMCA3 pump

Staining with the antibody against isoform 3 was restricted to defined regions in the rat brain, where it was mainly detected in the region of the soma. Positive reactions to isoform 3 were detected in the fourth ventricle, the cerebellum, the cerebral cortex and in three nuclei in the hindbrain (Table 1). Moderate staining was detected in the structures of the choroid plexus of the fourth ventricle (Fig. 3D) and in the cell bodies of the gigantocellular nucleus and of the solitary tract nucleus (Fig. 3E). Weak staining was found in the cell bodies of Purkinje cells and granular cells (Fig. 2E) and in the deep cerebellar nuclei. Very weak coloration was found in pyramidal cells of layer V of the cerebral cortex (Fig. 2F) and in hippocampal cells.

4. Discussion

The antibodies showed signals at the expected molecular mass (130–135 kDa) when tested on rat brain membrane preparations. No additional signals were detected with the antibodies against PMCA1 and 2, whereas that against PMCA3 revealed an additional band of molecular mass of about 160 kDa. Since the specificity of the signals was determined by preabsorbing the antibodies with the peptides against which they were generated, it is very likely that the band found using the PMCA3 antibody represented an aggregation product of PMCA3. Two signals at 130–135 kDa were detected using the antibody against isoform 2, probably representing alternatively spliced forms of isoform 2, as already observed in neuronal tissues at the mRNA level [5,6,18,28].

The immunolocalization work showed that only neurons were PMCA positive. No consistent labeling was detected in glial cells using any of the antibodies, with the exception of some astrocytes in the optic nerve.

The expression pattern of the three isoforms in rat brain showed great regional differences. PMCA1 was expressed widely, whereas PMCA2 and 3 were only found in some well defined regions, e.g., the cerebellum (PMCA2) or the choroid plexuses (PMCA3). Differences were also found in the subcellular distribution of the isoforms, 1 and 3 being found in the soma and in the neuropil (dendrites and/or axons), isoform 2 almost exclusively in the neuropil: isoform 2 may therefore be neuropil specific. Isoform 1 was expressed ubiquitously in all tissues and can thus be considered as a housekeeping isoform [18,25,28].

Particularly high amounts of PMCA2 were detected in the plasma membrane of Purkinje cells, clustered in the dendritic spines. Thus, the strong immunoreactivity found by others in the molecular layer of the cerebellum using an antibody recognizing all isoforms [9] was presumably due to isoform 2, since the present work showed that much lower amounts of isoforms 1 and 3 were present in this layer (Fig. 2A,E). In the cerebral cortex only layers II–IV were found to be isoform 2 positive. The stained neuropil may thus originate from cells of the layers II–IV expressing PMCA2 [26] or from cells projecting into these regions.

The transcripts of PMCA3 were previously detected in high amounts mainly in choroid plexuses (in lower amounts in some additional regions [26]. On the protein level the same structures were found to be strongly PMCA3 positive. These results are consistent with the findings of a recent study using a PMCA3 specific antibody [10]. Additionally PMCA3 was also found in some regions in the hindbrain, where this isoform had so far not been described, e.g., the gigantocellular nucleus and cells of the solitary tract nucleus (see Table 1).

The distribution patterns of the three isoforms corresponded well to those of their mRNA [26]. However, differences were also evident; and could have more than one cause, e.g. the different cellular localization of the mRNA and the proteins. The mRNAs are clustered around the nucleus and, therefore, the signals are easily correlated to a certain cell. The corresponding proteins could be distributed over the whole cell, resulting in a weaker overall signal. Another reason could be the unequal translation efficiency of the mRNA in different brain regions.

In the different compartments of a neuronal cell, Ca^{2+} ions may be involved in distinct processes, e.g., in the dendrites they are responsible for the generation and the transport of excitatory or inhibitory signals to the soma, whereas in the presynapse they modulate the release of neurotransmitters. Interestingly, the regulation of the Ca^{2+} concentration in compartments such as the dendrites is virtually independent from that in the remainder of the cell [17]. Thus different subsets of Ca^{2+}-binding and Ca^{2+}-transporting proteins could be present in different regions of the cell. In the dendrites of Purkinje cells, more specifically in their spines, the inositol 1,4,5-trisphosphate receptor [23,24], the endoplasmic reticulum Ca^{2+} pump (SERCA2) [17] and various Ca^{2+}-binding proteins (parvalbumin, calsequestrin, calreticulin) [8,17,21] are present in very high amounts. It is of great interest that structures like the P-type voltage-dependent Ca^{2+} channels [20] and the PMCA2 pump are also highly concentrated in these compartments. Although, the high Ca^{2+} affinity of the plasma membrane Ca^{2+}-ATPase qualifies it ideally for the restoring of the resting Ca^{2+} level in neuronal cells

and more specifically in the Purkinje cell dendrites, the reason for the presence of a specific isoform (PMCA2) is unknown. One could cautiously speculate that the various PMCA isoforms differ in basal activity and in a way they are regulated by calmodulin [14]. Isoform 2 was found to have a much higher affinity for calmodulin than other isoforms and thus becomes fully activated at a lower concentration of the Ca^{2+}-calmodulin complex. PMCA2 could therefore be better suited than other isoforms to control the Ca^{2+} concentration in a region characterized by its high turnover. It is pointed out, however, that this isoform has not been found in compartments of other cells also known to use intensive calcium signaling, e.g. the presynaptic region or dendritic spines of other neurons [16].

PMCA1 is distributed ubiquitously and may therefore be responsible for the housekeeping regulation of the Ca^{2+} homeostasis. The Ca^{2+} extrusion that follows a Ca^{2+} signal in the soma of pyramidal neurons has been shown to be due to the activity of PMCA [1,2]. The high amounts of PMCA1 found in these neurons suggest that this isoform may be responsible for it.

No data are available on the activity and regulation properties of PMCA3. One could speculate that the high amounts of PMCA3 in the choroid plexus may be related to the regulation of the extracellular (cerebrospinal fluid) Ca^{2+} concentration.

Acknowledgements

We thank C. Dumas and D. Uldry (University of Fribourg) for expert technical help. This work was supported by the Swiss National Science Foundation, Grant Nos. 31-30858.91 (to E.C.) and 31-36483.92 (to M.R.C.).

References

[1] Benham, C.D., Evans, M.L. and McBain, C.J., Ca^{2+} Efflux mechanisms following depolarization evoked calcium transients in cultured rat sensory neurons, *J. Physiol. (Lond.)*, 455 (1992) 567–583.

[2] Bleakman, D., Roback, J.D., Wainer, B.H., Miller, R.J. and Harrison, N.L., Calcium homeostasis in rat septal neurons in tissue culture, *Brain Res.*, 600 (1993) 257–267.

[3] Borke, J.L., Caride, A.J., Yaksh, T.L., Penniston, J.T. and Kumar, R., Cerebrospinal fluid calcium homeostasis: evidence for a plasma membrane Ca^{2+}-pump in mammalian choroid plexus, *Brain Res.*, 489 (1989) 355–360.

[4] Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 72 (1976) 248–254.

[5] Brandt, P., Neve, R.L., Kammesheidt, A., Rhoads, R.E. and Vanaman, T.C., Analysis of the tissue-specific distribution of mRNAs encoding the plasma membrane calcium-pumping ATPases and characterization of an alternately spliced form of PMCA4 at the cDNA and genomic levels, *J. Biol. Chem.*, 267 (1992) 4376–4385.

[6] Brandt, P. and Neve, R.L., Expression of plasma membrane calcium-pumping ATPase mRNAs in developing rat brain and adult brain subregions: evidence for stage-specific expression, *J. Neurochem.*, 59 (1992) 1566–1569.

[7] Carafoli, E., The plasma membrane calcium ATPase: fifteen years of work on the purified enzyme, *FASEB J.*, 8 (1994) 993–1002.

[8] Celio, M.R., Calbindin D-28k and parvalbumin in the rat nervous system, *Neuroscience*, 35 (1990) 375–475.

[9] De Talamoni, T.N., Smith, C.A., Wasserman, R.H., Beltramino, C., Fullmer, C.S. and Penniston, J.T., Immunocytochemical localization of the plasma membrane calcium pump, calbindin-D28k, and parvalbumin in Purkinje cells of avion and mammalian cerebellum, *Proc. Natl. Acad. Sci. (USA)*, 90 (1993) 11949–11953.

[10] Eakin, T.J., Antonelli, M.C., Malchiodi, E.L., Baskin, D.G. and Stahl, W.L., Localization of the plasma membrane Ca^{2+}-ATPase isoform PMCA3 in rat cerebellum, choroid plexus and hippocampus, *Mol. Brain Res.*, 29 (1995) 71–80.

[11] Enyedi, A., Verma, A.K., Heim, R., Adamo, H.P., Filoteo, A.G., Strehler, E.E. and Penniston, J.T., The Ca^{2+} affinity of the plasma membrane Ca^{2+} pump is controlled by alternative splicing, *J. Biol. Chem.*, 269 (1994) 41–43.

[12] Greeb, J. and Shull, G.E., Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skelatal muscle, *J. Biol. Chem.*, 264 (1989) 18569–18576.

[13] Heim, R., Hug, M., Iwata, T., Strehler, E.E. and Carafoli, E., Microdiversity of human-plasma-membrane calcium-pump isoform 2 generated by alternative RNA splicing in the N-terminal coding region, *Eur. J. Biochem.*, 205 (1992) 333–340.

[14] Hilfiker, H., Guerini, D. and Carafoli, E., Cloning and expression of isoform 2 of the human plasma membrane Ca^{2+} ATPase, *J. Biol. Chem.*, 269 (1994) 26178–26183.

[15] Hsu, S.M., Raine, L. and Fanger, H., Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures, *J. Histochem. Cytochem.*, 29 (1981) 577–580.

[16] Jaffe, D.B., Fisher, S.A. and Brown, T.H., Confocal laser scanning microscopy reveals voltage-gated calcium signals within hippocampal dendritic spines, *J. Neurobiol.*, 25 (1994) 220–233.

[17] Johnson, R.J., Pyun, H.Y., Lytton, J. and Fine, R.E., Differences in the subcellular localization of calreticulin and organellar Ca^{2+}-ATPase in neurons, *Mol. Brain Res.*, 17 (1993) 9–16.

[18] Keeton, T.P., Burk, S.E. and Shull, G.E., Alternative splicing of exons encoding the calmodulin-binding domains and C-termini of plasma membrane calcium-ATPase isoforms 1, 2, 3, and 4, *J. Biol. Chem.*, 268 (1993) 2740–2748.

[19] Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227 (1970) 680–685.

[20] Llinás, R., Sugimori, M., Hillman, D.E. and Cherksey, B., Distribution and functional significance of the P-type voltage-dependent Ca^{2+} channels in the mammalian central nervous system, *Trends Neurosci.*, 15 (1992) 351–355.

[21] Sadowski, Z., Pietrzyk, E., Baraniewski, K., Piszczek, I., Proniewsa, W., Swiatecka, G., Sczaniecka, O., Curylo, A., Dziduszko Fedorko, E., Prasal, M. et al., Thrombolytic therapy of myocardial infarction. Prognostic value of early reduction of elevation of ST segment, *Kardiol. Pol.*, 36 (1992) 6–12.

[22] Schatzmann, H.J., ATP-dependent Ca^{2+} extrusion from human red blood cells, *Experientia*, 22 (1966) 364–368.

[23] Sharat, P.A.H., Dawson, T.M., Ross, C.A., Fothi, M., Mourey, R.J. and Snyder, S.H., Inostol 1,4,5-trisphosphate receptors: immunohistochemical localization to discrete areas of rat central nervous system, *Neuroscience*, 53 (1993) 927–942.

[24] Sharat, P.A.H., McPherson, P.S., Dawson, T.M., Aoki, C., Campbell, K.P. and Snyder, S.H., Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca^{2+} release channels in rat brain, *J. Neurosci.*, 13 (1993) 3051–3063.

[25] Shull, G.E. and Greeb, J., Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. Struc-

tural and functional domains exhibit similarity to Na^+, K^+ and other cation transport ATPases, *J. Biol. Chem.*, 263 (1988) 8646–8657.

[26] Stahl, W.L., Eakin, T.J., Owens, J.W.M.J., Breininger, J.F., Filuk, P.E. and Anderson, W.R., Plasma membrane calcium-ATPase isoforms: distribution of mRNAs in rat brain by in situ hybridization, *Mol. Brain Res.*, 16 (1992) 223–231.

[27] Stahl, W.L., Keeton, T.P. and Eakin, T.J., The plasma membrane Ca^{2+}-ATPase mRNA isoform PMCA4 is expressed at high levels in neurons of rat piriform cortex and neocortex, *Neurosci. Lett.*, 178 (1994) 267–270.

[28] Stauffer, T.P., Hilfiker, H., Carafoli, E. and Strehler, E.E., Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes, *J. Biol. Chem.*, 268 (1993) 25993–26003.

[29] Stauffer, T.P., Guerini, D. and Carafoli, E., Tissue distribution of the four gene products of the plasma membrane Ca^{2+} pump: a study using specific antibodies, *J. Biol. Chem.*, 270 (1995) 12184–12190.

[30] Strehler, E.E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A.G., Penniston, J.T. and Carafoli, E., Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocyte membrane, *J. Biol. Chem.*, 265 (1990) 2835–2842.

[31] Towbin, H., Staehlin, T. and Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. (USA)*, 76 (1979) 4350–4354.

[32] Verma, A.K., Filoteo, A.G., Stanford, D.R., Wieben, E.D., Penniston, J.T., Strehler, E.E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler, M.-A., James, P., Vorherr, T., Krebs, J. and Carafoli, E., Complete primary structure of a human plasma membrane Ca^{2+} pump, *J. Biol. Chem.*, 263 (1988) 14152–14159.

[33] Zacharias, D.A., Dalrymple, S.J. and Strehler, E.E., Transcript distribution of the plasma membrane Ca^{2+} pump isoforms and splice variants in the human brain, *Mol. Brain Res.*, (1995) in press.