The Plasma Membrane Ca\textsuperscript{2+}-ATPase Isoform 4 Is Localized in Lipid Rafts of Cerebellum Synaptic Plasma Membranes*

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Here we describe the association of the synaptosomal plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) from pig cerebellum with cholesterol/sphingomyelin-rich membrane domains (rafts). The PMCA4 was localized exclusively in rafts prepared by floatation in Nycodenz density gradients of ice-cold Brij 96 extracts. This was corroborated by its colocalization with the raft markers cholesterol, ganglioside GM1, and PrP\textsuperscript{c}. The remaining PMCA isoforms were found in the detergent-soluble fractions, with the majority of the membrane proteins. Activity assays confirmed the bimodal distribution of the PMCA isoforms in the density gradient, with a lower activity for PMCA4 and greater stimulation by calmodulin than for the other isoforms. By providing an ordered membrane microenvironment, lipid rafts may contribute to the interaction of PMCA4 with proteins involved in Ca\textsuperscript{2+} signaling at discrete functional positions on the synaptic nerve terminals.

The Ca\textsuperscript{2+}-ATPases from the plasma membrane (PMCA\textsuperscript{3}) provide an important pathway for the removal of the intracellular Ca\textsuperscript{2+} excess by pumping this ion out of the cell. PMCA isoforms are encoded by at least four different genes resulting in four basic isoforms (PMCA1–4). Additional pumping this ion out of the cell. PMCAs are encoded by at least four proteins involved in Ca\textsuperscript{2+} homeostasis maintenance and participate in the local regulation of Ca\textsuperscript{2+} signaling. Moreover specific demands for Ca\textsuperscript{2+} regulation must influence the preferential targeting of PMCA isoforms to specific locations in the plasma membrane through a yet unknown mechanism.

The organization of biological membranes into specialized subdomains, known as lipid rafts, is an emergent source of research given their involvement in many essential cellular processes such as signal transduction (2–4). Lipid rafts are characterized by their high content of cholesterol and glycosphingolipids and serve as suitable platforms for the assembly of protein complexes involved in signaling pathways (3). In neurons, lipid rafts have been found in dendrites where they sustain a variety of postsynaptic protein complexes (5). In fact, their disruption with hydroxylamine reduces its floatation properties. The differences observed in the membrane-domain segregation are likely to relate to the PMCA isoform specificity of the role that they play in cell signaling and other molecular processes. The fact that different proteins involved in neurotransmitter release have been associated with lipid rafts in the nerve terminal suggests a direct role of PMCA4 in these processes.

EXPERIMENTAL PROCEDURES

Preparation of Synaptosomal Plasma Membrane Vesicles—SPM from adult pig cerebellum were prepared by osmotic lysis of synaptosomes as described by Salvador and Mata (17) and stored at −80 °C until use. The protein content was measured using the Bradford method (18) with bovine serum albumin as standard.

Preparation of Detergent-resistant Membranes by Floatation—The floatation assay was performed as described by Naslavsky et al. (19) but substituting Brij 96 (Fluka) for Triton X-100 to diminish domain mixing (20). Essentially, 0.75 ml of SPM (7–10.5 mg of protein) in 25 mM Tris, pH 7.4, 150 mM NaCl was treated with 0.5% Brij 96 (0.5% (w/v) final concentration) for 1 h at 4 °C with constant stirring and then enriched in 35% (w/v) Nycodenz (Sigma). A volume of 1.5 ml was placed on the bottom of an Ultracentrifuge tube, and then overlayed with 214 μl each of 25, 22.5, 20, 18, 15, 12, and 8% Nycodenz prepared in 25 mM Tris, pH 7.4, 150 mM NaCl containing 0.5% Brij 96. After 4 h of ultracentrifugation at 260,000 × g (rotor MLS-55, OptimaMax, Beckman Instruments) at 4 °C, 13 fractions of 230 μl were collected from the top.

Electrophoresis and Immunoblots—Equal volumes of each fraction were separated in 6.5% (PMCA analysis) or 13.5% (PrP\textsuperscript{c} and flotillin analysis) polyacrylamide gels according to Laemmli’s procedure (21). Proteins were either visualized with Coomassie Brilliant Blue or electrotransferred to polyvinylidene difluoride membranes using a Trans-Blot\textsuperscript{®} SD semidyed electrotransfer system (Bio-Rad). After blocking for

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§ The abbreviations used are: PMCA, plasma membrane Ca\textsuperscript{2+}-ATPase; PrP\textsuperscript{c}, cellular prion protein; SPM, synaptosomal plasma membrane; TBS, Tris-buffered saline; pAb, polyclonal antibody; mAb, monoclonal antibody; MAGUK, membrane-associated guanylate kinase; HRP, horseradish peroxidase.
30 min in Tris-buffered saline (TBS) containing 2% (v/v) nonfat dry milk (TBS-milk), the membranes were incubated with the primary antibody for 3 h at room temperature in TBS-0.05% Tween 20 (TBST). The primary antibodies used were: pAb anti-PMCA1 (1/500, Affinity Bioreagents), pAb anti-PMCA2 (1/500, Affinity Bioreagents), pAb anti-PMCA3 (1/500, Affinity Bioreagents), mAb antibody anti-PMCA4 (1/500, Affinity Bioreagents), mAb 6H4 anti-PrP (1/3000, Prionics AG), and mAb anti-flotillin-2/ESA (1/500, BD Transduction Laboratories). The anti-PMCA4a pAb CR4a (1/200) and the anti-PMCA4b mAb JA3 (1/1000) were kindly provided by A. G. Filoteo and E. E. Strehler (Mayo Clinic, Rochester, MN). After washing, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody and stained with 4-methoxy-1-naphthol. Immunoreactive bands were quantified with the TINA software (Raytest, Straubenhardt, Germany).

**Ganglioside GM1 Detection**—For the lipid raft-associated ganglioside GM1, 0.5 μl of each gradient fraction was spotted onto a nitrocellulose membrane sealed on a dot-blot set-up (Bio-Rad). After washing with TBS-0.5% Tween 20, the membrane was incubated with 6 M guanidine chloride for 5 min at room temperature and then washed extensively.
with TBS-0.5% Tween 20. After blocking with TBS containing 3% (w/v) defatted milk for 2 h at room temperature, membranes were incubated with the cholera toxin B subunit-HRP (1/20,000, Calbiochem) overnight at 4 °C. The detection was performed by chemoluminescence (Bio-Rad Immune-Star HRP substrate kit). The intensities of spots were quantified with the TINA software.

Cholesterol Determination—Cholesterol content was determined colorimetrically using the ferric chloride reaction in a sulfuric acid-acetic acid solution (22). Briefly, 10 μl of each fraction was mixed with 80 μl of chloroform/methanol (2/1, v/v), and the phase containing the lipids was transferred onto a glass tube and dried with nitrogen. Cholesterol was extracted by the addition to each tube of 0.75 ml of acetic acid and 0.5 ml of ferric chloride reagent (prepared by mixing 1 ml of 2.5% (w/v) FeCl3 in 85% H3PO4 with 11.5 ml of concentrated H2SO4). The samples were incubated for 30 min, and the absorbance was read at 550 nm. Cholesterol standard solutions were used for calibration.

Ca2+-ATPase Activity—The enzymatic activity was measured in 7.5 μl of each fraction by using a coupled enzyme assay as described previously (23). The activities were measured in the presence of 100 nM thapsigargin and 5 mM sodium azide to inhibit the contamination of the endoplasmic reticulum Ca2+-ATPase (SERCA) and other ATPases. Calmodulin (0.32 μg/ml) was added when indicated. The membrane vesicles were permeabilized with 0.01% saponin to obtain the activity of all PMCA molecules. The reaction was started by the addition of 1 mM ATP. The PMCA activity of these fractions was obtained after subtraction of the Mg2+-ATPase activity, measured in the presence of 6 mM EGTA, and was given in μmol P1/min·mg−1.

Determination of Hydroxylamine Sensitivity—SPM (10 mg protein) were treated with 1M hydroxylamine (pH 7.4) or 1M Tris (pH 7.4) as a control for 2 h at 4 °C under constant stirring. After 1 h of ultracentrifugation at 260,000 × g at 4 °C, the membrane pellets were resuspended in 25 mM Tris, pH 7.4, 150 mM NaCl, cold-extracted with Brij 96, and fractioned on a Nycodenz gradient as described above.

RESULTS

To investigate the differential membrane domain segregation of PMCA isoforms we used cold Brij 96-extracts of SPM vesicles isolated from pig cerebellum fractioned by ultracentrifugation along a Nycodenz
FIGURE 5. Alignment of the amino acid sequences of the four PMCA isoforms. The sequences of PMCA isoforms (Swiss-Prot accession numbers P20020, Q01814, P16720, and P23634 of hPMCA1, -2, -3, and -4, respectively) were analyzed using the BLAST algorithm. Gaps were introduced into the sequences to maintain the alignment. Amino acids conserved in PMCA1, -2, and -3, but not in PMCA4, are displayed as bold letters in gray boxes. The transmembrane domains (TMD) are underlined, and the cytoplasmic (C) and extracellular (E) loops are indicated by arrows. The C terminus of hPMCA4a is shown in italics and denoted by an asterisk. The PDZ-binding motif and the potentially palmitoylated Cys (Œ) are labeled. The insert depicts a schematic view of the PMCA domains.
PMCA4 Association with Lipid Rafts of Cerebellum Membranes

The choice of Brij 96 instead of Triton X-100 as detergent for the cold extraction was based on the reported improved preservation of raft entities (20). Regarding the density gradient for the floatation fractionation, Nycodenz instead of sucrose was used to reduce the centrifugation time needed to achieve the density equilibrium, thus favoring the preservation of the functional integrity of the membrane proteins.

Fractions from SPM Brij 96 extracts were first analyzed for the lipid raft markers, sphingolipid GM1 and cholesterol (Fig. 1). As described (19, 20) the GM1 distribution showed the highest levels at the top of the gradient (fractions 1–4), indicating that these low density fractions were raft-enriched fractions (Fig. 1A). Similarly, cholesterol was found mainly in low density fractions 2–4 at the top of the gradient, with very low levels in the remaining fractions (Fig. 1B). As protein markers of rafts, we used both the cellular prion protein (PrP<sup>C</sup>) and flotillin (24, 25). Fig. 2A shows that the vast majority of PrP<sup>C</sup> appeared in low density fractions 2–6, separated from most membrane proteins (Fig. 2C). Flotillin showed a broader distribution, with its highest content found in fractions 3 and 4 (Fig. 2B). This floatation distribution of markers was preserved for SPM cold detergent extractions performed at protein concentrations in the 9–14 mg/ml range.

The presence and distribution of PMCA in the gradient were first analyzed with an anti-PMCA antibody that recognizes all isoforms, giving a broad staining in all fractions (result not shown). Hence we used isoform-specific antibodies to identify the four PMCA isoforms along the gradient (Fig. 3A). PMCA1, -2, and -3 were detected in the non-raft high density fractions, with stronger staining for PMCA1 and -3 than for PMCA2. Interestingly, the anti-PMCA4 antibody recognized two protein bands at the top of the gradient, around low density fractions 2–4.

To test whether the observed bands corresponded to both splicing variants a and b, Western blots of SPM were probed with specific anti-PMCA4a (CR4a) and anti-PMCA4b (J43) antibodies (26, 27) (Fig. 3B). It can be seen that each antibody identified only one band corresponding to each splice variant, whereas the anti-PMCA4 antibody stained both variants in the SPM. The quantization of PMCA1–4 bands (Fig. 3C) pointed out more precisely the bimodal distribution of PMCA isoforms along the Brij 96-Nycodenz gradient, with one peak corresponding to PMCA4 located in the low density raft fraction 3 and another peak corresponding to the other PMCA isoforms located in the high density non-raft fraction 9. Furthermore, similar, if not identical, bimodal distribution was obtained from SPM isolated from pig cerebrum (data not shown).

Measurements of Ca<sup>2+</sup>-ATPase activity in all fractions (Fig. 4) showed two pools of PMCA activity that overlapped with those obtained by immunoreactivity. The incubation of membranes from each fraction with 0.32 μg/ml calmodulin showed stimulation of the activity just around fraction 3.

The preferential ability of PMCA4, among all of the isoforms, to associate with lipid rafts could be the result of an intrinsic propensity; or this ability could be driven indirectly by the existence of modular partners that could either promote it, in the case of PMCA4, or preclude it, in the remaining isoforms. The fact that PMCA4b binds to several PDZ (PSD-95/Dlg/ZO-1) proteins of the MAGUK family (28, 29), some of which are raft-associated (12, 30–33), could support an indirect raft association. In fact, a ETSV (PMCA4b) to ETSL (PMCA1s 1b, 2b, and 3b) sequence change in the class I PDZ-binding motif found at the C terminus could explain the distinct raft segregation among PMCA b splicing variants (28, 34). However, this unique PDZ-mediated association with lipid rafts cannot explain the association capacity of PMCA4a and the lack of association of PMCA2b. In this sense, PMCA4a has a shorter C-terminal domain, lacking the PDZ-binding motif (35), and PMCA2b has been described to interact with MAGUK proteins (29) and does not appear as a raft-associated protein.

To explain this controversy we considered the possibility of raft association as an intrinsic property of PMCA4 isoform. If this would be the case, then PMCA4 should contain in its sequence a divergent difference compared with the rest of the PMCA isoforms (Fig. 5). Among the sequence differences found, we identified two regions in which PMCA4 contains differentially a Cys residue (GLQRIEQ in the C2 of PMCA4a/b, VDCNQ in the C6 of PMCA4b) compared with a Ser in the remaining isoforms. These Cys residues could sustain a covalent modification such as palmitoylation, which has been essential for the lipid raft localization of several transmembrane proteins (36–38). To test this possibility we investigated the sensitivity of PMCA4 buoyancy to 1 m hydroxylamine, which is known to reverse protein palmitoylations (39). Fig. 6 shows that a pretreatment with 1 m hydroxylamine shifted the maximum of the floatation distribution of PMCA4 from fraction 3 to 9 compared with the control performed with 1 m Tris, whereas that of PMCA2 and the other isoforms remained unchanged at fraction 9.
DISCUSSION

This study shows for the first time that the cerebellar PMCA4 isoform, but not the other PMCA isoforms, is specifically associated with the low density membrane domains from SPM that exhibit high cholesterol and GM1 content and similar flotation properties to the raft-associated proteins PrPC and flotillin. Interestingly, this association is sensitive to hydroxylamine, indicating protein palmitoylation as a driving force.

Membrane microdomains, such as the so-called lipid rafts, function as segregation platforms to concentrate different molecules and assemble them into efficient signal transduction machineries (2, 41, 42). An increasing number of kinases and other proteins appear to use this strategy for best working in signal cascades (43). In neurons, lipid rafts were first thought to be restricted to the axonal compartment (44), although later it was shown that several postsynaptic proteins located in dendrites are also associated to rafts (5). We have recently shown that in pig cerebellum the PMCA is present in the plasma membrane of the soma of Purkinje cells as well as in areas enclosing many synapses, such as in cerebellar glomeruli and dendritic spines of Purkinje cell arborization (23). However, this pattern was not discriminative regarding the specific isoforms because the analysis was done with an antibody that recognized all PMCA isoforms.

The presence of lipid rafts in SPMs was evidenced in the low density fractions of a gradient ultracentrifugation of cold detergent extract, on the basis of the high content of the lipid markers cholesterol and ganglioside GM1 and the protein markers PrPC and flotillin (19, 20, 45). The immunodetection of PMCA proteins using specific antibodies showed that only the PMCA4 isoform appears associated in raft, whereas the PMCA isoforms 1, 2, and 3 emerge in the high density non-raft fractions located closer to the bottom of the gradient, where the majority of the proteins were found. The distribution of PMCA isoforms in two different portions of the gradient was corroborated by functional assays. The lower PMCA activity found in fraction 3 with respect to fraction 9 and its stimulation by calmodulin correlates with the presence of PMCA4 in that fraction, because this isoform has the lowest basal activity and the greatest stimulation by calmodulin among isoforms (46, 47). The low level or even the lack of calmodulin stimulation found in fractions 3 and 9, respectively, could be expected, provided that PMCA isoforms are already activated by the presence of a high content of phosphatidylycerine in SPM from the cerebellum as it has been reported in brain (17). It has been reported that the synaptosomal PMCA activity from pig brain is ganglioside-dependent (48), as the activity is reduced by GM1 and to a greater extent by GM2 and GM3. Thus, the high content of gangliosides in the same fraction as PMCA4 (Figs. 1 and 3) may also account for the lower activity found in fraction 3 with respect to fraction 9. Our activity results about the corresponding decrease of PMCA activity with the increase of sphingomyelin and cholesterol content in reconstituted pig brain PMCA are in good agreement with those reported by Pang et al. (49). The distribution pattern of PMCA isoforms was observed in SPM isolated not only from cerebellum but also from cerebral cortex, giving a more general than regional view on the process.

Screening for molecular determinants of raft association in PMCA4 revealed the presence of isoform-specific Cys residues in regions corresponding to the C2 and C6 cytoplasmic loops, susceptible a priori of being palmitoylated. The key role of this covalent modification was evidenced in the reduction of PMCA4 buoyancy after treatment with 1 M hydroxylamine. However, it must be kept in mind that the accurate attribution of this modification to PMCA4 instead to a second, yet unidentified, partner will require further experimentation.

Although the physiological relevance of PMCA4 in rafts has not been established, it is tempting to speculate on the advantages of this process. Given that, among all isoforms, PMCA4b has the lowest basal activity but the highest stimulation by calmodulin (50), it has been suggested that PMCA4b might be actively involved in the development, shaping, and duration of the Ca\(^{2+}\) signal (47). Such a scenario agrees with a preferential localization at a definite membrane domain as rafts (see Fig. 7) in which a Ca\(^{2+}\) signaling event must be a very tightly regulated process.

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PMCA4 Association with Lipid Rafts of Cerebellum Membranes

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