Altered Ca^{2+} dependence of synaptosomal plasma membrane Ca^{2+} -ATPase in human brain affected by Alzheimer's disease

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ABSTRACT High-affinity Ca^{2+} transport ATPases play a crucial role in controlling cytosolic Ca^{2+} . The amyloid β -peptide ($\text{A}\beta$) is a neurotoxic agent found in affected neurons in Alzheimer's disease (AD) that has been implicated in dysregulation of Ca^{2+} homeostasis. Using kinetic assays, we have shown that the Ca^{2+} dependencies of intracellular Ca^{2+} -ATPase (SERCA and SPCA) activity are the same in human AD and normal brain but that of plasma membrane Ca^{2+} -ATPase (PMCA) is different. The addition of $\text{A}\beta$ to normal brain decreases the PMCA activity measured at pCa 5.5, resulting in the same Ca^{2+} dependency as that seen in AD brain, whereas the addition of $\text{A}\beta$ to AD brain has no effect on PMCA activity. $\text{A}\beta$ also decreases the activity of PMCA purified from pig cerebrum, the effect being isoform specific. The level of inhibition of purified PMCA caused by $\text{A}\beta$ is reduced by cholesterol, and the level of inhibition of PMCA activity by $\text{A}\beta$ in the raft fraction of pig synaptosomal membranes is lower than for the nonraft fraction. We conclude that the effect of $\text{A}\beta$ on PMCA activity could be important in amyloid toxicity, resulting in cytoplasmic Ca^{2+} dysregulation and could explain the different Ca^{2+} dependencies of PMCA activity observed in normal and AD brain.—Berrocal, M., Marcos, D., Sepúlveda, M. R., Pérez, M., Ávila, J., Mata, A. M. Altered Ca^{2+} dependence of synaptosomal plasma membrane Ca^{2+} -ATPase in human brain affected by Alzheimer's disease. *FASEB J.* 23, 1826–1834 (2009)

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CALCIUM PLAYS A KEY ROLE IN brain functions such as learning and memory, and it is also implicated in neuron survival and death. These events require a precise regulation of the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$. A protein family crucially involved in high-affinity Ca^{2+} regulation is that of the adenosine triphosphate (ATP)-driven Ca^{2+} pumps. There are three types of Ca^{2+} -ATPase: the plasma membrane Ca^{2+} -ATPase (PMCA), which pumps excess Ca^{2+} out of

the cell, and two intracellular pumps that accumulate Ca^{2+} in the sarco/endoplasmic reticulum (SERCA) and the secretory pathway (SPCA), respectively. The three types of pump are encoded by separate genes, giving rise to four major PMCA isoforms, three SERCA isoforms, and two SPCA isoforms (1–3). Additional isoform diversity is generated by alternative splicing of their primary transcripts.

An inability of neurons to regulate Ca^{2+} homeostasis is a feature of Alzheimer's disease (AD) and other neurodegenerative disorders that appear to be closely involved in the damage and death of neurons (4–5). The amyloid β -peptide ($\text{A}\beta$) has been reported to be involved in the disruption of cellular Ca^{2+} homeostasis in affected neurons in AD (5), although the mechanism of the effect is a matter of debate. Some findings suggest that $\text{A}\beta$ induces oxidative stress, leading to an elevation of basal $[\text{Ca}^{2+}]_i$ (6, 7). Other findings suggest that $\text{A}\beta$ causes Ca^{2+} influx by forming a cation-selective ion channel in membranes (8, 9). It has also been reported that the Na^+/K^+ -ATPase and other ion motive ATPases are inhibited by $\text{A}\beta$ (6, 7). An important feature of the pathogenesis of AD is the interaction of $\text{A}\beta$ with membrane lipids (10), cholesterol being a recognized modulator of $\text{A}\beta$ fibrillogenesis and neurotoxicity (11, 12). Further, processing of $\text{A}\beta$ (13) and other proteins (14, 15) involved in neurodegenerative diseases seems to be associated with cholesterol-enriched membrane microdomains (lipid rafts) that can be isolated from neuronal plasma membranes (16) and other membranes based on their resistance to solubilization in nonionic detergents. Although the full-length $\text{A}\beta$ contains 39 to 43 amino acid residues, it has been shown that the amyloid β -fragment 25–35 ($\text{A}\beta_{25-35}$) represents the biologically active region of the full peptide and exhibits the same neurotoxic effect as the full-length peptide (17). Here, we relate changes in

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Ca²⁺-ATPase function in human brain affected by AD to effects of A β on the three types of Ca²⁺-ATPases.

MATERIALS AND METHODS

Materials

Bovine brain calmodulin, phosphatidylserine (PS) from bovine brain, phosphatidylcholine (PC) type XI-E from egg yolk, thapsigargin, ammonium vanadate, and calmodulin-agarose were obtained from Sigma (Madrid, Spain). Peptides A β 25-35, A β 35-25, and A β 1-40 were obtained from BioNova (Madrid, Spain). The PMCA1-4 specific antibodies were obtained from Affinity Bioreagents (Golden, CO, USA), and the secondary antibodies conjugated with peroxidase were obtained from Bio-Rad (Madrid, Spain). The sarcoplasmic reticulum SERCA1 from rabbit was kindly supplied by Dr. Fernando Henao (University of Extremadura, Badajoz, Spain). All other reagents were of the highest purity available.

Preparation of membrane vesicles from human tissue

Crude membranes were prepared as detailed by Sepulveda *et al.* (18), from human brain tissue (medium temporal gyrus from cerebral cortex, highly affected in AD) obtained at autopsy from 8 patients diagnosed as having AD (age 79 $\pm$ 2 yr) and from 7 age-matched non-AD control subjects. Samples were carefully conserved and supplied by Dr. R. Ravid and Dr. P. van't Klooster from the Netherlands Brain Bank (Amsterdam, The Netherlands). Membranes from a nonvulnerable region, such as human cerebellum, of three AD and three non-AD subjects were kindly provided by Dr. Alberto Rábano [Alzheimer's Disease Research Unit, Centro de Investigación de Enfermedades Neurológicas (CIEN) Foundation, Madrid, Spain]. Tissues were homogenized in 10 mM HEPES/KOH, pH 7.4; 0.32 M sucrose; 0.5 mM MgSO₄; 0.1 mM phenylmethanesulfonyl fluoride (PMSF); 2 mM 2-mercaptoethanol; and protease inhibitor cocktail solution (Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged for 10 min at 1500 g, and the supernatant was centrifuged for 45 min at 100,000 g. The final pellet was resuspended in 10 mM HEPES/KOH, pH 7.4, containing 0.32 M sucrose.

Preparation of synaptosomes and purified plasma membrane Ca²⁺-ATPase from pig cerebrum and cerebellum

Synaptosomes were prepared from adult pig (5 mo old) cerebrum, as described by Salvador and Mata (19). Briefly, one fresh cerebrum was homogenized in 10 vol of 10 mM HEPES/KOH, pH 7.4; 0.32 M sucrose; 0.5 mM MgSO₄; 0.1 mM PMSF; and 2 mM 2-mercaptoethanol. After two centrifugation steps at low and high speeds, respectively, the pellet was layered onto a 40–20% (w/v) discontinuous sucrose gradient, and synaptosomes were obtained at the interface and kept in 10 mM HEPES/KOH, pH 7.4, and 0.32 M sucrose. Synaptosomal plasma membranes (SPMs) were obtained by osmotic lysis of synaptosomes. The plasma membrane Ca²⁺-ATPase (PMCA) was obtained by subsequent solubilization of SPM with 0.6% (w/v) Triton X-100 and loading the solubilized proteins onto a calmodulin affinity column. The fraction containing PMCA was eluted free of lipids with a buffer containing 15% glycerol, 0.06% Triton X-100, and 2 mM EDTA. The purified PMCA from pig cerebellum was obtained in the same way.

Cell culture and DNA transfection

Human embryonic kidney 293 (HEK293) cells were seeded in 100-mm culture plates at a density of 2.5×10^6 cells per plate and in coverslips with gelatin at a density of 3×10^4 cells per coverslips. Cells were transfected with the full-length cDNA of PMCA isoforms 2b, 3b and 4b cloned in the pMT2 expression vector (kindly provided by Dr. E. E. Strehler, Mayo Clinic, Rochester, MN, USA), and of SERCA 2a and 2b isoforms cloned in the pSV45 expression vector (by Dr. F. Wuytack, Katholieke Universiteit Leuven, Leuven, Belgium), using GenJuice transfection reagent (Merck, Darmstadt, Germany). After incubation for 60 h at 37°C and 5% CO₂, microsomes were prepared from the cells harvested from plates, as described by Verboomen *et al.* (20) and used for ATPase activity assays, while the cells from the coverslips were used for immunocytochemistry assays.

Immunocytochemistry

Cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 4 min. After blocking with 3% BSA in PBS for 1 h, the overexpressed-PMCA isoforms were stained by incubation for 2 h with rabbit anti-PMCA2 (1:500), rabbit anti-PMCA3 (1:500), or mouse anti-PMCA4 (1:500) antibodies (BioNova), followed by incubation with Alexa488 goat anti-rabbit or Alexa594 goat anti-mouse (1:750) antibodies (Molecular Probes, Eugene, OR, USA) for 1 h. All the incubation steps were performed at room temperature. Nuclei were visualized by staining with 10 μ M 4',6-diamidino-2-phenylindole (DAPI). Finally, sections were covered with FluorSave mounting medium and analyzed using a fluorescence microscope.

Ca²⁺-ATPase activity

The enzymatic activity of each type of Ca²⁺-ATPase from brain and from HEK cells was measured in 10 μ g of microsomes or 40 μ g of synaptosomes using a coupled enzyme assay, as described in detail by Sepulveda *et al.* (21). Briefly, membranes were incubated for 2 min at 37°C with different concentrations of A β freshly prepared from a 188 μ M stock solution in distilled water, in 50 μ l final volume, and were then added to the standard assay medium containing, in a volume of 1 ml, 50 mM HEPES/KOH (pH 7.4), 100 mM KCl, 2 mM MgCl₂, 5 mM Na₃N, 3.16 μ M free Ca²⁺ (pCa 5.5), 0.22 mM NADH, 0.01% saponin (to disrupt both the plasma and intracellular membranes, allowing the access of substrates to all protein molecules in membrane vesicles), 0.42 mM phosphoenolpyruvate, 10 IU pyruvate kinase, and 28 IU lactate dehydrogenase, and incubated for a further 2 min. The contributions of SERCA, PMCA, and SPCA activities to the total Ca²⁺-ATPase activity were evaluated, as detailed in Sepulveda *et al.* (21). In brief, independent activity measurements were performed after the addition to the medium of 1 mM ATP, to evaluate total (Ca²⁺+Mg²⁺)-ATPase activity, or with 100 nM thapsigargin to inhibit SERCA activity, 2 μ M vanadate to selectively inhibit PMCA activity, or 3 mM EGTA, to measure the Mg²⁺-ATPase activity. The total Ca²⁺-ATPase activity was obtained after subtraction of the Mg²⁺-ATPase activity. The SERCA activity was calculated by subtracting the activity measured in the presence of thapsigargin, which includes PMCA, SPCA, and Mg²⁺-ATPase activities, from the total (Ca²⁺+Mg²⁺)-ATPase activity. The SPCA activity was calculated by subtracting the Mg²⁺-ATPase activity from the activity in the presence of thapsigargin and vanadate. The PMCA activity was calculated by subtracting the Mg²⁺-

ATPase and SPCA activity from the activity in the presence of thapsigargin.

To assay the effect of A β on the activity of PMCA, the delipidated, purified PMCA (2.5 μ g) containing 0.06% Triton X-100 was mixed with 13.32 μ g of lipid and cholesterol previously dried to a thin film under a N $_2$ atmosphere, as described by Palacios *et al.* (22), and with the required concentration of A β , in a final volume of 50 μ l. The mixture was incubated for 2 min at 37°C and then diluted directly into the assay medium, in 1 ml final volume. The sample was incubated for a further 2 min before starting the reaction with 1 mM ATP. When required, bovine brain calmodulin was added directly into the reaction medium to a final concentration of 0.32 μ g/ml. The Ca $^{2+}$ -ATPase activity of SERCA1 from rabbit sarcoplasmic reticulum was measured using the same coupled enzyme assay, as described by Mall *et al.* (23), in the presence of 4% (w/w) Ca $^{2+}$ ionophore calcimycin (A23187), to make the vesicles leaky to Ca $^{2+}$.

Data fitting and statistical analysis

Data were fitted to the appropriate equations using the nonlinear least-squares routine in the SigmaPlot 10 package (SPSS Inc., Chicago, IL, USA). The data were analyzed by unpaired Student's *t* test. Statistical significance was accepted for $P \leq 0.001$.

Cholesterol and protein concentration

Cholesterol was determined colorimetrically as described in Sepulveda *et al.* (16). Protein content was determined using the bicinchoninic acid method with the BCA protein assay kit (Pierce, Rockford, IL, USA).

Preparation of detergent-resistant membranes by floatation

Detergent-resistant membrane fractions were prepared from pig cerebrum by floatation in Nycodenz density gradients of ice-cold Brij 96 extracts, as described by Sepulveda *et al.* (16).

Electrophoresis and immunoblotting

Electrophoresis was performed in 6.5% (w/v) Laemmli gels (24). Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes and blocked in Tris-buffered saline (TBS) containing 2% (w/v) of nonfat dry milk (TBS-milk). Immunostaining reactions were performed by incubating the PVDF membranes for 3 h at room temperature with the polyclonal antibodies anti-PMCA1, anti-PMCA2, and anti-PMCA3 (1:500 dilution), and the monoclonal antibody anti-PMCA4 (1:1000). Membranes were then incubated with peroxidase-conjugated secondary antibodies (1:3000) and stained with chemiluminescent (ECL) substrate (Pierce).

RESULTS

Ca $^{2+}$ -ATPase activity in human AD brain

It has been shown that Ca $^{2+}$ dysregulation is involved in the mechanism of A β toxicity and in neuronal degeneration in AD and other neurodegenerative disorders (6, 17, 25). Because of the importance of the Ca $^{2+}$ -ATPases in Ca $^{2+}$ homeostasis, we explored the possibility that Ca $^{2+}$ -ATPase activity was altered in human postmortem temporal gyrus from AD brains. **Figure 1**

compares the calcium dependence of total Ca $^{2+}$ -ATPase activity in crude membranes from control and AD brains. Significant differences were found. Control membranes showed the typical bell-shaped Ca $^{2+}$ -dependent ATPase activity, with stimulation of ATPase activity by Ca $^{2+}$ in the range of pCa values between 7.5 and 5.5, attributed to Ca $^{2+}$ binding to the transport sites on the Ca $^{2+}$ -ATPase, with inhibition at higher Ca $^{2+}$ concentrations, between pCa values of 5.5 and 3.0 (Fig. 1). A maximum activity of $0.15 \pm 0.02 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ was observed at 3.16 μM free Ca $^{2+}$ (pCa 5.5). Similar results have been reported for total Ca $^{2+}$ -ATPase activity in pig cerebrum and cerebellum (22, 26). However, a very different profile for the Ca $^{2+}$ dependence of activity was observed in brain AD membranes, with the activity reaching a Ca $^{2+}$ maximal value of $0.30 \pm 0.04 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 0.316 μM free Ca $^{2+}$ (pCa 6.5) and no inhibition until the pCa value reached ~ 3.75 . Thus the affinity of the stimulatory site for Ca $^{2+}$ increases in the AD brain and that for the inhibitory site decreases.

When the activities of SERCA, SPCA and PMCA were determined separately in the human brain (**Fig. 2**), we found that the Ca $^{2+}$ sensitivities of SERCA and SPCA were very similar in control and AD brains, and similar to that for PMCA in control brain, whereas PMCA in the AD brain showed the abnormal Ca $^{2+}$ dependence, with the same activity when measured at pCa 4.5, 5.5, and 6.5, suggesting that the abnormal Ca $^{2+}$ dependence of Ca $^{2+}$ -ATPase activity in AD brain could be due to an effect on PMCA. An analogous experiment performed in a nonvulnerable AD region, such as cerebellum, showed that PMCA, SERCA, and SPCA activities were very similar in their Ca $^{2+}$ dependence in both control and AD human cerebellar membranes (see Supplemental Fig. S1).

Figure 1. Ca $^{2+}$ dependence of total Ca $^{2+}$ -ATPase activity in membranes from human postmortem brain of AD patients and non-AD controls. Total Ca $^{2+}$ -ATPase activity was measured as described in Materials and Methods. Free Ca $^{2+}$ concentration, expressed as pCa, was adjusted by the appropriate addition of CaCl $_2$ and BAPTA. Results are presented as percentage of activity determined at pCa 5.5 and represent means $\pm$ SD (bars) of 10 experiments performed with different membrane preparations from 8 AD patients (●) and 7 age-matched non-AD controls (○). The 100% activity corresponded to 0.36 ± 0.01 and $0.30 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for AD and control membranes, respectively.

Figure 2. Ca²⁺ dependence of PMCA, SERCA, and SPCA activity in membranes from human control and AD brains in the absence and presence of Aβ. Ca²⁺-ATPase activity of each pump was measured as described in Materials and Methods, in the absence (open symbols) or presence (solid symbols) of 2.5 μM Aβ₂₅₋₃₅ (●), Aβ₁₋₄₀ (▲), and Aβ₄₀₋₁ (☆). Data represent means ± SD (bars) of 10 experiments performed with different membrane preparations from 8 AD patients and seven age-matched non-AD controls.

As shown in Fig. 2, the addition of 2.5 μM Aβ₂₅₋₃₅ or Aβ₁₋₄₀ to control brain inhibited PMCA activity measured at pCa 5.5, with little effect on the activity measured at pCa 6.5 or 4.5, resulting in a profile similar to that observed for AD brain. The addition of 2.5 μM Aβ₂₅₋₃₅ or Aβ₁₋₄₀ to AD brain had no effect on PMCA activity. No effect of Aβ was seen on SERCA or SPCA activity in either control or AD brains (Fig. 2).

Aβ peptides inhibit the activity of PMCA in pig cerebrum

Aβ₂₅₋₃₅ and Aβ₁₋₄₀ result in marked inhibition of PMCA activity in pig cerebrum synaptosomes at pCa 5.5, with smaller levels of inhibition at pCa 6.5 and 4.5 (Fig. 3). The result is that the activity of the inhibited PMCA varies little with pCa in the range 4.5–6.5, as was observed for PMCA from AD brain (Fig. 2). A similar effect was seen for Aβ₂₅₋₃₅ and Aβ₁₋₄₀ on purified PMCA in the presence of phosphatidylserine (PS).

The effect of Aβ peptides was then characterized in detail, in pig brain, in which PMCA has been extensively studied (18, 19, 21, 22, 26, 27). As shown in Fig. 4A, Aβ₂₅₋₃₅ inhibits the Ca²⁺-ATPase activity of PMCA at pCa 5.5 in a dose-dependent manner, giving a maximum inhibition of 61%, with an IC₅₀ value (concentration producing half-maximum inhibition) of 2.5 μM. Notably,

however, Aβ₂₅₋₃₅ does not inhibit either of the intracellular Ca²⁺ pumps, SERCA, or SPCA (Fig. 2).

The studies with synaptosomes reported above relied on the use of inhibitors to separate PMCA activity from total Ca²⁺-ATPase activity. To confirm the validity of these results, the effects of Aβ were also studied on PMCA purified from pig cerebrum. As shown in Fig. 4B, effects of Aβ on the purified PMCA at pCa 5.5 were very similar to those observed with synaptosomes (Fig. 4A). For PMCA reconstituted in phosphatidylcholine (PC), a maximum inhibition of 75% was observed with Aβ₂₅₋₃₅, with an IC₅₀ of 1 μM. As expected for PMCA, higher activity was observed in the presence of calmodulin, but again Aβ₂₅₋₃₅ showed a maximum inhibition of 88% with a similar IC₅₀. The activity of PMCA reconstituted in phosphatidylserine (PS) was higher than that in PC (19) but again Aβ₂₅₋₃₅ results in 89% inhibition with an IC₅₀ of 1 μM. Inhibition of PMCA in PC or PS bilayers by the full-length peptide Aβ₁₋₄₀ was identical to that seen with Aβ₂₅₋₃₅ (Fig. 4B). The scrambled peptide Aβ₃₅₋₂₅ had no effect on the activity of PMCA in PS bilayers. In contrast to the effects seen at pCa 5.5, at pCa 4.5 and 6.5, Aβ₁₋₄₀ had no effect on PMCA activity at concentrations of peptide up to 10 μM (Fig. 4B). In all cases, as shown by the solid lines in Fig. 4B, the inhibition data fitted well to a simple inhibition curve $V/V_{\max} = K_I/(I + K_I)$, where $V_{\max}$ and V are the rates of ATP hydrolysis in the absence and presence of Aβ at a concentration I , respectively, and K_I is the apparent dissociation constant for Aβ. Estimated K_I values were 2.312, 1.216, and 1.218 μM, for PMCA reconsti-

Figure 3. Effect of Aβ on Ca²⁺ dependence of PMCA activity in synaptosomes and purified PMCA from pig cerebrum. PMCA activity was measured for pig cerebrum synaptosomes and for purified PMCA reconstituted in PS, as detailed in Materials and Methods, at the indicated pCa, in the absence (○) and presence of 2.5 μM of Aβ₂₅₋₃₅ (●), Aβ₁₋₄₀ (▲), Aβ₃₅₋₂₅ (□) or Aβ₄₀₋₁ (△). Data represent means ± SD of 4 experiments performed with 3 membrane preparations.

Figure 4. Effect of Aβ on Ca²⁺-ATPase activity. *A*) PMCA (○), SERCA (□), and SPCA (Δ) ATPase activities were measured at pCa 5.5, in 10 μg of pig synaptosomes incubated with the indicated concentrations of Aβ₂₅₋₃₅ as specified in Materials and Methods. *B*) PMCA activity was measured at pCa values of 4.5 (+), 6.5 (×) and 5.5 (other symbols), for 2.5 μg delipidated, purified PMCA from cerebrium, reconstituted with 13.3 μg of PS (○, ●, ☆, ×, +) or PC (Δ, ▽) and the indicated concentrations of Aβ₂₅₋₃₅ (●, Δ, □), Aβ₁₋₄₀ (○, Δ, ■, ×, +) or Aβ₃₅₋₂₅ (☆). Calmodulin (CaM) was added to PC reconstituted PMCA (□, ■). Solid lines show best fits to simple inhibitor binding, giving values for the dissociation constants *K*₁ of 1.216, 2.312, and 1.218 μM, respectively. *C*, *D*) SERCA activity was assayed in 10 μg of SR from rabbit (◆) (*C*) and microsomes overexpressing SERCA2a (□) and SERCA2b (■) (*D*), as detailed in Materials and Methods.

tuted in PC, PS, and PC in the presence of calmodulin, respectively.

In cerebrium, the SERCA subtype is predominantly SERCA2 (28). To confirm that the lack of effect of Aβ on SERCA activity from cerebrium shown in Fig. 2 is also true for other SERCA subtypes, effects of Aβ were also studied on SERCA1 from rabbit skeletal muscle (Fig. 4*C*) and SERCA2a and SERCA 2b overexpressed in COS-7 cells (Fig. 4*D*). As seen, Aβ₁₋₄₀ did not affect the activity of any of the SERCA isoforms studied.

The presence of cholesterol in reconstituted membranes containing purified PMCA modulates the inhibitory effect of Aβ in PMCA activity

The effects of Aβ have been widely reported to be cholesterol dependent (29-32). To test whether chole-

sterol might affect the interaction of Aβ with PMCA, we tested the effects of cholesterol on purified PMCA from pig cerebrium reconstituted in PS, the lipid in which the enzyme shows the highest activity. ATPase activity was measured at pCa 5.5 after incubation with 2.5 μM Aβ₂₅₋₃₅ or Aβ₁₋₄₀ (Fig. 5*A*). In the absence of cholesterol, the level of inhibition of cerebrium PMCA was 60%, but levels of inhibition decreased with increasing concentrations of cholesterol, so that at a cholesterol:PS molar ratio of 0.75:1, 2.5 μM Aβ₂₅₋₃₅ had no effect on activity. In the absence of Aβ₂₅₋₃₅, the addition of cholesterol had only a small effect on the activity of PMCA. Cholesterol was, however, unable to provide full protection at higher concentrations of Aβ₂₅₋₃₅ and Aβ₁₋₄₀ (Fig. 5*B*).

Endogenous cholesterol protects PMCA activity from its inhibition by Aβ in cholesterol-enriched lipid microdomains

The effect of endogenous cholesterol on the interaction of Aβ peptides with PMCA was also analyzed in cholesterol-enriched microdomains (rafts) isolated from synaptosomal membranes. It has been shown that after

Figure 5. PMCA activity of purified pig PMCA from cerebrium in the presence of both cholesterol and Aβ. *A*) ATPase activity was measured in 2.5 μg of PMCA purified from cerebrium, reconstituted with 13.3 μg PS and cholesterol at the given molar ratio of cholesterol to PS, in the absence (open symbols) or presence (solid symbols) of 2.5 μM Aβ₂₅₋₃₅ (○, ●) or Aβ₁₋₄₀ (□, ■). *B*) PMCA activity was measured for 2.5 μg PMCA from cerebrium reconstituted with 0.75 mol cholesterol/mol PS at the indicated concentrations of Aβ₂₅₋₃₅ or Aβ₁₋₄₀. Data are means ± SD (bars) of 4 experiments from 4 different preparations. **P* < 0.0001 *vs.* control.

treatment of membranes with nonionic detergents, raft-associated lipids and proteins remain insoluble and can be separated in low-density sucrose gradients (33). Lipid rafts were prepared from pig cerebrium synaptosomes, as described by Sepúlveda *et al.* (16). As expected, the low-density Brij 96-insoluble fraction (raft fraction) contained ~4-fold more cholesterol than that of the nonraft fraction (Table 1). Table 1 also compares effects of Aβ25-35 and Aβ1-40 on PMCA activity in intact synaptosomes and in the raft and nonraft fractions. Whereas 2.5 M Aβ25-35 or Aβ1-40 caused about a 50% inhibition of PMCA activity in intact synaptosomes and in the nonraft fraction, it resulted in no inhibition of PMCA activity in the raft fraction, consistent with the higher cholesterol content of the raft fraction.

Effect of Aβ on cerebellar PMCA and on PMCA isoforms

As shown in Fig. 6, 2.5 μM Aβ25-35 had no effect on the activity of PMCA purified from cerebellum and reconstituted into PS. The presence of cholesterol resulted in a small decrease in PMCA activity, but again no inhibition by 2.5 μM Aβ25-35 was observed at any level of cholesterol up to a 1:1 M ratio with PS.

To determine whether the differential effect of Aβ in cerebrum and cerebellum could be related to specific PMCA isoforms in each tissue, we checked the isoform content in synaptosomes from pig cerebrum and cerebellum (Fig. 7A). Although all PMCA isoforms (PMCA1-4) were present in both tissues, isoform 2 was more abundant in the cerebellum than in the cerebrum, and isoform 4 was more abundant in the cerebrum than in the cerebellum. A similar distribution was seen in PMCA purified from cerebrum and cerebellum (Fig. 7B).

The lack of inhibition of PMCA purified from cerebellum and reconstituted in PS could therefore represent an isoform specificity. This was checked by preparing microsomes from HEK293 cells overexpressing PMCA isoforms 2, 3, and 4 (Fig. 8). As shown in Fig. 8A, all overexpressed PMCAs were correctly targeted to the plasma membrane. However, whereas the activity of overexpressed PMCA4 in a microsomal fraction of the HEK293 cells was inhibited by Aβ25-35 and Aβ1-40, there was no inhibition of the activity of PMCA2; a

Figure 6. PMCA activity of purified pig PMCA from cerebellum in the presence of both cholesterol and Aβ. A) PMCA activity was measured for 2.5 μg of PMCA purified from cerebellum, reconstituted with 13.3 μg PS and cholesterol at the given molar ratio of cholesterol to PS, in the absence (open symbols) or presence (solid symbols) of 2.5 μM Aβ25-35 (Δ, ▲). Data are means ± SD (bars) of 4 experiments from 4 different preparations.

slight inhibition of the activity of PMCA3 was observed (Fig. 8B). Inhibition of the activity of PMCA4 was structurally specific; no inhibition was observed with the peptide Aβ35-25 (Fig. 8B). Further, as shown in Fig. 8B, incubation of the microsomal fraction with cholesterol prevented inhibition of PMCA4 by Aβ25-35 and Aβ1-40 and also prevented inhibition of PMCA3.

DISCUSSION

There is much evidence to suggest that a failure to regulate internal Ca²⁺ concentrations plays a role in AD (4, 5). A comparison of total Ca²⁺-ATPase activity in normal and AD human brain shows that the response to Ca²⁺ is markedly different in AD brain from that in normal brain, with stimulation of activity at lower concentrations of Ca²⁺ in AD brain and less inhibition at high concentrations of Ca²⁺ (Fig. 1). Evidence using inhibitors to distinguish among PMCA, SERCA, and SPCA activities suggests that the differences in Ca²⁺ sensitivity lie only with PMCA, as the Ca²⁺ dependencies of SERCA and SPCA activity are the same in normal and AD brain (Fig. 2). The addition of Aβ to normal

TABLE 1. PMCA-dependent Ca²⁺-ATPase activity and cholesterol content in intact synaptosomes, synaptosomes treated with Brij96 + Nycodenz, and detergent-resistant membranes (raft fraction) or detergent-soluble membranes (nonraft fraction) prepared by floatation in the presence of cold Brij 96 from pig cerebrum

Component	PMCA activity (μmol min ⁻¹ mg ⁻¹)			Cholesterol (nmol/μg protein)
	-Aβ	+Aβ25-35	+Aβ1-40	
Intact synaptosomes	0.164 ± 0.003	0.089 ± 0.005	0.087 ± 0.007	0.25 ± 0.02
Synaptosomes with Brij96 + Nycodenz	0.093 ± 0.006	0.043 ± 0.002	0.049 ± 0.002	0.25 ± 0.02
Raft fraction	0.031 ± 0.003	0.030 ± 0.002	0.035 ± 0.002	0.51 ± 0.07
Nonraft fraction	0.034 ± 0.004	0.015 ± 0.002	0.018 ± 0.002	0.15 ± 0.01

Figure 7. Identification of PMCA isoforms in synaptosomes and purified PMCA from pig cerebrum (Cr) and cerebellum (Cb). Thirty micrograms of protein from synaptosomes (A) or 2.5 μ g of purified PMCA (B) was electrophoresed in a 6.5% SDS-polyacrylamide gel, electrotransferred to PVDF membranes, and immunostained with the indicated PMCA isoform-specific antibodies. Blot is representative of 5 assays performed with different preparations of synaptosomes or purified PMCA.

brain results in a Ca^{2+} dependency of PMCA activity that resembles that observed in AD brain, whereas the addition of $\text{A}\beta$ to AD brain has no effect on PMCA activity (Fig. 2). These results obviously point to the possibility that the altered Ca^{2+} sensitivity observed for PMCA in the AD brain is due to the presence of $\text{A}\beta$ in the AD brain.

The inhibitory effects of $\text{A}\beta$ on PMCA were confirmed in studies of the effects of $\text{A}\beta$ on the activity of PMCA purified from pig cerebrum and reconstituted into PS. In the presence of $\text{A}\beta$, the Ca^{2+} dependence of PMCA activity (Fig. 3) was the same as that observed for PMCA activity in human AD brain or for the PMCA activity in normal human brain in the presence of $\text{A}\beta$ (Fig. 2). The change in Ca^{2+} dependence for PMCA activity in the presence of $\text{A}\beta$, for either purified PMCA, or in normal human brain or pig cerebral synaptosomes follows from a decrease in ATPase activity at pCa 5.5, with little effect at pCa 4.5 or 6.5, resulting in an ATPase activity that does not change with Ca^{2+} concentration in the range 4.5–6.5 (Figs. 2 and 3). The reaction mechanism of the Ca^{2+} -ATPase is complex (34), and it is not possible from the results presented here to say whether the observed effects of $\text{A}\beta$ are on Ca^{2+} binding or release steps of the reaction sequence, or on rates of phosphorylation or dephosphorylation. However, the addition of high concentrations of spermine to SERCA results in a change in Ca^{2+} dependency rather similar to that observed here for $\text{A}\beta$ on PMCA (35), and the effect of spermine on SERCA activity followed largely from a decrease in the rate of dissociation of Ca^{2+} from the phosphorylated intermediate. It is possible, therefore, that $\text{A}\beta$ has a similar effect on PMCA.

The observed effects of $\text{A}\beta$ were specific for PMCA, with no effect on SPCA (Fig. 2) or SERCA2, the most abundant isoform in the brain (28), or on SERCA1, SERCA2a, or SERCA2b (Figs. 2 and 4). They were also subtype specific, with large effects on PMCA4, smaller

effects on PMCA3, and no effect on PMCA2 (Fig. 8). It has been suggested that $\text{A}\beta$ interacts with lipids (29, 36). However, the effects reported here are likely to follow from direct interaction with PMCA, since the effects of $\text{A}\beta$ on reconstituted PMCA are similar in the presence of PC and PS (Fig. 4B). Nevertheless, the effects of $\text{A}\beta$ are modulated by the presence of cholesterol. Thus, $\text{A}\beta$ had no effect on the activity of purified PMCA in the presence of high levels of cholesterol (Fig. 5) and no effect on PMCA activity of the raft fraction isolated from pig cerebrum membranes, in which the cholesterol content is high (Table 1). It has been reported that $\text{A}\beta$ 1-40 binds with higher affinity to cholesterol than to other lipids (29, 37) and that cholesterol facilitates binding of $\text{A}\beta$ to the membrane (37), results that might suggest that the presence of cholesterol would decrease the effect of $\text{A}\beta$ on PMCA. An alternative possibility is, therefore, that binding of cholesterol to PMCA results in a conformational change on PMCA, masking the binding site for $\text{A}\beta$.

The result of the changed Ca^{2+} dependence of PMCA activity in AD brain and in the presence of $\text{A}\beta$ would be that the rate of removal of Ca^{2+} would be independent of the free Ca^{2+} concentration in the range from 0.316 to 31.6 μ M. This is a consequence of

Figure 8. Location of overexpressed PMCA isoforms in human embryonic kidney 293 (HEK293) cells and effects of $\text{A}\beta$ and cholesterol on their Ca^{2+} -ATPase activity. A) HEK293 cells transfected with the cDNA of PMCA isoforms 2b (green), 3b (green), and 4b (red) were incubated with the corresponding specific PMCA antibodies, as described in Materials and Methods. As shown, each isoform is found in the plasma membrane of the cell. Nuclei (blue) were stained with DAPI. B) Microsomes (10 μ g) from HEK293 cells overexpressing specific isoforms of PMCA from human (h) and rat (r), were incubated for 2 min at 37°C with 2.5 μ M $\text{A}\beta$ 25-35, $\text{A}\beta$ 35-25, and $\text{A}\beta$ 1-40, in the absence or presence of 0.18 μ mol of cholesterol, in a final volume of 50 μ l. After dilution into the assay medium, PMCA activity was assayed by starting the reaction with 1 mM ATP. Data are means $\pm$ SD (bars) of 4 experiments from two different preparations. * P < 0.0001 *vs.* control.

the particular inhibition of Ca^{2+} transport activity at pCa 5.5 (3.16 μM) and a low efficiency of Ca^{2+} homeostasis.

The effect of A β on synaptosomal PMCA activity at pCa 5.5 was much more marked than that reported for total Ca^{2+} -ATPase activity (6), $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity (6, 14), and Cl^{-} -ATPase activity (39). Also, a protective effect of vitamin E on the inhibition of $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity by A β 25-35 has been shown (6), suggesting a role for reactive oxygen species (ROS) in these effects. We have also observed similar a protective effect (unpublished results), indicating that an oxidative modification of Ca^{2+} -ATPase by A β would also be responsible for PMCA inhibition.

Therapeutic strategies arise from understanding the molecular basis of AD and focus on the specific neurotoxic cascade suggested to be induced by amyloid. The observation reported here that A β inhibits PMCA activity suggests that this protein could be involved in the intracellular Ca^{2+} dysregulation observed in AD. Extensive evidence confirms a cause-effect relationship between altered Ca^{2+} homeostasis and neuronal dysfunction in AD. From the study reported here, it can be concluded that the toxic amyloid peptide interacts with PMCA, affecting its function as a high-affinity Ca^{2+} regulator. In particular, these data demonstrate a specific alteration in the functioning of PMCA with respect to the Ca^{2+} dependence of ATPase activity in AD patients as compared to control subjects, which may result or be a consequence of dysregulation in neuronal Ca^{2+} homeostasis in AD brains. In conclusion, these results suggest a potential role for the PMCA pump in AD. FJ

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