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Calmodulin antagonizes amyloid-β peptides-mediated inhibition of brain plasma membrane Ca^{2+}-ATPase

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ABSTRACT

The synaptosomal plasma membrane Ca^{2+}-ATPase (PMCA) plays an essential role in regulating intracellular Ca^{2+} concentration in brain. We have recently found that PMCA is the only Ca^{2+} pump in brain which is inhibited by amyloid-β peptide (Aβ), a neurotoxic peptide implicated in the pathology of Alzheimer's disease (AD) [1], but the mechanism of inhibition is lacking. In the present study we have characterized the inhibition of PMCA by Aβ. Results from kinetic assays indicate that Aβ aggregates are more potent inhibitors of PMCA activity than monomers. The inhibitory effect of Aβ could be blocked by pretreating the purified protein with Ca^{2+}-calmodulin, the main endogenous activator of PMCA, and the activity of truncated PMCA lacking the calmodulin binding domain was not affected by Aβ. Dot-overlay experiments indicated a physical association of Aβ with PMCA and also with calmodulin. Thus, calmodulin could protect PMCA from inhibition by Aβ by burying exposed sites on PMCA, making them inaccessible to Aβ, and also by direct binding to the peptide. These results suggest a protective role of calmodulin against neuronal Ca^{2+} dysregulation by PMCA inhibition induced by Aβ.

1. Introduction

The plasma membrane Ca^{2+}-ATPases (PMCAs) are P-type ATPases with very high affinity for Ca^{2+}, transporting Ca^{2+}out of the cell coupled to the hydrolysis of ATP. PMCA proteins are encoded by four genes (*ATP2B1–4*), with more than 20 variants generated by alternative splicing [2]. These pumps are predicted to contain 10 transmembrane α-helices with two major cytosolic loops involved in PMCA function and regulation. They also contain a C-terminal tail, missing in intracellular Ca^{2+} pumps, that plays a key role in regulation of PMCA activity [3–5]. Thus, this tail is involved in protein autoinhibition, binds to calmodulin, acidic phospholipids and ethanol, and provides sites for modification by phosphorylation. A strong interaction of the C-terminal tail with the transduction and catalytic domains of PMCA results in an autoinhibitory conformation by burying the catalytic core of the ATPase [6–8]. Calmodulin, the ubiquitous intracellular Ca^{2+} sensor, binds to a calmodulin-binding domain located in the C-terminal tail, and removes PMCA autoinhibition by changing the pump conformation and stimulating activity. The high affinity of PMCA for calmodulin has been widely used to purify PMCA from a variety of tissues, including brain [9]. The purified protein is obtained in a delipidated, inactive form but can be partially or fully activated by reconstitution in phosphatidylcholine (PC) or phosphatidylserine (PS), respectively, as previously described [9]. It has been shown that calmodulin can fully activate the PC-reconstituted PMCA in the presence of Ca^{2+}, but that it does not affect the activity of the PS-reconstituted PMCA. Besides, the removal of the autoinhibitory domain by proteolysis or mutagenesis drives the protein to maximal activity [10–12].

PMCAs are important in Ca^{2+} homeostasis and signal transduction in the nervous system (reviewed in [13]) where Ca^{2+} dyshomeostasis is a critical feature in many neurodegenerative diseases. In the Ca^{2+} hypothesis of Alzheimer's disease (AD), disruption of Ca^{2+} signaling is a key factor in neuronal dysfunction [14,15] and we have recently presented evidence for an impairment of PMCA activity in AD linked to an inhibitory effect of the neurotoxic amyloid-β peptide (Aβ) [1]. However, a clear understanding of the mechanism of inhibition is lacking. On the other hand it has been shown that calmodulin content in AD affected brains was significantly reduced (66% of that found in brains of patients who died of a dementia process other than AD and in normal control brains [16]). Taking into account these considerations, in this work, we have characterized the functional effect of Aβ on PMCA and its relationship with calmodulin. We analyzed the effect of Aβ length and aggregation on PMCA inhibition, and described a protective role of calmodulin on Aβ-mediated PMCA inhibition. Thus, calmodulin may be an important mediator of Ca^{2+} dysregulation in AD due to its interaction with both Aβ and with PMCA.

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; PBS, Phosphate buffered saline; PC, Phosphatidylcholine; PMCA, Plasma membrane Ca^{2+}-ATPase; TBS, Tris buffered saline

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2. Material and methods

2.1. Materials

Bovine testes calmodulin, phosphatidylcholine (PC) type XI-E from egg yolk, calmodulin-agarose, chymotrypsin and anti-βtubulin antibody were obtained from Sigma. Calpain (μ-calpain) and biotinylated-calmodulin were from Calbiochem. Peptides Aβ25-35, and Aβ1-40 were obtained from AnaSpec and Aβ1-42 and scrambled Aβ42-1 were synthesized by StabVida.

2.2. Preparation of fresh and aggregated Aβ peptides

Aβ25-35 was dissolved in distilled water to make a 2 mg/ml stock solution. Aβ1-40, Aβ1-42 and Aβ42-1 were dissolved in 70 μl of 1% NH_4OH to give a clear solution, and then diluted with 100 mM Hepes/KOH (pH 7.4) to prepare a 4 mg/ml stock solution. Peptides were used immediately or were incubated at 37 °C to prepare aggregates. An optimal time of 2 h incubation was typically used (and referred as "aged" peptide).

2.3. Preparation of purified plasma membrane Ca^{2+}-ATPase from pig cerebrum

The pig brain plasma membrane Ca^{2+}-ATPase (PMCA) was purified as described by [9]. Briefly, fresh pig brain from adult pig (5 months-old), was homogenized in 10 volumes of 10 mM Hepes/KOH (pH 7.4), 0.32 M sucrose, 0.5 mM $MgSO_4$, 0.1 mM phenylmethylsulfonylfluoride and 2 mM 2-mercaptoethanol. After two centrifugations at low and high speed, respectively, synaptosomes were obtained by discontinuous 20–40% (w/v) sucrose gradient centrifugation and lysed in a hypotonic medium. Synaptosomal plasma membrane vesicles were obtained by centrifugation of the pellet and were solubilized in a medium containing 20 mM Hepes/KOH (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, 15% glycerol, 2 mM 2-mercaptoethanol, and 0.6% (w/v) Triton X-100. The solubilized fraction was applied to a calmodulin-agarose column and PMCA was eluted with a buffer containing 15% glycerol, 0.06% (w/v) Triton X-100 and 2 mM EDTA instead of calcium. Samples were stored at −80 °C until use.

The protein content was evaluated by the Bradford method [17], using bovine serum albumin as a protein standard.

2.4. Ca^{2+}-ATPase activity

The activity of purified PMCA from pig brain was measured with a coupled enzyme assay, as described in [9], using 2.5 μg of delipidated protein reconstituted with PC (13.3 μg). The effects of Aβ and/or pretreatment with calmodulin were assayed by incubating PMCA for 3 or 10 min at 25 °C with Aβ and/or calmodulin, in a final volume of 25 μl. The mixture was then diluted directly into the assay medium, containing 50 mM Hepes/KOH (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 5 mM Na_3N, 3.16 μM free Ca^{2+} (pCa 5.5), 0.22 mM NADH, 0.42 mM phosphoenolpyruvate, 10 IU pyruvate kinase, and 28 IU lactate dehydrogenase, in 1 ml final volume. The activity was measured after triggering the reaction with 1 mM ATP and, when indicated, after addition of 200 nM calmodulin to the reaction medium. Thus, results will be referred to calmodulin and Aβ final concentrations in 1 ml activity assay.

The enzymatic activity of overexpressed PMCA from COS-7 cells was measured in 10 μg of membrane vesicles and the effects of Aβ and/or pretreatment with calmodulin were assayed by incubating membranes as above, with 30 μM peptide and/or 400 nM calmodulin, respectively. The mixture was added to 1 ml of standard assay medium which also contained 0.01% saponin (to disrupt both the plasma and intracellular membranes, allowing the access of substrates to all protein molecules in membrane vesicles). The reaction was started with 1 mM ATP, and further activation by calmodulin was checked by adding 400 nM calmodulin to the medium.

2.5. Dot-blot overlay binding assay

Aβ peptides or purified PMCA were loaded on nitrocellulose membrane by using a Bio-Dot® Microfiltration Apparatus (BioRad). The membrane was then blocked with PBS-1% Tween 20 containing 2% (w/v) low-fat milk for 1 h at RT and incubated with 0.3 μg/μl biotinylated PMCA in PBS-0.05% Tween 20 (PBS-T) or with 0.5 μg/μl biotinylated calmodulin (Calbiochem) in PBS-T in the absence or presence of 0.5 mM $CaCl_2$ for 2 h at 37 °C. After several washing steps with PBS-T to remove unbound biotinylated protein, the membrane was incubated with ExtrAvidin-Peroxidase (1:2000, Sigma) in PBS-T for 1 h at RT. After one wash with PBS-T and two washes with PBS, the membrane was developed with ECL substrate and spots were visualized with a Chemidoc™ XRS + Imaging System (BioRad), and quantified with Image Lab™ software 3.0 (BioRad).

2.6. PMCA biotinylation

A 38:1 molar ratio of biotin to PMCA (equivalent to 0.33 mol of biotin per lysine) was used. Briefly, a 0.1 mg/ml stock solution of Sulfo-NHS biotin (ProteoChem) was prepared in 50 mM sodium bicarbonate (pH 8.0) buffer immediately prior to use. Then 2.7 μg of biotin was mixed with 22.5 μg of PMCA and incubated for 2 h on ice. Free biotin was removed through a 50 kDa Cut-off microcone centrifuge filter (Millipore).

2.7. Negative staining and transmission electron microscopy

Aβ1-40 and 1–42, and the corresponding Aβ 40–1 and 42–1 scrambles were diluted to 188 μM in 100 mM Hepes/KOH (pH 7.4). Aβ fibrillogenesis was induced by incubation at 37 °C for 30 min, 2 h and 3 days. For negative staining, carbon-coated grids were floated on the peptide solutions for 5 min, dried and stained with 2% uranyl acetate for 1 min. After washing in milliQ water, grids were air dried and visualized by transmission electron microscopy (JEM1010 Jeol).

2.8. Digestion with proteases

Purified pig brain PMCA (2.5 μg) was incubated with 0.025 μg chymotrypsin or 0.5 μg calpain in buffer containing 100 mM KCl, 3.16 μM free Ca^{2+}, 25 mM Hepes/KOH (pH 7.4), in 25 μl final volume, on ice. Proteolysis was stopped after the indicated time by addition of the protease inhibitor phenylmethyldulfonyl fluoride (PMSF) to a final concentration of 2 mM. Samples were then incubated in the presence or absence of 30 μM 2 h-aged Aβ1-42 for 3 min at 25 °C for activity assays.

Alternatively, PMCA digestion was stopped by precipitation with 8% trichloroacetic acid. Samples were centrifuged at 15,000 ×g for 15 min at 4 °C and pellets were washed with 200 μl of 10 mM Hepes/KOH (pH 7.4) and solubilised in 20 μl Laemmli's buffer [18]. The samples were then subjected to electrophoretic analysis on 6.5% gels followed by staining with Coomasie Blue.

2.9. Construction of truncated hPMCA4b and expression in COS-7 cells

hPMCA4b cDNA cloned in pMM2 was kindly provided by Dr. E. E. Strehler (Mayo Clinic, Rochester, MN, USA). Using this vector as template, a site-directed mutagenesis PCR was carried out using 5′-CATGCTGAGATGGAGTGACGCCGAGGCCAGATCC-3′ and 5′- GGATCTGGCCTCGGCGTCACTCCATCTCAGCATG-3′. The reverse primer contains a stop codon (underlined) to truncate the protein immediately after

leucine 1086 (hPMCA4b-L1086*). The mutant lacked 120 amino acids at the C-terminus, including the 28-residue calmodulin-binding inhibitory domain. The construct was checked by sequencing. The wild-type and mutant cDNAs cloned in pMM2 were used for transfection in COS-7 cells using GenJuice transfection reagent (Novagen). After incubation for 60 h at 37 °C and 5% CO_2, membrane vesicles were prepared from cells harvested from the plates, as described by [19] and used for ATPase activity assays.

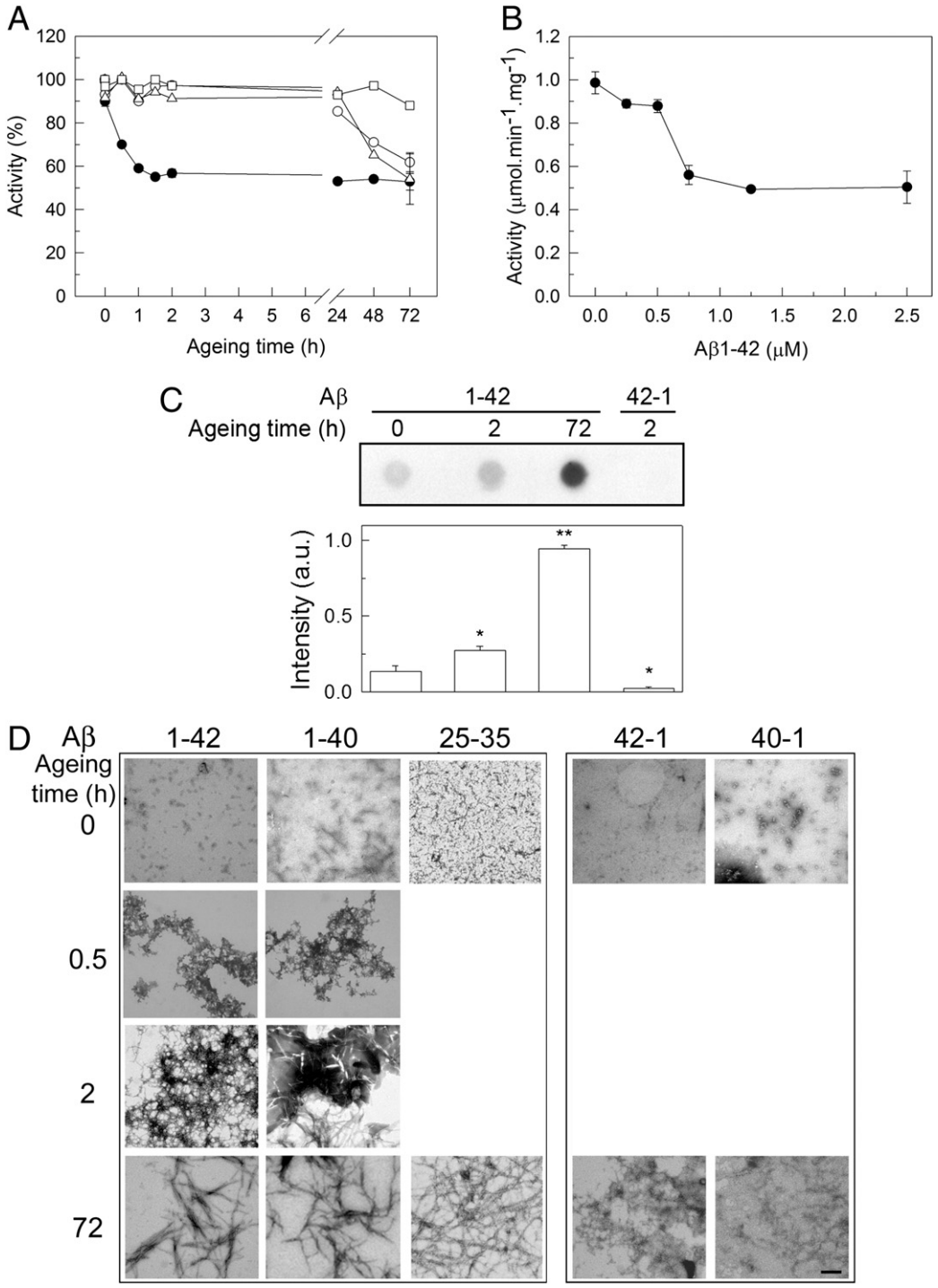


Fig. 1. Ageing of Aβ peptides and their effects on PMCA activity. (A) Effect of Aβ ageing on Ca^{2+}-ATPase activity. Pig cerebrum purified PMCA (2.5 μg) reconstituted in PC was incubated for 2 min at 37 °C in a volume of 25 μl, with 30 μM of Aβ1-42 (●), Aβ1-40 (△), Aβ25-35 (○), or Aβ42-1 (□) peptides, freshly prepared or after incubation at 37 °C for the indicated times. Then incubates were diluted to 1 ml with the assay medium, giving a final Aβ concentration of 0.75 μM. Ca^{2+}-ATPase activity was then measured as described in the Methods section. (B) Effect of Aβ concentration on PMCA activity. 2.5 μg of PMCA reconstituted in PC was incubated in a volume of 25 μl, with increasing concentrations of 2 h-aged Aβ1-42 for 2 min. The sample was then transferred to 1 ml of assay medium, giving the indicated concentrations of Aβ. Ca^{2+}-ATPase activity was measured as described in the Methods section. (C) Aβ-PMCA binding by Dot-blot overlay assay. Aβ1-42 (0.5 μg) freshly prepared, 2 h-aged and 3 days-aged, and 2 h-aged scrambled Aβ42-1 (0.5 μg) as a negative control, were spotted on Dot-blot nitrocellulose membrane and incubated with biotinylated PMCA as described in the Methods Section. The relative signal intensity of each spot was calculated and represented as mean ± SD from four different experiments (*, $p \leq 0.05$; **, $p \leq 0.005$, respect to 0 h ageing). (D) Negative staining electron microscopy analysis of Aβ peptides and the scrambled sequences before and during ageing at 37 °C. Different aggregation stages are shown. All electron micrographs were recorded at a magnification of × 100 000. Scale bar: 0.2 μm.

2.10. Statistical analysis

Significant differences were determined by an unpaired Student *t*-test using the SigmaPlot v10 software (SPSS Inc, Chicago, IL). A $p \leq 0.05$ value was considered statistically significant.

3. Results

3.1. Inhibition of PMCA activity by Aβ peptide

In vitro aggregation of Aβ was induced by incubation at 37 °C and was followed for 3 days. Fig. 1A shows that Aβ inhibits the Ca^{2+}-ATPase activity of purified PMCA reconstituted in PC, the degree of inhibition increasing with Aβ length and aggregation. Thus, the highest PMCA inhibition was obtained with 0.75 μM Aβ1-42, a concentration which inhibits ATPase activity by 50%, after 2 h ageing (Fig. 1B). Scrambled peptide Aβ42-1 did not inhibit enzyme activity (Fig. 1A). Similar results were found after reconstitution in PS (results not shown). Dot blot assays (Fig. 1C), using Aβ1-42 or the scrambled peptide with biotinylated PMCA were performed to prove Aβ-PMCA binding. As shown, binding increased with peptide aggregation. The inverted amino acid sequence of the 2 h-aged scrambled peptide did not bind to PMCA (Fig. 1C). Negative-staining technique and electron microscopy were also performed to follow Aβ aggregation (Fig. 1D). This revealed filament structures for all peptides after 3 days incubation, with Aβ1-42 aggregates forming an amorphous structure after 2 h incubation. The scrambled peptides did not form fibrils.

Considering that calmodulin is the main activator of PMCA pump in the presence of PC, as described in the Introduction, we analyzed effects of calmodulin on PMCA reconstituted in PC. First (Fig. 2), it was observed that inhibition of PMCA activity by 0.75 μM 2 h-aged Aβ1-42 was incubation-time dependent. Second, 2-fold activation of the protein was observed on addition of calmodulin to the assay medium (at saturating concentrations of calmodulin) at all times of incubation with peptide. Both curves fit to an exponential decay curve, showing that Aβ produced similar rates of inhibition in basal and calmodulin-activated PMCA activities, with 50% inhibition in both cases after 1.3 min incubation.

3.2. Protection of PMCA activity from Aβ inhibition

The effect of Aβ on PMCA was also analyzed after previous incubation of the protein with increasing concentrations of calmodulin. As seen in Fig. 3A, 0.75 μM Aβ1-42 caused a 50% decrease of PMCA basal activity but this level of inhibition was reduced by pre-treatment of PMCA with calmodulin. Significant protection of PMCA activity against inhibition by Aβ was observed at calmodulin concentrations above 0.35 nM, corresponding to a 1/2140 molar ratio of calmodulin/Aβ. The effect of the peptide was completely blocked at concentrations of calmodulin above 5 nM, equivalent to a molar ratio of 1/150. These results show that PMCA has a much higher affinity for calmodulin than for Aβ. The protective effect of calmodulin was only seen when calmodulin was activated by Ca^{2+}; in the presence of EGTA the apo-calmodulin was unable to protect PMCA from its inhibition by Aβ (Fig. 3B).

Similar experiments were performed using fixed concentrations of calmodulin and increasing concentration of peptide (Fig. 4). Data agree with those in Fig. 3A, showing that 5 nM calmodulin prevent the inhibitory effect of 0.75 μM Aβ on PMCA activity. The results reveal that Aβ affinity for PMCA decreased when the protein was pre-treated with calmodulin. Thus, the half maximal inhibitory concentration (IC_{50}) of Aβ increased from 0.62 μM in the absence of calmodulin, to 0.65, 0.68 and 0.9 μM, in the presence of 0.35, 1.25 and 5 nM calmodulin, respectively. However, high concentrations of peptide suppress the protective effect of calmodulin (insert in Fig. 4).

These results raise the possibility that the calmodulin-binding domain of PMCA may be directly involved in its interaction with Aβ. To analyze this hypothesis, PMCA was digested with chymotrypsin or calpain, two proteases that cut the protein preferentially at the carboxyl terminus containing the calmodulin-binding domain [11,20,21]. Functional assays were then carried out with the resulting fragments to explore the sensitivity of digested PMCA to Aβ. The time course of

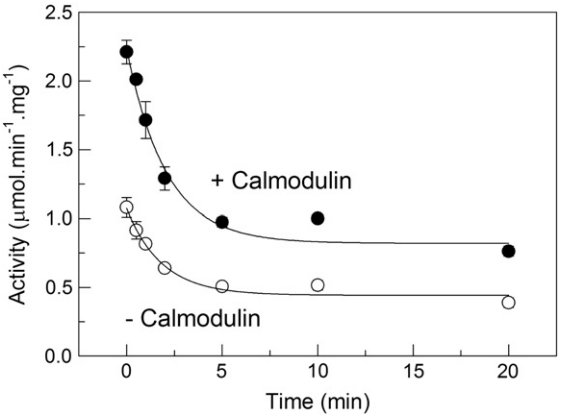


Fig. 2. Kinetics of PMCA inhibition by Aβ. 2.5 μg of PMCA reconstituted in PC were incubated with 30 μM 2 h-aged Aβ1-42 for the indicated times at 25 °C in a volume of 25 μl, and then added to the reaction mixture (1 ml total volume). Ca^{2+}-ATPase activity was measured as described in the Methods section, in the absence (○) or presence of 200 nM calmodulin (●). Data are mean ± SD values from three experiments performed in duplicate and with three different preparations.

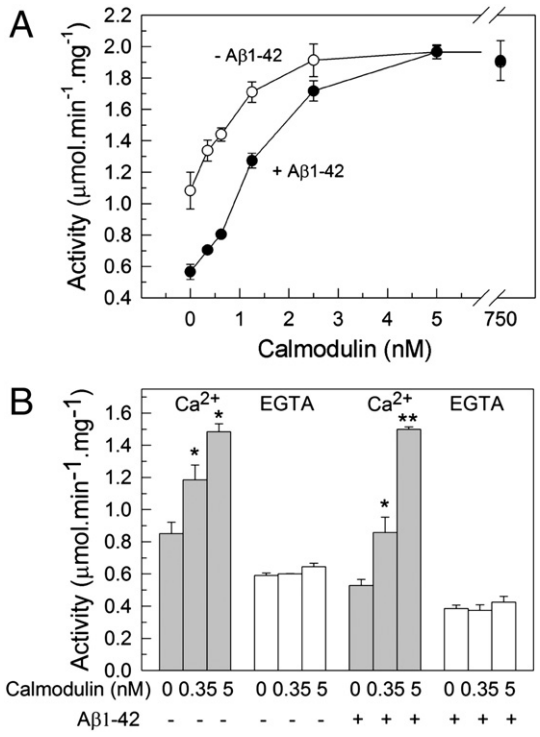


Fig. 3. Protective effect of calmodulin on PMCA inhibition by Aβ. (A) 2.5 μg of PMCA reconstituted in PC were incubated for 10 min at 25 °C, in a volume of 25 μl, with increasing concentrations of calmodulin in the presence of 3.16 μM free Ca^{2+}. For the samples containing Aβ (●), 30 μM 2 h-aged Aβ1-42 (1 μl) was then added and incubated for 3 min. Finally, incubates were diluted to 1 ml with assay medium, giving 0.75 μM Aβ, and the indicated calmodulin final concentrations. The Ca^{2+}-ATPase activity was measured as described in the Method section. (B) Assays were performed as in A, using 14 and 200 nM calmodulin (giving final concentrations of 0.35 and 5 nM in 1 ml final volume) in the presence of 3.16 μM free Ca^{2+} (grey bars) or 2 mM EGTA (white bars). Data represent mean ± SD values from three experiments performed in duplicate and with three different preparations (*, $p \leq 0.05$; **, $p \leq 0.005$).

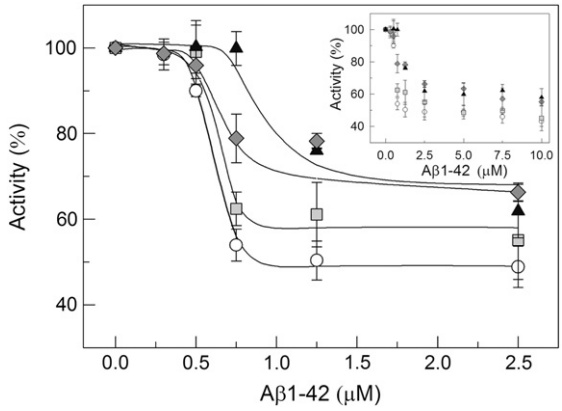


Fig. 4. Concentration-dependence of PMCA inhibition by Aβ1-42 after pretreatment with calmodulin. 2.5 μg PMCA were reconstituted in PC and incubated without (○) and with 14 nM (□), 50 nM (◇) and 200 nM (△) calmodulin for 10 min at 25 °C, in the presence of 3.16 μM free Ca^{2+} in 25 μl total volume. The mixture was then incubated with increasing concentrations of 2 h-aged Aβ1-42 (1 μl) for 3 min followed by dilution to 1 ml with assay medium, giving the indicated Aβ final concentrations. Ca^{2+}-ATPase activity was measured after addition of 1 mM ATP. 100% activities correspond to 0.941 ± 0.12, 1.29 ± 0.07, 1.53 ± 0.12, and 1.78 ± 0.17 μmol min^{-1} mg^{-1}, in the absence or presence of 0.35 nM, 1.25 nM and 5 nM calmodulin (in 1 ml final volume), respectively. Data represent mean ± SD values from four experiments performed in duplicate and with three different preparations. The insert shows effects of Aβ over a larger concentration range.

PMCA limited digestion at 25 °C showed a gain of catalytic activity in the absence of calmodulin (Fig. 5A), reaching V_{max} after 20 min digestion; addition of calmodulin increased the activity of intact PMCA and PMCA digested for less than 20 min, to the maximum rate. These results show that the calmodulin binding site of PMCA was being gradually removed by both proteases. Inhibition of ATPase activity by Aβ was also seen to decrease with increasing time of digestion, with minimal inhibition being seen after ca. 20 min digestion (Fig. 5A). The extent of proteolysis was followed by SDS-PAGE (Fig. 5B), showing degradation of the 140 kDa-native protein to a main 124 kDa fragment after chymotrypsin and calpain treatments, which corresponds to PMCA products lacking their calmodulin-binding domain, as a consequence of 20 min incubation with proteases [22].

We have previously shown that hPMCA4b overexpressed in COS cells can be specifically inhibited by Aβ [1]. In order to analyze the putative involvement of the C-terminal calmodulin-binding domain of PMCA in Aβ binding, we used the hPMCA4b-L1086* mutant lacking the C-terminal calmodulin-binding domain. The mutant was prepared as described in the Methods and its kinetics properties with respect to calmodulin and Aβ were compared to those of the wild type (Fig. 6). The basal activity of wild-type PMCA4b was inhibited by Aβ, and also was further activated by addition of calmodulin to the assay medium (see white bars). It could also be completely activated when it was preincubated with calmodulin or after removing its autoinhibitory calmoduling-binding domain with calpain. The activated forms

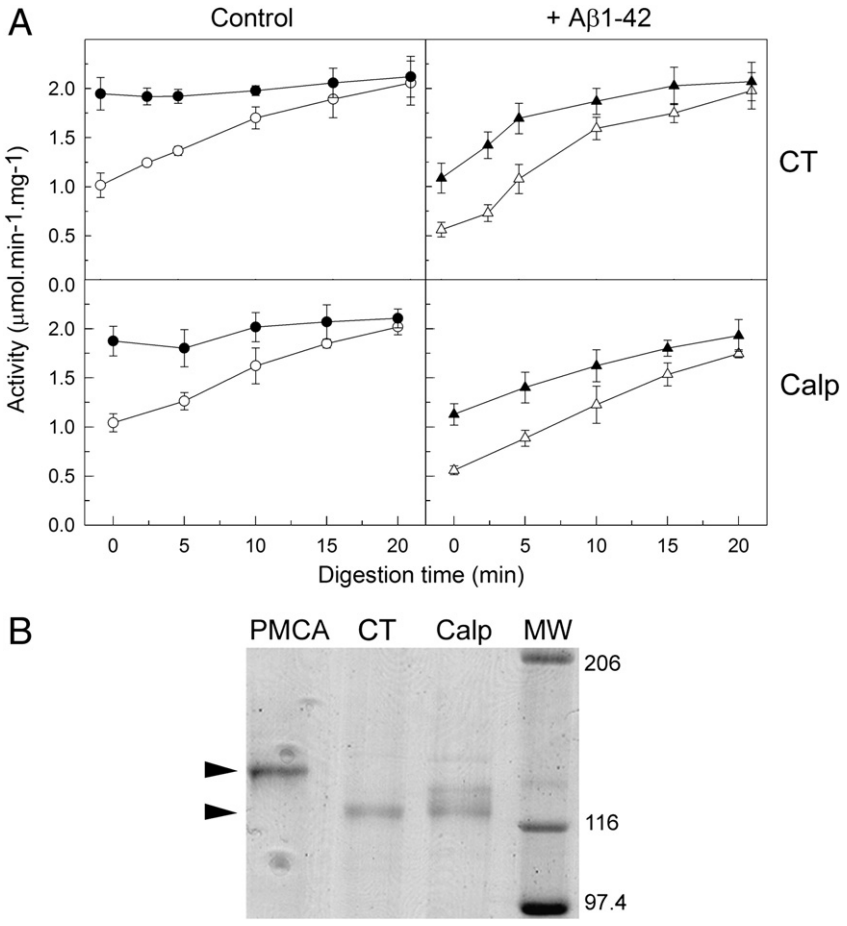


Fig. 5. Effects of calmodulin and Aβ on Ca^{2+}-ATPase activity of proteolyzed PMCA. (A) 2.5 μg of PMCA were digested, as described in the Methods, with 0.025 μg chymotrypsin (CT) or 0.5 μg calpain (Calp) for the indicated times, in a volume of 25 μl, on ice. Proteolysis was stopped by addition of 2 mM PMSF and immediately afterwards, the samples were incubated without (○,●) and with (△,▲) 30 μM 2 h-aged Aβ1-42 for 10 min at 37 °C. Incubates were added to assay medium (1 ml) and Ca^{2+}-ATPase activity was measured in the absence (open symbols) or presence of 200 nM calmodulin (filled symbols). Data are mean ± SD values from three experiments performed in duplicate and with three different preparations. (B) Electrophoretic analysis of intact PMCA (lane 1) and fragments generated by 20 min digestion with chymotrypsin (lane 2) and calpain (lane 3).

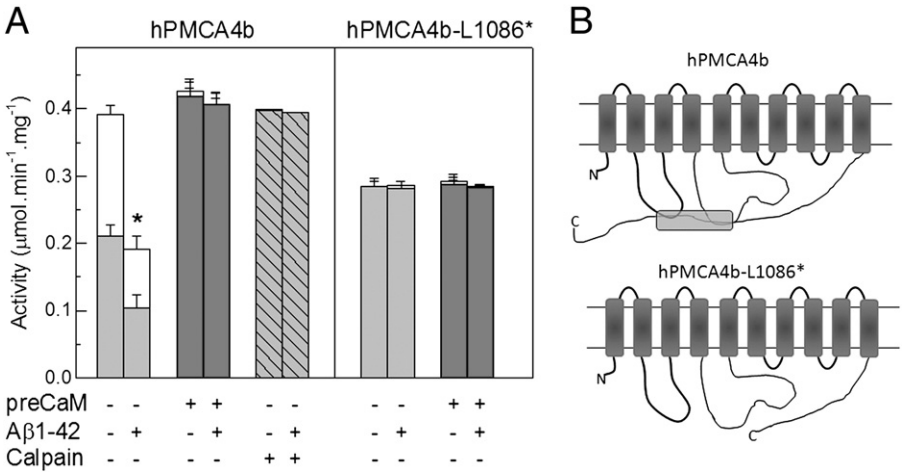


Fig. 6. Effects of preincubation with calmodulin and Aβ peptide on Ca^{2+}-ATPase activity of full-length PMCA4b isoform and a truncated isoform lacking the calmodulin binding domain. (A) Intact membrane vesicles (10 μg) from COS-7 cells overexpressing full-length hPMCA4b or the truncated mutant hPMCA4b-L1086* were incubated as indicated without or with 400 nM calmodulin (preCaM) plus 3.16 free Ca^{2+} for 10 min at 25 °C in a volume of 25 μl, and then 30 μM aged Aβ1-42 (1 μl) was added and further incubated for 3 min at 37 °C. Membrane vesicles (10 μg) were also digested with 2 μg of calpain in the presence of 3.16 free Ca^{2+} during 20 min on ice (striped bars) and afterwards incubated in the absence or presence of Aβ1-42 as above. Incubates were added to assay medium (1 ml) and PMCA activity was measured as described in the Methods section also in the absence of calmodulin or after addition of calmodulin up to 400 nM (white bars). Data represent mean ± SD values from four experiments performed in duplicate and with three different preparations (*, $p \leq 0.005$). (B) Schematic representation of the membrane topography of the full-length and truncated PMCA4b.

were not further inhibited by Aβ. hPMCA4b-L1086* had a higher basal activity than the wild-type enzyme, due to the loss of its autoinhibitory calmoduling-binding domain and could not be activated by addition of calmodulin or be inhibited by Aβ. Further, incubation of the mutant with calmodulin did not increase its activity, although calmodulin did increase the activity of the wild-type PMCA. These results strongly support those revealed by proteolysis experiments and suggest that Aβ may bind to a site close to or at the calmodulin binding site which is lacking in both digested PMCA and truncated mutant.

An alternative hypothesis to explain the protective effect of calmodulin on PMCA inhibition by Aβ, is that calmodulin could also bind to Aβ, reducing or even blocking the binding of Aβ to PMCA. To test for a possible interaction between Aβ and calmodulin we performed binding studies by Dot-blot overlay assays, using Aβ1-42 or the scrambled peptide and biotinylated calmodulin (Fig. 7). Peroxidase-conjugated ExtrAvidin staining showed that calmodulin did indeed interact with Aβ1-42. PMCA-calmodulin binding was used as a positive control. An increase in dot intensity was seen when Aβ and PMCA were mixed before being spotted onto the membrane. This increase was due to calmodulin binding to both Aβ and PMCA. Additional test were carry out by exploring the sensitivity of

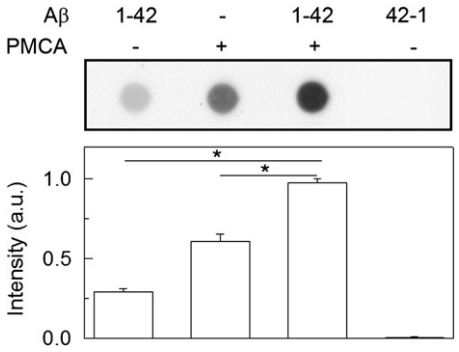


Fig. 7. Dot-blot overlay assay showing Aβ-calmodulin interaction. Two μg of 2 h-aged Aβ1-42, and 0.2 μg of purified PMCA (as positive control) were spotted on nitrocellulose membranes, separately and after mixing for 10 min. The membrane was incubated with of biotinylated calmodulin, and treated as indicated in the Methods. Aβ42-1 was spotted as a negative control. Dot intensities were represented as mean ± SD of four different experiments (*, $p \leq 0.005$).

intrinsic fluorescence in Aβ, calmodulin and PMCA to conformational changes induced by Aβ binding. Emission fluorescence spectra upon exciting at 280 nm (which excites only Tyr residues) of each protein and peptide were recorded independently and after pre-mixing the peptide with PMCA or/and calmodulin (Supplementary Fig. S1). It was found that when the Aβ peptide was preincubated with PMCA or calmodulin the maximum of fluorescence reached was lower than the combined fluorescence (calculated as the sum of fluorescence peak values of each molecule). This indicated that Tyr residues on Aβ and calmodulin were perturbed by the close interaction when they were incubated together or/and with PMCA.

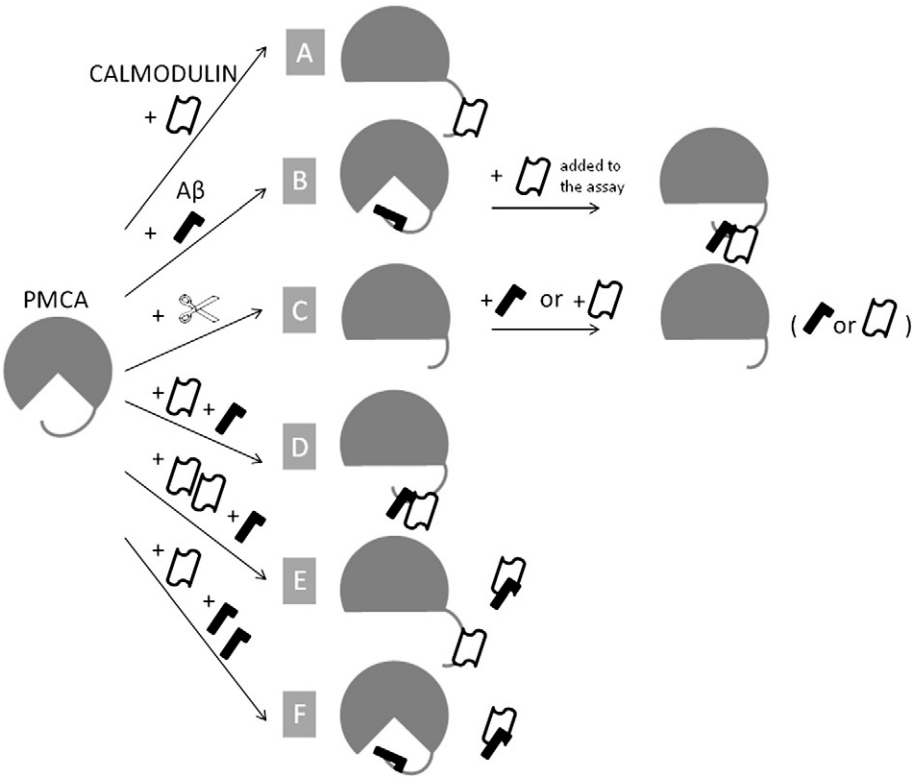
4. Discussion

The Aβ peptide, a major component of senile plaques in patients with AD, is believed to facilitate the progressive neurodegeneration of this disease. Early studies have shown that Aβ1-42 is more insoluble than the more abundant Aβ1-40, and that it is the most abundant Aβ species found in senile plaques [23]. The shorter Aβ25-35 peptide is not found *in vivo*, but it represents the main toxic part of the full length Aβ peptides [24]. In this work, we show that synaptosomal PMCA is inhibited by neurotoxic Aβ peptides in a length and aggregation-dependence manner. The longest Aβ1-42 peptide produced the highest inhibition on PMCA activity, increasing this effect with peptide aggregation (Fig. 1). This effect is related to the capacity of this peptide to form more toxic fibrils and faster than Aβ1-40 [25]. Besides, we found that binding of Aβ1-42 to PMCA is not proportional to inhibition of PMCA activity. The maximum inhibitory effect was obtained after reaching a certain oligomerization stage (as the one formed at 2 h ageing) and not higher inhibition was found with further aggregation stages. Thus, binding of 2 h- or 72 h-aged Aβ1-42 to PMCA may produce a similar conformational change that hinder Ca^{2+} binding sites and then reduces PMCA activity. This fact could be supported by recent Aβ-structural studies showing that residues 1–10 are unstructured in oligomers and fibrils while the other amino acids adopt a β-turn-β folding [26,27]. Thus, unstructured N-terminal sites of Aβ could be involved in PMCA inhibition, becoming inhibition not proportional to binding. Impairment of PMCA activity by Aβ peptides appears to be important in amyloid toxicity, because it might result in cytoplasmic Ca^{2+} dysregulation and explain the different Ca^{2+}-dependencies of PMCA activity observed in normal

and AD brains [1]. Here we show that effects of Aβ on PMCA are modulated by calmodulin and suggest a mechanism for the observed effects that is summarized in Scheme I.

PMCA contains a calmodulin-binding domain in its C-terminal tail that, in an enriched neutral lipidic environment, acts as an endogenous inhibitor [28] by establishing intramolecular contacts with two cytosolic loops of the pump, which most probably prevent the access of substrates to the active site [8]. Ca^{2+}-calmodulin activates the pump by removing the C-terminal tail from its intramolecular binding sites [3], as shown in Scheme IA. Interestingly, the inhibitory effect of Aβ on PMCA shown in this work does not impede further calmodulin binding to the pump and its activation (Fig. 2). This suggests that Aβ binding to PMCA occurs in a protein region that interferes with its catalytic domain and slows the reaction cycle, thus inhibiting PMCA activity, but without altering calmodulin binding and subsequent activation (Scheme IB). This region may be located at the C-terminal tail of PMCA, in or close to its calmodulin-binding domain. This is suggested by the results obtained by limited proteolysis (Fig. 5). It is well established that controlled proteolysis of the pump produces PMCA fragments lacking the autoinhibitory calmodulin binding domain and which are therefore fully active and cannot be further stimulated by calmodulin [12,20,21,29–31]. In agreement, the PMCA fragments obtained here by limited proteolysis with chymotrypsin and calpain showed higher activity than the native protein (Fig. 5). This increase in activity was linked to a decrease in both its activation by calmodulin and its inhibition by Aβ. After 20 min treatment with either protease, the PMCA protein lost completely its calmodulin-binding domain and was insensitive to the effects of calmodulin and Aβ. Similar results were obtained with the PMCA4b-L1086* truncated mutant (Fig. 6). Thus, truncation of the protein by proteolysis or by site-directed mutagenesis prevents the inhibitory effect of Aβ (and activation by calmodulin) on PMCA activity (Scheme IC).

Interestingly, pretreatment of PMCA with low concentrations of calmodulin reduces the inhibitory effect of Aβ and blocks it totally at high concentrations of calmodulin (Figs. 3A and 4). This suggests that high affinity binding of calmodulin to PMCA produces a conformational change in PMCA, masking its binding site(s) for Aβ. The protective effect of calmodulin is concentration-dependent because calmodulin could not protect PMCA from its inhibition by high concentrations of Aβ (Fig. 4). These results suggest that the peptide could also bind to calmodulin. In fact, Dot-blot overlay assays using biotinylated calmodulin (Fig. 7) and measurements of intrinsic fluorescence (Fig. S1) point out a calmodulin-Aβ interaction. Then, it is possible that the successive preincubation of PMCA with calmodulin and Aβ resulted in the peptide binding to PMCA and/or calmodulin, depending on the Aβ/calmodulin molar ratio (Scheme ID-F). Thus, binding of Aβ to calmodulin would result in lowering the concentration of free Aβ available to bind to PMCA. However, high concentration of peptide binds to both calmodulin and PMCA, thus preventing further binding of calmodulin to PMCA. Interestingly, it has been reported that PMCA can be activated not only by full length Ca^{2+}/calmodulin but also by the C-terminal lobe of calmodulin alone [32–34], leaving the N-terminal lobe of calmodulin free for other interactions. Thus Aβ could also bind to its N-terminus inducing a conformational change in calmodulin, thus preventing calmodulin binding to PMCA. Analysis of the Aβ1-42 sequence reveals a 1-8-14 motif (DAEFRHDSGYEVHHQK**L**VFFAED**V**GSNKG**A**IIGLMVGGVVIA) characteristic of calmodulin targets [32]. In fact, it has been proposed a



Scheme I. Calmodulin and putative Aβ binding to PMCA based in activity assays. (A) shows binding of calmodulin to the C-terminal tail of PMCA leading to reduced interaction of the tail with the catalytic core of PMCA and the formation of an active conformation. (B) shows binding of Aβ to the C-terminal tail of PMCA leading to loss of activity. Subsequent addition of calmodulin to the assay results in calmodulin binding to PMCA and activation, as resulted in Fig. 2. (C) shows the active fragment of truncated PMCA that loss the binding sites for calmodulin and Aβ. (D) PMCA preincubated with low concentrations of calmodulin and with Aβ. It shows binding of calmodulin and Aβ to PMCA with partial loss of activity. (E) represents PMCA preincubated with high concentrations of calmodulin and Aβ. It shows binding of calmodulin to PMCA leading to the active conformation of PMCA and binding of Aβ to free calmodulin, thus preventing the inhibitory effect of the peptide on PMCA activity. (F) PMCA preincubated with calmodulin and high concentrations of Aβ. The peptide binds to calmodulin and free peptide also binds to PMCA, resulting in inhibition of PMCA activity.

calmodulin connection with Alzheimer's disease (AD), based on the presence of calmodulin binding domains in several AD-related proteins, opening new ways to explore on this devastating disease [35].

Although identification of PMCA specific residues involved in Aβ binding is complicated by the absence of reliable tertiary structural characterization of the protein, it cannot be discarded that the inhibitory effects of Aβ follow from protein oxidation. First, Aβ itself has a free radical-generating capacity [36] and Aβ induces peroxide accumulation in cultured cells and antioxidants can protect against Aβ-induced toxicity [37,38]. Second, PMCA has been found to be highly susceptible to oxidative stress and its exposure to oxidants leads to structural changes that result in loss of activity [39]. Third, it has been reported that calmodulin binding to PMCA produces a conformational change that might bury some residues susceptible to oxidation [40] since the susceptibility of PMCA to the oxidant H_2O_2 is reduced by prior exposure to calmodulin [41]. Moreover, results from our lab show that antioxidants such as vitamin A or C completely protect PMCA activity from Aβ inhibition (unpublished results). Further studies are required to fully characterize the mechanism of inhibition of PMCA by Aβ.

Several studies have shown that a feature of aged brain is a reduction in calmodulin levels and/or calmodulin oxidation [42,43] as well as oxidative damage to PMCA [40,44]. Reduced concentrations of calmodulin has also been shown in an age-related neurodegenerative disorders such as AD [16]. This would result in a reduction or even a lack of protective effect of calmodulin on PMCA inhibition by the neurotoxic Aβ.

The finding that protective effect of calmodulin on PMCA inhibition by Aβ is dependent of peptide concentration involves a physiological relevance in AD, since Aβ distribution is not homogeneous in AD-affected brain; Aβ is highly concentrated in senile plaques that appear to be local focus of neurotoxicity [45]. We propose calmodulin as a new target in the study of AD, where it can play a protective role at early stages of the disease. At advanced stages of AD, Aβ goes on to form larger aggregates in plaques; then, high concentration of Aβ would suppress the physiological protection by calmodulin on Aβ-mediated PMCA inhibition, resulting in a decreased clearance of intracellular Ca^{2+}. Our work goes a step further, pointing out, by *in vitro* assays, the protective use of exogenous calmodulin to target the neurotoxic Aβ peptide, e.g. blocking the Aβ inhibitory effect on PMCA or catching free Aβ and then reducing its neurotoxic effect. This would result in restore the Ca^{2+} levels after Ca^{2+} dysfunction induced by Aβ in neurodegenerative disorders. This study could establish a basis to perform further *in vivo* studies with exogenous calmodulin in animal models of AD.

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