Ca\(^{2+}\)-independent binding and cellular expression profiles question a significant role of calmyrin in transduction of Ca\(^{2+}\)-signals to Alzheimer’s disease-related presenilin 2 in forebrain

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Abstract

The interaction between the EF-hand Ca\(^{2+}\)-binding protein calmyrin and presenilin 2 (PS2) has been suggested to play a role in Alzheimer’s disease (AD). We now report that calmyrin binds specifically endogenous PS2 and not PS1. However, binding appears to be Ca\(^{2+}\)-independent and calmyrin does not exhibit a Ca\(^{2+}\)-dependent translocation to intracellular membranes as demonstrated in a Ca\(^{2+}\)-myristoyl switch assay. Moreover, calmyrin is only present at very low levels in brain areas associated with the onset of AD. In rat, forebrain calmyrin is localized only in a subset of principal neurons, similarly as in human forebrain. Finally, subcellular fractionation demonstrates only a limited overlap of calmyrin and PS2 at neuronal membranes. We therefore conclude that calmyrin will not contribute significantly as a Ca\(^{2+}\)-sensor that transduces Ca\(^{2+}\)-signaling events to PS2 in forebrain.

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1. Introduction

The pathogenesis of AD is associated with loss of neurons in limbic and cortical regions of forebrain, characterized by extracellular deposits of insoluble \(\beta\)-amyloid peptide (A\(\beta\)) called senile plaques and intracellular aggregates composed mainly of the cytoskeletal tau protein known as neurofibrillary tangles. Another feature of the disease is dysregulation of intracellular Ca\(^{2+}\)-signaling events [1,2]. Accordingly, a variety of neuronal Ca\(^{2+}\)-binding proteins including calbindin, sorcin, calmyrin, and calp/kchip4 [3–6] have been identified that potentially can trigger altered Ca\(^{2+}\)-signaling pathways to key molecules for the onset of the disease. Such molecules include presenilins (PS1 and PS2), which are integral membrane protein components of \(\gamma\)-secretase complexes responsible for the generation of \(\beta\)-amyloid peptides as well as for the intramembranous proteolysis of other proteins [7]. Presenilins by themselves can regulate intracellular Ca\(^{2+}\)-concentrations. Part of this scheme is the idea that intracellular Ca\(^{2+}\)-levels will regulate these interactions and that a disturbance in Ca\(^{2+}\) homeostasis underline neurodegeneration [2]. Unfortunately, however, the molecular underpinnings for most of these interactions are not clear.

The Ca\(^{2+}\)-binding protein calmyrin, also termed CIB [8] or KIP [9] has been shown to interact with PS2 and it was suggested that this interaction might play a role in the pathogenesis of AD [5]. Along these lines, we have recently investigated the distribution of calmyrin in human forebrain during normal aging and in AD [10]. Calmyrin was found to be present in a subset of forebrain neurons and also accumulated in senile plaques, hallmarks of AD [10]. Immunostainings and Western blot analyses, however, indicated that calmyrin is not a particularly abundant neuronal protein in the brain regions affected in AD [10]. To learn more about the nature of its
potential role in the pathogenesis of AD, we investigated whether binding to PS2 is Ca\(^{2+}\)-dependent and tried to identify cellular and subcellular structures in the rat brain where the proposed interaction could happen in vivo.

2. Materials and methods

2.1. Antibody production

Recombinant calmyrin was generated as described previously [10]. Purified protein was used to generate two anti-calmyrin polyclonal antisera in rabbits (Innovagen, Sweden, and Biogenes, Germany). The antisera from Biogenes was affinity-purified using standard technology and employed for immunohistochemical analyses.

2.2. Calmyrin affinity chromatography

Recombinant calmyrin (10 mg) was covalently attached to cyanogen bromide activated Sepharose 4B (Fluka) according to the manufacturer’s instructions. Immortalized human lymphocytes were grown in RPMI supplemented with 10% FBS. Cells were homogenized in 20 mM Tris–HCl, pH 7.5, 1 mM 2-mercaptoethanol (2-ME), and protease inhibitor cocktail (Roche) with a Dounce homogenizer and centrifuged at 1000 × g for 15 min at 4 °C. The supernatant was homogenized again and centrifuged at 100,000 × g for 1 h at 4 °C. The resulting supernatant was centrifuged again at 100,000 × g for 1 h at 4 °C and both pellets were combined. This crude lyophilized membrane fraction was solubilized in buffer A (20 mM Tris–HCl, pH 7.5, 1% CHAPS, 0.1 mM CaCl\(_2\) or 2 mM EGTA, 1 mM 2-ME, Roche protease inhibitor cocktail), spun at 100,000 × g for 1 h at 4 °C and the supernatant was applied to the column equilibrated with the same buffer. Elution of proteins was monitored by continuous absorbance measurements at 280 nm. The flow-through, a fraction eluted with buffer A supplemented with 150 mM NaCl (buffer B), followed by the fraction eluted with the buffer B supplemented with 2 mM EGTA (only in the presence of 0.1 mM CaCl\(_2\)) and finally with buffer B supplemented with 1 M NaCl were concentrated (Vivaspin, Sigma), and analyzed by SDS-PAGE and immunoblotting.

2.3. Pull-down assay

HeLa cells were lysed in TBS, pH 7.5, 0.3% Triton X-100, complete inhibitor cocktail (Roche), and 2 mM EGTA or 0.1 mM CaCl\(_2\) for 1 h at 4 °C and centrifuged at 23,600 × g for 30 min. 500 μl of the supernatant containing ~0.5 mg total protein were incubated overnight at 4 °C with 4 μg of recombinant calmyrin covalently attached to Sepharose 4B according to manufacturer’s instruction (Fluka). After extensive washing with TBS, SDS-PAGE sample buffer was added to the matrix to a final amount of 60 μl. 20 μl of the pull-down fraction was subjected to Western blotting.

2.4. Transfection and coinmunoprecipitation

For transient transfection, the pCaMy EGFP N1 or pEGFP N1 plasmid (4 μg, Clontech) was transfected with Polyfect reagent (Qiagen) according to the manufacturer’s protocol into COS-7 cells seeded in 10 cm plates. Two days after transfection, cells were harvested in TBS and centrifuged at 600 × g at 4 °C for 5 min. The supernatant was discarded and the pellet was homogenized in the lysis buffer (TBS, pH 7.5, 0.2% Triton X-100, 2 mM EGTA, complete inhibitor cocktail (Roche)). The lysed cells were incubated on ice at 4 °C for 1 h and centrifuged at 11,500 × g at 4 °C for 30 min to remove cell debris. Aliquots of cell lysates were precleared with Sepharose A (Amersham) at 4 °C for 30 min and then immunoprecipitated using rabbit polyclonal antibodies anti-GFP (Abcam) and anti-PS2 (NTF, Sigma). Normal rabbit IgG (Santa Cruz) was used as a negative control. After overnight incubation at 4 °C and extensive washing with TBS, SDS-PAGE sample buffer was added, samples were separated on 5–20% gradient gels and analysed by Western blotting.

2.5. Isolation of subcellular protein fractions from rat brain

Brains from 16 adult rats were homogenized with Dounce homogenizer (12 strokes at 900 rpm) in 10 ml homogenization buffer (5 mM HEPES, pH 7.4, 320 mM sucrose, and protease inhibitor cocktail from Roche). Cell debris and nuclei were removed by 1000 × g centrifugation and the supernatant was spun for 20 min at 13,000 × g, resulting in the supernatant and the pellet P2 (crude membrane fraction). The P2 pellet was homogenized with Dounce homogenizer and fractionated by centrifugation at 85,000 × g for 2 h at 4 °C in a sucrose step gradient (0.85:1.0:1.2 M Sucrose). Myelins, microsomes, and synaptosomes were separated and collected. For isolation of the synaptic membranes containing synaptic junctional proteins, the synaptosomal fraction was incubated with 1 mM Trit–HCl, pH 8.1 (hypo-osmotic shock) for 30 min at 0 °C and centrifuged for 30 min at 33,000 × g. After centrifugation, the pellet containing synaptosomal membranes was collected. SDS-PAGE sample buffer was added to the all collected subcellular fractions which were next analyzed by Western blotting with anti-calmyrin and anti-PS2 antibodies.

2.6. Western blot analysis

Protein extracts of lymphocytes, HeLa cells and COS-7 cells were obtained as described above. Homogenates of different rat tissues and subcellular fractions were subjected to Western blotting as described previously [10]. Anti-calmyrin antibodies were diluted 1:3000, antibodies against N- and C-terminus of PS2 (Sigma) and PS1 (R&D System) were diluted 1:200 and 1:500, respectively. Mouse anti-GFP polyclonal antibody (Babco) was diluted 1:5000. Detection was performed with the ECL detection kit (Amersham).

2.7. Ca\(^{2+}\)-myristoyl switch assay

COS-7 cells were grown on poly-D-lysine coated glass coverslips to 50–70% confluency. Transient transfection was done as described above. Time-lapse microscopy of COS-7 cells was performed two days after transfection as described previously [11]. Translocation of VILIP-1-GFP or calmyrin-GFP was monitored after raising the intracellular calcium concentration by addition of 2 μM ionomycin (Calbiochem).

2.8. RT-PCR

Perfused adult rat brains were dissected and mRNA extracted according to the manufacturer’s protocol (Oligotex, Qiagen). Due to the low abundance of the calmyrin transcript in brain, mRNA was reverse transcribed using random primers and a 3’-calmyrin gene-specific primer (Sensicient Reverse Transcription, Qiagen). PCR amplification was performed using Taq DNA polymerase (Qiagen) and calmyrin-specific primers (Forward primer: 5’ agt cgc 3’, reversed primer: 5’ cag gag ata ctt aag aaa gag agc tgt 3’). PCR conditions: 40 cycles with annealing at 58 °C, 30 s; extension at 72 °C, 1 min; denaturation at 95 °C, 2 min, and final extension at 72 °C, 5 min.

2.9. Animals and immunostainings of rat brain sections

12-week-old male Wistar rats kept under normal laboratory conditions (12-12 h dark-light cycle at a relative humidity of 50–60% and 22 °C with food and water available) were used in an institutional review-board approved protocols. Different areas of rat brain without perfusion were frozen for Western blots. Other rats were transcardially perfused with PBS followed by 4% formaldehyde in PBS. Perfused brains were dissected, fixed by 4% formaldehyde in PBS, embedded in 30% sucrose, and serial sections cut in cryostat were subjected to immunohistochemical staining. Immunocytochemical labeling of calmyrin, PS2, and MAP2 was performed according to standard diaminobenzidine/nickel immunoperoxidase protocols as described previously [12]. Primary antibody [rabbit anti-calmyrin antibody affinity purified (1:100 and 1:1000), rabbit anti-PS2 antibody (Sigma, 1:100) and mouse anti-MAP2 antibody (Sigma, 1:1000)] were diluted in 10% normal goat serum, 0.3% Triton X-100 supplemented with 0.1% NaN\(_3\), and incubated for 24 h at 4 °C. The specificity of the immunoreaction was controlled by incubation with buffer and omitting the primary antibody (no staining was observed). Additionally, antibody against
calmyrin was preabsorbed with different concentrations of recombinant protein. These procedures abolished or drastically attenuated the immunosignals proportionally to the antibodies preabsorption.

3. Results

3.1. Calmyrin binds to endogenous PS2 in a Ca\(^{2+}\)-independent manner

Western blot comparison of calmyrin expression level in main parts of rat brain and in human blood cells suggested that lymphocyte membranes contain high amounts of both calmyrin and PS2 (Fig. 1A) and are easily accessible. We have therefore chosen these cells to characterize the Ca\(^{2+}\)-requirements for binding of endogenous PS2 to calmyrin. The solubilized lymphocyte membrane fraction containing PS2 was transferred to calmyrin-affinity resin. Affinity chromatography of solubilized proteins from the membrane fraction showed that PS2 was retarded on the calmyrin-bound resin (Fig. 1B). Noteworthy, PS1 did not bind to the calmyrin-affinity column (Fig. 1B), indicating that the PS2/calmyrin interaction was specific. In the presence of 0.1 mM Ca\(^{2+}\), endogenous PS2 was retained on the column, and was not released in the presence of the Ca\(^{2+}\)-chelator EGTA until a buffer of high ionic strength was used (Fig. 1B). PS2 from the solubilized membrane fraction bound to the calmyrin-resin also in the presence of EGTA and was only eluted in the presence of 1 M NaCl (Fig. 1B). Thus, although the specific interaction between calmyrin and native PS2 could be confirmed, these data suggest that the binding is not Ca\(^{2+}\)-dependent. Also, no difference in binding to wild type PS2 and PS2 with recently identified mutation Q228L [13] was observed (data not shown). To further substantiate these observations, we performed calmyrin/PS2 pull-down assays. Sepharose-coupled calmyrin pulled down endogenous PS2 from HeLa cells while matrix without calmyrin did not (Fig. 1C). Binding of PS2 occurred both in the presence of 0.1 mM Ca\(^{2+}\) and 2 mM EGTA, suggesting that Ca\(^{2+}\) concentrations do not regulate the PS2/calmyrin binding. In addition, we referred to the previous report that calmyrin interacts with the loop region located in the C-terminal part of PS2 in Yeast 2 Hybrid assay [5]. As PS1 and PS2 exist in the cell not only as holoproteins, but also as their N-terminal and C-terminal peptides, we have analyzed Ca\(^{2+}\) requirements of calmyrin binding to the endogenous C-terminal PS2 fragment (PS2 CTF). As depicted in Fig. 1D, calmyrin pulled-down PS2 CTF in the presence of EGTA while PS1 CTF was not bound. This experiment sustains additional verification of the specific, Ca\(^{2+}\)-independent interaction of recombinant calmyrin with the endogenous C-terminal peptide of PS2.

Further evidence for the existence of calmyrin/PS2 complexes in the absence of Ca\(^{2+}\) in the cell was provided by the coimmunoprecipitation assay performed in COS-7 cells. As shown in Fig. 2A, endogenous PS2 levels in COS-7 cells are rather high. Since calmyrin expression in these cells was undetectable, we transiently transfected COS-7 cells with GFP or calmyrin-GFP coding plasmid providing free N-terminal calmyrin end for myristoylation (intracellular myristoylation of calmyrin-transfected cells was demonstrated previously in [5]). Cell lysates prepared in the presence of 2 mM EGTA were subjected to immunoprecipitation (Fig. 2B, C). In this experiment, anti-PS2 antibody coimmunoprecipitated calmyrin-GFP and not GFP, suggesting specific calmyrin/PS2 interaction which occurs in the absence of Ca\(^{2+}\). Control
experiment carried out using normal IgG at a high concentration did not result in GFP or calmyrin immunoprecipitation, confirming that the interaction between calmyrin and PS2 is specific. Among various attempts of calmyrin/PS2 coimmunoprecipitation undertaken by others, so far only one assay, based on the detection of 3H-myristoylated calmyrin in cotransfected HeLa cells, proved successful [5]. Our data provide therefore first evidence that intracellular calmyrin has the ability to interact with physiological levels of PS2 and that this interaction does not require Ca2+.

3.2. Calmyrin does not exhibit a Ca2+-myristoyl switch

Studies on recoverin have shown that the myristoyl group is sequestered in the Ca2+-free form of the protein and following Ca2+-binding extrusion of the myristoyl group follows the substantial conformational change in the EF-hand structures [14]. This so-called Ca2+-myristoyl switch is thought to be a mechanism for anchoring Ca2+-binding proteins to intracellular and plasma membranes in response to elevated Ca2+ levels. Calmyrin has been shown to be myristoylated at its N-terminus [5], and a Ca2+-dependent translocation to intracellular membranes might provide another mechanism for a Ca2+-dependent trigger of a target interaction with PS2. To test this possibility, we transfected COS-7 cells with a wild-type calmyrin–GFP fusion protein. As a positive control, we used a similar VILIP-1 construct, a neuronal calcium sensor protein, for which a Ca2+-myristoyl switch as a mechanism for a Ca2+-mediated translocation to cellular membranes has been documented [11]. Accordingly, the translocation of VILIP-1-GFP could be confirmed in COS-7 cells after stimulation with 2 μM ionomycin (Fig. 3C, D). In contrast, no translocation to membranes was seen in response to ionomycin stimulation in calmyrin–GFP transfected cells (Fig. 3A, B). Thus, the postulated function of calmyrin as a Ca2+-dependent sensor triggering Ca2+ signals to PS2 seems also not supported by a Ca2+-myristoyl switch within the intramolecular structure of calmyrin.

3.3. Calmyrin is of low abundance in rat forebrain and its expression in subcellular membranes shows limited overlap with PS2

Western blotting and RT-PCR experiments confirmed the presence of calmyrin in rat brain areas (Fig. 4A, B). However, the protein expression level compared to β-actin seems to be lower than in some other tissues and lower than PS2 level. Calmyrin subcellular localization in rat brain membranes showed limited overlap with that of PS2 and particularly its low levels were detected in synaptosomes (Fig. 4C).

Since the onset of AD is associated with functional and structural changes in the forebrain, we examined the distribution of calmyrin in more details by means of immunohistochemistry in this brain region. As reported previously for human brain [10], staining of a subset of pyramidal neurons was observed in rat cortices (Fig. 5A). As compared to the staining pattern obtained with PS2 or MAP2, the immunolabel was, however, much more restricted (Fig. 5A–C), indicating that the vast majority of cortical neurons do not express...
calmyrin. Weak staining was observed in the hippocampus (Fig. 5D, E). Interestingly, in contrast to the human cerebellum, calmyrin-immunolabel was also present in Purkinje cells and deep cerebellar nuclei of rat brain (Fig. 5F, I). The most intense neuronal calmyrin staining in rat brain was found in neurons of medulla, where somata, neurites, and axonal processes were labelled (Fig. 5H).

4. Discussion

In the present study, we have shown that calmyrin binds to PS2 but not to PS1 and that this interaction appears to be Ca\(^{2+}\)-independent, what suggests it will largely depend on local protein levels. However, no Ca\(^{2+}\)-induced translocation of calmyrin to intracellular membranes indicative for a Ca\(^{2+}\)-myristoyl switch was observed. Moreover, the calmyrin/PS2 interaction was originally identified in a Yeast 2 Hybrid screen [5], a protein–protein interaction assay where N-terminal myristoylation is not involved. Accordingly, we also found in the present study binding of endogenous PS2 holoprotein and its separate C-terminal peptide to recombinant non-myristoylated calmyrin. These data implicate that the interaction with PS2 is not dependent on the presence of a myristic acid in calmyrin’s N-terminal part. Moreover, coimmunoprecipitation of endogenous PS2 with calmyrin in the absence of Ca\(^{2+}\) suggests that calmyrin myristoylation does not change lack of Ca\(^{2+}\) requirements for calmyrin binding to PS2. Finally, in agreement with our previous data in human [10], calmyrin transcript and protein levels in rat forebrain proved to be very low and its subcellular localization differs from that of PS2. We therefore conclude that calmyrin is not a major Ca\(^{2+}\)-sensor for PS2 in the forebrain neurons and in proposed scheme of Ca\(^{2+}\)-dysregulation during the onset of AD.

Recently, Zhu et al. [15] reported that point mutations of crucial residues for Ca\(^{2+}\)-binding in calmyrin’s EF-hands 3
and 4 abolish its interaction with PS2. Such EF-hand mutation experiments have to be, however, taken with some caution. EF-hand surfaces are not only high-affinity binding sites for Ca\(^{2+}\), but are concomitantly interfaces for protein-protein interactions. Thus, deleting acidic surface exposed amino acids does not exclusively affect Ca\(^{2+}\)-binding, but will also interfere with protein-protein interactions irrespective of the bound Ca\(^{2+}\) ion. A variety of basic and hydrophobic \(\alpha\)-helical binding structures have been identified that confer to this scenario (see for instance [16]).

Calmyrin ability to bind with its cellular target in a Ca\(^{2+}\)-independent manner seems not only restricted to PS2, but was also recently demonstrated for calmyrin interaction with the cytoplasmic domain of platelet integrin \(\alpha\)IIb \(\beta\) [17]. Target binding of calmyrin independent of an intracellular Ca\(^{2+}\) stimulus distinguishes this protein from many related Ca\(^{2+}\)-sensors, but is not unique in this protein family; some protein interactions of typical Ca\(^{2+}\)-sensors like calmodulin or caldendrin are also controlled by other means than intracellular Ca\(^{2+}\)-levels [18,19]. Similarly, calmyrin binding with PS2 may not be constitutive in vivo but regulated, for example, by other proteins that are known to associate with PS2 overlapping calmyrin binding site in the C-terminal PS2 fragment. Such protein interactions seem to underlie involvement of PS2 in vital cellular processes including apoptosis and Ca\(^{2+}\) homeostasis [20], so the cross-talk between these processes may determine availability of PS2 pools for its binding partners. As the dissociation of other PS2 target proteins might be a prerequisite to PS2/calmyrin interaction, such scenario would explain lack of requirements for prior calmyrin translocation to the membranes observed in our studies. Moreover, while the paradigm of Ca\(^{2+}\)-dependent binding seems not applicable to calmyrin/PS2 complex formation, some Ca\(^{2+}\)-dependent regulation of the preformed calmyrin/PS2 is not excluded and seems probable in the light of recent findings showing that Ca\(^{2+}\)-binding to calmyrin in the calmyrin/\(\alpha\)IIb complex induces subtle structural changes that could further modulate the activity of \(\alpha\)IIb [17]. This type of Ca\(^{2+}\)-regulation was already described for troponin complex in vertebrate skeletal muscle, where Ca\(^{2+}\)-binding to troponin C constitutively associated with troponin I induces conformational changes that activate the complex [21]. Therefore, it should be clarified in future studies whether calmyrin binding to PS2 can contribute to signalling events downstream of PS2 and whether Ca\(^{2+}\)-binding to calmyrin in calmyrin/PS2 complex can affect its structure and known PS2 activities.

Our studies indicate that while calmyrin and PS2 are colocalized in some cell types such as lymphocytes, a significant regulatory function of calmyrin for PS2 signaling is hard to conceive in most brain neurons. In the light of low calmyrin levels and limited overlap of the two protein expression pattern, a probability for physical interaction of the two proteins in forebrain neurons seems low. This is in contrast to another PS2-interacting Ca\(^{2+}\)-binding protein calsenilin (called also Downstream Regulatory Element Antagonist Modulator, DREAM or Potassium Channel Interacting Protein, KChIP3), which expression pattern parallels PS2 staining in cerebellar and hippocampal neurons and is consistent with calsenilin possible role as PS2 neuronal binding partner [22]. Interestingly, association of endogenous calsenilin and PS2 is also Ca\(^{2+}\)-independent [23], leaving the question regarding mechanism of a putative Ca\(^{2+}\)-regulation of PS2 in forebrain neurons open.

Another question that will be interesting to address in the future arises from our finding that calmyrin is prominently present in neurons of the medulla. Based on the relatively high level of expression, it can be predicted that calmyrin major function may be associated with this particular cell type and its functional role deserves further investigation.

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