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ORIGINAL ARTICLE

AKAP79/150 interacts with the neuronal calcium-binding protein caldendrin

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Abstract

The A kinase-anchoring protein AKAP79/150 is a postsynaptic scaffold molecule and a key regulator of signaling events. At the postsynapse it coordinates phosphorylation and dephosphorylation of receptors via anchoring kinases and phosphatases near their substrates. Interactions between AKAP79 and two Ca^{2+} -binding proteins caldendrin and calmodulin have been investigated here. Calmodulin is a known interaction partner of AKAP79/150 that has been shown to regulate activity of the kinase PKC in a Ca^{2+}-dependent manner. Pull-down experiments and surface plasmon resonance biosensor analyses have been used here to demonstrate that AKAP79 can also interact with caldendrin, a neuronal calcium-binding protein implicated in regulation of Ca^{2+} -influx and release. We demonstrate that calmodulin and caldendrin compete for a partially overlapping binding site on AKAP79 and that their binding is differentially dependent on calcium. Therefore, this competition is regulated by calcium levels. Moreover, both proteins have different binding characteristics suggesting that the two proteins might play complementary roles. The postsynaptic enrichment, the complex binding mechanism, and the competition with calmodulin, makes caldendrin an interesting novel player in the signaling toolkit of the AKAP interactome.

Keywords: calcium sensor protein, calmodulin, postsynaptic density, surface plasmon resonance.

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The postsynaptic density (PSD) provides the structural basis of functional synaptic transmission at excitatory synapses. A key component involved in the organization of this electron-dense network of proteins is the A kinase-anchoring protein AKAP79/150, which constitutes a platform anchoring cAMP-dependent protein kinase (PKA), protein kinase C (PKC) (Klauck *et al.* 1996; Faux *et al.* 1999), as well as their counterparts, the protein phosphatases 2B/Calcineurin (PP2B/CaN) (Coghlan *et al.* 1995; Dell'Acqua *et al.* 2002) and PP1 at the PSD (Le *et al.* 2011). Species orthologues of the corresponding gene *akap5* differ in their apparent molecular weight as indicated by their names (bovine AKAP75, human AKAP79, and rodent AKAP150). The anchoring of kinases and phosphatases by AKAP79/150 has been shown to be required for the regulation of α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA) receptors during processes of synaptic plasticity (Rosenmund

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Abbreviations used: ADRB1/2, Beta-adrenergic receptor 1, 2; AKAP79/150, A kinase-anchoring protein79/150; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid; CaBP1, Ca^{2+}-binding protein 1, shorter splice isoform of caldendrin; CaM, Calmodulin; $Ca_V1.2$, L-type voltage-gated Ca^{2+}channel 1.2; CDD, Caldendrin; GFP, Green fluorescent protein; GST, Glutathione S transferase; HEK293, Human embryonic kidney cell line 293; KCNQ, Voltage-gated K^+ channel, KQT-like subfamily; LTD, Long-term depression; LTP, Long-term potentiation; LZ, Leucine zipper; MAGuK, Membrane-associated guanylate kinase; NMDA, N-methyl d-aspartate; PD, Pull down; PIP_2, Phosphatidylinositol-4,5-bisphosphate; PKA, cAMP-dependent protein kinase/protein kinase A; PKC, Protein kinase C; PP1, Protein phosphatase 1; PP2B/CaN, Protein phosphatase 2B / Calcineurin; PSD, Postsynaptic density; PSD-95/SAP90, Postsynaptic density protein 95/synapse-associated protein 90; SAP97, Synapse-associated protein 97; SPR, Surface plasmon resonance.

et al. 1994; Snyder *et al.* 2005; Dell'Acqua *et al.* 2006; Tavalin 2008). AKAP79/150 binds to the membrane-associated guanylate kinases (MAGuKs) PSD-95 (SAP90) and SAP97, which constitute a bridge between AKAP79/150-anchored proteins and the AMPA- and N-methyl d-aspartate (NMDA)-type glutamate receptors (Colledge *et al.* 2000), but also with beta-1-adrenergic receptors (ADRB1) (Gardner *et al.* 2007). In contrast to ADRB1, the beta-2-adrenergic receptor (ADRB2) is directly bound by AKAP79/150 (Fraser *et al.* 2000). Furthermore, the M-type voltage-gated K^+ channel KCNQ2/3, (Hoshi *et al.* 2003; Higashida *et al.* 2005), and the L-type voltage-gated Ca^{2+}channel $Ca_V1.2$ (Oliveria *et al.* 2007) interact with AKAP79/150, the latter via a modified leucine-zipper motif at the very C-terminus of AKAP79/150 (Fig. 1a).

Localization of AKAP79/150 to postsynaptic sites is realized via three mainly α-helical polybasic domains at the N-terminus (A, B, and C domains; see Fig. 1a). Via these domains, AKAP79/150 associates with the phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) at the plasma membrane (Dell'Acqua *et al.* 1998), the actin cytoskeleton (Li *et al.* 1996; Gomez *et al.* 2002) and cadherin adhesion molecules (Gorski *et al.* 2005). Localization of AKAP79/150 to lipid rafts is achieved via palmitoylation of cysteine resides C36 and C129 in the A and C domains, respectively (Delint-Ramirez *et al.* 2011).

The N-terminal region of AKAP79/150 furthermore interacts with adenylyl cyclase (AC) isoforms 5 and 6 (Efendiev *et al.* 2010), and the Ca^{2+} sensor calmodulin (CaM) (Faux and Scott 1997). Already upon its discovery AKAP75, the bovine orthologue of AKAP79/150, was found to co-purify with CaM (Sarkar *et al.* 1984). Later, the existence of two independent CaM-binding sites in AKAP79/150 was postulated (Faux and Scott 1997). CaM has been shown to compete with PKC for binding at the more N-terminally located site, and binding is regulated via intracellular Ca^{2+} levels (Faux and Scott 1997). The second predicted CaM binding sequence is located downstream of the first one (Fig. 1a, in red letters; according to the CaM Target Database http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html). This interaction site, however, has not yet been verified experimentally. In summary, a signalosome-like protein assembly is formed and anchored via AKAP79/150 in the PSD. This assembly is capable of regulating various signaling events, most of which are dependent on the intracellular Ca^{2+} concentration.

The neuronal Ca^{2+} -binding protein caldendrin is the closest relative of CaM in brain (Seidenbecher *et al.* 1998; Mikhaylova *et al.* 2011). Caldendrin has a bipartite structure, with an N-terminal half highly enriched in basic amino acids and non-homologous to any other known protein (Seidenbecher *et al.* 1998), and a rather acidic C-terminus. The latter shares high homology with CaM, also containing four EF-hand motifs, although the second EF hand in caldendrin is cryptic and does not bind Ca^{2+} (Fig. 1b) (Seidenbecher *et al.* 1998; Laube *et al.* 2002). Both EF hand pairs are separated by an α-helical region, which is four amino acids longer as compared with CaM. These four amino acids correspond to one α-helical turn, possibly making the EF-hand region of caldendrin bigger and more flexible as compared with CaM (Seidenbecher *et al.* 1998; Haeseleer *et al.* 2000; Laube *et al.* 2002). Because of alternative splicing, there are two shorter isoforms of caldendrin, caldendrin-S1 (S-CaBP1) and caldendrin-S2 (L-CaBP1), which exhibit unrelated shorter N-termini (Haeseleer *et al.* 2000; Seidenbecher *et al.* 2002). However, in contrast to caldendrin, which is expressed in many mature neurons of forebrain regions with laminar structure, the shorter CaBP1 isoforms are only abundant in the retina (Haeseleer *et al.* 2000; Seidenbecher *et al.* 2002).

Because of their similarity, caldendrin/CaBP1 and CaM share some binding partners. Interestingly, in some cases the consequences of binding of the two Ca^{2+} sensors to the same protein are quite different. For example, the L-type voltage-gated Ca^{2+} channel $Ca_V1.2$ shows Ca^{2+} -dependent facilitation, prolonged Ca^{2+} currents, and prevented Ca^{2+} -dependent inactivation after the binding of CaBP1, but stronger Ca^{2+} -dependent inactivation after CaM binding (Zhou *et al.* 2004). Moreover, also caldendrin and CaBP1 binding to $Ca_V1.2$ channels have different consequences on channel gating activity (Tippens and Lee 2007).

The second major synaptic function of caldendrin at synapses involves the Ca^{2+} -dependent anchoring of the synapto-nuclear protein messenger Jacob (Dieterich *et al.* 2008). In this case CaM does not compete for the binding site on Jacob, and a caldendrin protein knockdown leads to more efficient redistribution of Jacob to the nucleus (Dieterich *et al.* 2008). However, other synaptic caldendrin-binding partners have not yet been identified so far.

Based on the postsynaptic co-enrichment of caldendrin and AKAP79/150 and on the fact that AKAP79/150 has CaM binding motifs, it is conceivable that caldendrin might be an AKAP79 binding partner. To address this question we implemented pull down and co-immunoprecipitation approaches as well as Surface Plasmon Resonance to show that caldendrin directly interacts with AKAP79/150. Furthermore, we compare AKAP79 binding to caldendrin and to CaM and show competition between both binding partners.

Materials and methods

Materials

The CaM and CaM sepharose were obtained from Sigma-Aldrich (Taufkirchen, Germany), glutathione sepharose beads from GE Healthcare (Munich, Germany), ProBond beads from Invitrogen (Darmstadt, Germany), and protease inhibitor (PI) complete tablets EDTA-free from Roche (Mannheim, Germany). All other chemicals were generally purchased in p.A. quality from Roth (Karlsruhe, Germany).

Plasmids and constructs

For construction of green fluorescent protein (GFP) fusion constructs, human AKAP79 was PCR-amplified from DNA extracted from human blood and cloned into EcoRI/BamHI restricted pEGFP-N1 (Clontech, Saint-Germain-en-Laye, France). Partial constructs of AKAP79 (see Fig. 1a) were cloned into EcoRI/SalI-restricted pEGFP-C2 (Clontech), the glutathione S transferase (GST) construct encompassing the AKAP79 B domain (aa 61–116) was cloned into EcoRI/SalI sites of pGEX-5X-1 (GE Healthcare), the AKAP79 B domain 6xHis-SUMO construct was cloned into pET-SUMO (Champion™ pET-SUMO TA Cloning kit, Invitrogen). The 6xHis-SUMO-caldendrin-Δ21 (lacking the first 21 amino acids) was subcloned by PCR from the full-length caldendrin-pEGFP-N1 construct. The GST-caldendrin-C-terminus (aa 137–298) was reported previously (Dieterich *et al.* 2008).

Antibodies

Rabbit anti-GFP (Abcam, Cambridge, UK), monoclonal mouse anti-GFP, mouse anti-GST (both Covance, Münster, Germany), goat anti-AKAP150-N19, goat anti-AKAP150-C20, and rabbit anti-CaM (all Santa Cruz, Heidelberg, Germany) were obtained from the commercial suppliers, and polyclonal rabbit anti-caldendrin and guinea pig anti-caldendrin antisera against the C-terminus of caldendrin were generated in the lab of M.R. Kreutz (Dr. Pineda Antibody-Service) and have been described previously (Dieterich *et al.* 2008).

Secondary horseradish peroxidase-labeled antibodies, goat anti-mouse, goat anti-rabbit, rabbit anti-guinea pig were obtained from Dianova-Jackson (Hamburg, Germany), and donkey anti-goat IgG was purchased from Santa Cruz (Heidelberg, Germany). For immunocytochemistry fluorochrome-labeled secondary antibodies Alexa 488 donkey anti-goat (Invitrogen), Cy3 donkey anti-rabbit (Dianova-Jackson), and Cy 5 donkey anti-mouse (Dianova-Jackson) were used.

Cell culture, transfection, and lysate preparation

Human embryonic kidney (HEK293) cells were used for the expression of GFP tagged proteins. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, GIBCO – Invitrogen, Darmstadt, Germany) supplied with 10% FKS, 2 mM glutamin, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (GIBCO – Invitrogen), at 37°C and under 5% CO_2.

Transfection was done using the calcium phosphate method at 50% confluency. A quantity of 2 – 4 μg of pDNA was mixed with 210 μl solution A (500 mM $CaCl_2$ in H_2O). A quantity of 210 μl solution B (140 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES), 1.5 mM Na_2PO_4 in H_2O, pH 7.05) was added and mixed by pipetting up and down. The mixture was incubated for 1 min at 22°C and then applied dropwise to the cell culture. Cells were harvested 48 h later in Tris-buffered saline (TBS), the pellet shock frozen in liquid nitrogen, then re-suspended in TBS with PI and 1% Triton X-100. After 30 min of incubation at 4°C shaking, the cell suspension was centrifuged 30 min at 20 000 *g* and the supernatant was used for pull-down experiments.

Expression and purification of GST and 6xHis-SUMO constructs

E. coli BL21 (DE3) bacteria were transformed with GST-fusion constructs or GST control plasmid. Expression and purification was done following standard protocols. Briefly, 10 ml of an overnight culture was used to inoculate 500 or 1000 mL fresh LB media. Cells were grown to an optical density of ∼0.6 at OD_{600}. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside for 4 h at 37°C. Bacteria were harvested by centrifugation at 3000 *g*, the pellets were suspended in ice-cold TBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl), and disrupted via passing through a French press at 20 000 psi. After adding protease inhibitors and 1% Triton X-100, the suspension was incubated at 4°C for 1 h shaking, and then centrifuged for 30 min at 4°C and 14 000 *g*. 500 – 1000 μl of glutathione sepharose beads were added to the supernatant and incubated at 4°C for 3 h shaking. Beads were washed three times with ice-cold TBS (50–100 times bed volume per wash step), transferred into a column (Biorad), and the proteins eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 7.6. Eluates were dialyzed against TBS and protein concentrations were determined by the colorimetric amidoblack assay as described by Popov *et al.* (Popov *et al.* 1975).

The 6xHis-SUMO constructs were expressed in *E. coli* BL21 as described above. After harvesting the cells, bacterial pellets were resuspended in native purification buffer (NPB, 25 mM NaH_2PO_4, 250 mM NaCl, pH 8.0) and passed though a French press. PI and Triton X-100 were added; the suspension was incubated and centrifuged as described earlier. A quantity of 3 mL of ProBond beads was added to the supernatant to capture the fusion proteins. The beads were first washed three-times in NPB, then once in NPB + 10 mM imidazole and once in NPB + 20 mM imidazole. They were subsequently transferred into a column, and the proteins were eluted by increasing concentrations of imidazole (from 100 up to 350 mM imidazole in NPB). Finally, the eluates were dialyzed, first against NPB and second against HEPES Buffer (10 mM HEPES, 150 mM NaCl, pH 7.4).

The three dimensional structure of the caldendrin-binding region in AKAP79 has been predicted using PEP-Fold (http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/)(Maupetit *et al.* 2009).

Pull-down assays

Purified GST or GST-fusion protein was coupled to 20 μl of glutathione sepharose beads in TBS containing 5% bovine serum albumin (BSA) for 3 h at 4°C. After that beads were washed, added to extracts of transfected HEK293T cells, and adjusted with TBS + 1 mM $MgCl_2$ and 2 mM $CaCl_2$ (Ca^{2+} buffer, final concentration) or TBS + 1 mM $MgCl_2$ and 2 mM ethyleneglycol bis(aminoethylether)tetraacetate (EGTA) (Ca^{2+} -free buffer, final concentration) to a final volume of 500 μl. The reactions were incubated overnight on a rotating wheel at 4°C. Supernatants containing unbound proteins were collected, beads washed three times in 1 ml of Ca^{2+} - or Ca^{2+} - free buffer, and bound proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer. Proteins in the original sample (inputs) as well as in the supernatant containing unbound protein and eluates with bound proteins were analyzed using standard immunoblotting protocols (Seidenbecher *et al.* 2004).

For pull-downs (PDs) with exclusively bacterially purified proteins, 8 μg of GST-AKAP79-B or GST were immobilized on sepharose beads as described above. The 6xHis-SUMO tag of caldendrin was cleaved by incubation of 6xHis-SUMO-caldendrin-Δ21 with 10 U of SUMO Protease (Invitrogen) in 1 × SUMO Protease Buffer without salt (Invitrogen) shaking at a temperature of 30°C for 6 h. The cleavage product was added to GST-AKAP79-B or GST beads, incubated shaking at 4°C for 3 h and processed as

described before. Approximately 38 μg of 6xHis-SUMO-caldendrin-Δ21 were cleaved and used per pull-down reaction.

The CaM sepharose pull-downs were performed as described for GST-fusion proteins, but with 10 μl CaM sepharose beads (7.5 μg CaM) per sample in either Ca^{2+} - or Ca^{2+} -free buffer.

To further characterize Ca^{2+} -dependency, after binding in Ca^{2+} -buffer beads were washed three times and incubated in 500 μl Ca^{2+} -free buffer containing 2 mM EGTA at 4°C for 3 h shaking. The EGTA-eluted proteins were harvested, the beads were washed three times, and bound proteins were eluted with SDS sample buffer. As a reference for EGTA elution, the third wash with Ca^{2+} buffer was collected.

Simultaneous binding of both calcium sensors was studied in competitive pull-down assays performed with 8 μg GST-AKAP79-B or GST immobilized on sepharose beads, with the addition of equimolar amounts of the calcium-binding proteins (38 μg cleaved CDD-fl and 16.5 μg purified bovine CaM) in Ca^{2+} buffer. Samples were incubated at 4°C on a rotating wheel for 3 h, and unbound and bound fractions were harvested and analyzed by immunoblotting as described above.

Disturbance of preformed protein complexes by the other binding partner was assessed in pull-down assays performed with 2 μg of CaM on CaM sepharose or 2 μg of GST-caldendrin-C-terminus coupled to GST sepharose beads. Incubation with extracts of GFP-AKAP79-B-transfected HEK293T cells (approx. 1.5 μg GFP-AKAP79-B per sample) was performed in Ca^{2+} buffer at 4°C on a rotating wheel overnight, unbound fractions were harvested and samples were washed three times. The third wash fraction was collected as a reference for later analysis. Subsequently, the respective competition partner was added, either 2 μg of untagged caldendrin-Δ21 or 3 to 6 μg of purified bovine calmodulin, and the samples were incubated in Ca^{2+} buffer at 4°C on a rotating wheel for 3 h. Eluted proteins were collected, the beads were washed three times and finally eluted with SDS sample buffer.

Preparation of rat brain extracts and immunoprecipitation

Membrane extracts from forebrains of female Wistar Hannoveraner rats (RjHan:Wi, Elevage Janvier, France) were prepared mainly as described in Tippens and Lee 2007; with some minor modifications (Tippens and Lee 2007). Brains were weighed out and homogenized with a Potter homogenizer (10 strokes at 900 rpm) in 10 volumes of 320 mM sucrose, 1 mM $MgCl_2$ in 4 mM HEPES, pH 7.4, PI, and either 2 mM $CaCl_2$ (high Ca^{2+}) or 2 mM EGTA (Ca^{2+} -free). Cell debris and nuclei were removed by centrifugation at 1,000 *g* for 5 min. The supernatant was ultra-centrifuged at 100 000 *g* for 30 min. The resulting membrane pellet was re-suspended in TBS with 1 mM $MgCl_2$, 1% Triton X-100, PI and either 2 mM $CaCl_2$ (high Ca^{2+}) or 20 mM EDTA, and 10 mM EGTA (Ca^{2+} -free), incubated for 30 min at 4°C shaking and ultra-centrifuged at 100 000 *g* for 30 min to remove insoluble material. The membrane extract was used for immunoprecipitation.

Rabbit anti-caldendrin-C-terminus antibodies or control rabbit IgGs (Santa Cruz) were added to 1 ml of rat brain membrane extracts and incubated on a rotating wheel at 4°C for 1 h. Protein A sepharose beads (30 μl per sample) were pre-blocked with 5% BSA in TBS for 30 min at 4°C, added to the membrane-antibody reaction and incubated overnight at 4°C with continuous shaking. Unbound fractions were harvested, beads washed three times in 1 ml TBS with 2 mM of $CaCl_2$ (high Ca^{2+}) or 2 mM of EGTA (Ca^{2+} -free) and bound proteins were eluted with SDS sample buffer.

Surface Plasmon Resonance-based interaction studies

Interactions were monitored employing Surface Plasmon Resonance (SPR) biosensor technology using a Biacore S51 instrument and CM5 Sensor chips (GE Healthcare, Uppsala, Sweden). Immobilization and interaction studies were performed at 25°C. 6xHis-SUMO-AKAP79-B was immobilized on sensor surfaces via standard amine coupling chemistry and was injected 5 min (5 μl min^{-1}) at a concentration of 0.1 mg min^{-1} in 10 mM Na-acetate, pH 5.5. An untreated sensor surface served as a reference. Caldendrin and CaM were analyzed in twofold diluted concentration series (Caldendrin: from 500 to 16 nM, CaM: from 250 to 8 nM) at a flow rate of 30 μL min^{-1} and analyses were repeated in triplicates on freshly prepared sensor surfaces. Running and sample buffer consisted of 25 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Tween-20, pH 7.4 (0.2 μm filtered). For regeneration of the sensor surface, a calcium-free buffer supplemented with 50 μM EGTA was injected for 30 s after each ligand injection.

Biacore T200 Evaluation Software 1.0 (GE Healthcare, Uppsala, Sweden) was used for data analysis. Signals from the reference surface were subtracted from the AKAP-surface. Average values from blank samples (without analyte) were subtracted from reference-subtracted data (reference = without ligand).

Immunofluorescence staining and confocal microscopy of primary hippocampal neuronal cultures

Rat hippocampal neurons were isolated at embryonic day 18, plated and cultured as described previously (Dieterich *et al.* 2008). To assess potential effects of intracellular Ca^{2+} conditions on the co-distribution of caldendrin and AKAP150, in some experiments neurons were either treated with 45 μM of BAPTA-AM for 30 min, with 1 μM of ionomycin for 1–5 min, or left untreated (control) prior to fixation. Neurons were fixed at 21 days *in vitro* (DIV) with 4% paraformaldehyde for 10 min at 22°C and washed three times with phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4). For permeabilization, PBS containing 0,25% Triton X-100 was added for 15 min. Then neurons were washed three times with PBS and blocked for 1 h in blocking buffer (2% BSA, 2% glycine, 0,2% gelatine, 50 mM NH_4Cl in PBS). Primary antibodies were diluted in blocking buffer in the following dilutions: rabbit anti-caldendrin-C-terminus 1 : 400, goat anti-AKAP150 1 : 250, mouse anti-PSD-95 1 : 800. Antibody solutions were added on the coverslips and incubated overnight at 4°C. Coverslips were washed five times for 10 min with PBS. Secondary antibodies were diluted 1 : 1000 in blocking buffer, ultracentrifuged for 30 min at 100 000 *g* and applied for 1 h at 22°C in darkness. Following extensive washing with PBS and double distilled water, slides were mounted in Mowiol (Merck, Darmstadt, Germany). Images were taken with 63 × oil objective as z-stacks (250 nM z step size) using a Leica DMRXE microscope (Wetzlar, Germany) equipped with a Krypton-Argon-Ion laser (488/568/647 nM) and an acousto-optic-tunable filter for selection and intensity adaptation of laser lines. Images were processed with ImageJ (NIH, USA).

Results

AKAP79 associates with the carboxy-terminal part of caldendrin

To test for a possible interaction between caldendrin and AKAP79, we first performed pull-down experiments with full length AKAP79-GFP and the GST-tagged carboxy terminus (C-term) of caldendrin (aa 137–298), containing the CaM-like EF-hand domains. The C-terminal part of caldendrin did indeed pull down full-length AKAP79-GFP from HEK293 cell extracts, whereas no binding in GFP or GST controls could be seen (Fig. 1c). In the next step, N-terminal and C-terminal parts of AKAP79 were tested separately in pull-down assays performed with GST-caldendrin-C-term. Binding was observed only with the N-terminal part of AKAP79 (Fig. 1d), which is in accordance with the predicted CaM-binding sites located in the A and the B subdomains (Fig. 1a). For a more refined mapping of the binding site, all three subdomains A–C were cloned and expressed as GFP fusion proteins. The C-terminus of caldendrin pulled down only the AKAP79-B subdomain, but not the AKAP79-A or AKAP79-C subdomains (Fig. 1e).

To verify these results and to ensure that AKAP also associates with full-length caldendrin and not only with the C-terminal part, we inverted the experiment, expressing the AKAP79-B domain as a GST construct and caldendrin as a full-length GFP fusion protein. As shown in Fig. 1f, caldendrin-full-length GFP is indeed found in the GST-AKAP79-B pull-downs from HEK293 cell extracts.

Summarizing these results, we found an association of AKAP79 and caldendrin, which is mediated by the polybasic AKAP79-B subdomain.

Comparison of the CaM and caldendrin interaction interfaces in the AKAP79-B domain

To further pinpoint the caldendrin-binding site, an analysis of the AKAP sequence was performed using the CaM Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html). The program predicted a CaM-binding sequence within the AKAP79-B domain (Fig. 1a; highlighted in red). Consequently, we decided to split the AKAP79-B domain into three peptides, an N-terminal (B1, aa 61–76), a central (B2, aa 75–97), and a C-terminal peptide (B3, aa 97–116; see Fig. 1a). These peptides were expressed as GFP fusion proteins in HEK293 cells and pull-down assays with GST-caldendrin-C-terminus and CaM were performed.

As expected, CaM pulled down the B2 polypeptide, containing the predicted binding site, while B1 and B3 did not bind to CaM (Fig. 2a).

Surprisingly, caldendrin did not bind to either of these individual peptides (Fig. 2b), showing that the CaM and caldendrin-binding interfaces in the AKAP79-B domain are not identical. We had slightly extended the AKAP79-B2 construct by adding three more residues (Fig. 1a, AKAP79-B2 aa 75–97), in accordance with CaM Target Database predictions. However, this extension was not sufficient to allow for caldendrin binding (Fig. 2b).

Therefore, two more constructs were made, combining the central peptide B2 encompassing the predicted binding motif with either the N- or C-terminally adjacent peptide (B1/2 and B2/3, respectively; Fig. 1a).

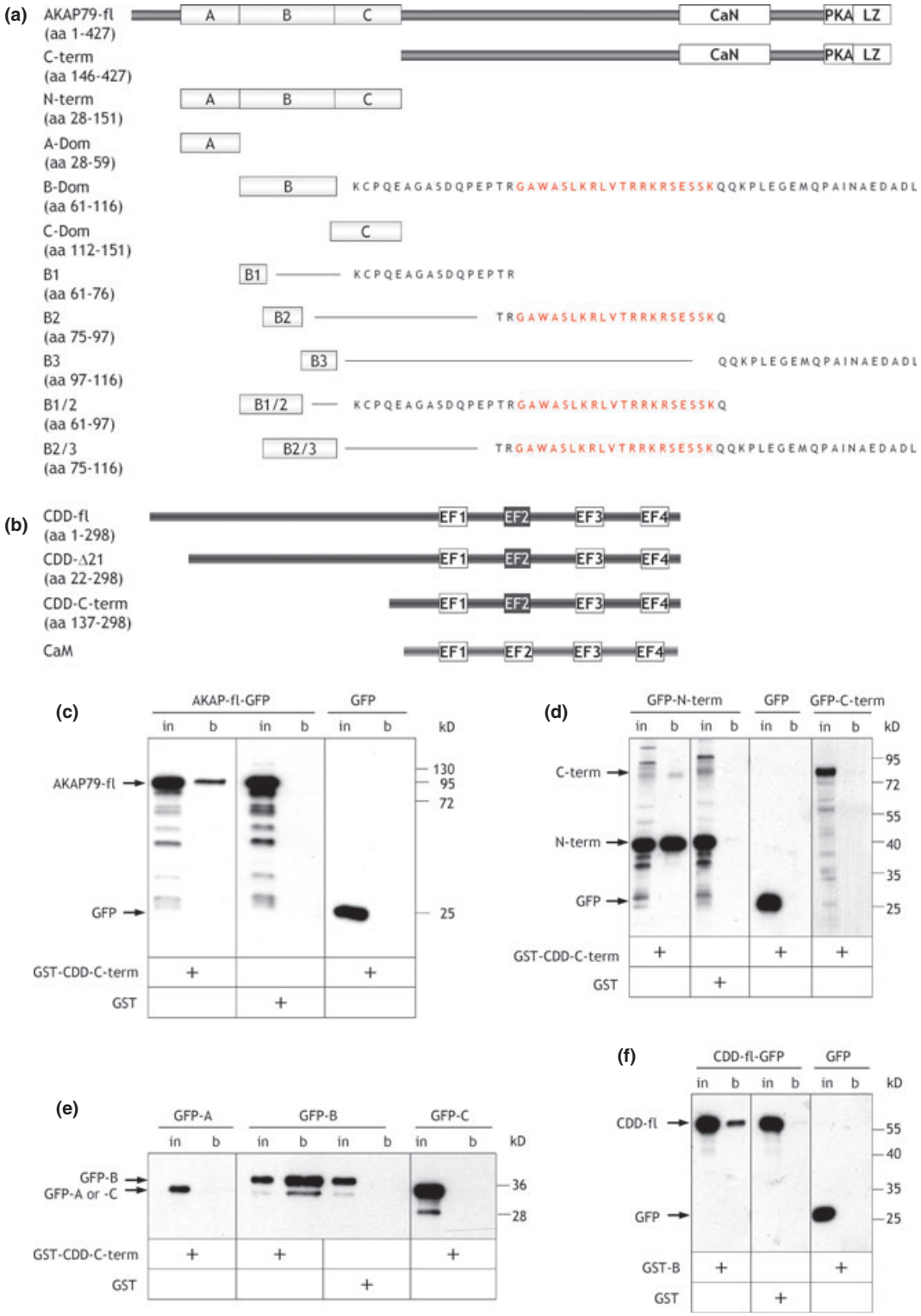
As expected, in the following pull-down assays, CaM precipitated each construct containing the B2 sequence (Fig. 2a). Caldendrin, however, only precipitated the B1/2 construct but not the C-terminally extended B2/3 polypeptide (Fig. 2b). Therefore, we concluded that CaM and caldendrin-binding interfaces overlap in the AKAP79-B domain, but caldendrin needs an N-terminally extended sequence for binding. According to protein structure prediction tools (PEP-Fold; http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/) this N-terminal extension does not form a helix but rather a loop structure (Fig. 3b).

The interaction between AKAP79-B and caldendrin is direct

Based on the experiments described above, we could not exclude that the observed association between AKAP79 and caldendrin might be indirect, i.e. mediated by endogenous

Fig. 1 Schematic representation of all proteins and fragments used in this study and identification of A kinase-anchoring protein AKAP79 fragments binding to caldendrin. (a) AKAP79 constructs. AKAP79-fl, AKAP79-full-length; C-term, C-terminal fragment; N-term, N-terminal fragment; A, B, C, basic-rich targeting domains; CaN, calcineurin/PP2B binding site; PKA, PKA binding site; LZ, modified leuzine zipper; B1, B2, B3, B1/2, B2/3, subfragments of the B domain. To show the predicted Calmodulin (CaM) binding site (depicted in red) and the composition of the B-domain subfragments, amino acid sequences of AKAP79-B, B1, B2, B3, B1/2, and B2/3 are given next to their schematic representation. (b) Schematic representation of caldendrin-full-length (CDD-fl), N-terminally truncated caldendrin (CDD-Δ21) and C-terminal half (CDD-C-term) in comparison to CaM. The caldendrin EF hand 2 (dark gray box) is cryptic and does not bind Ca^{2+}. (c–f) Binding between AKAP79 and caldendrin under high Ca^{2+} conditions is assessed in Glutathione S transferase (GST) pull-down assays. (c) AKAP-fl- Green fluorescent protein (GFP) binds to GST-caldendrin-C-term but not to GST. The GFP alone does not bind to GST-caldendrin-C-term. (d) GST-caldendrin-C-term pulls down GFP-AKAP79-N-term but not GFP-AKAP79-C-term or GFP alone. This indicates that the caldendrin-C-term binding site is located in the N-terminal part of AKAP79. (e) GFP-AKAP79-B (GFP-B) binds to GST-caldendrin-C-term in contrast to GFP-AKAP79-A (GFP-A) or -AKAP79-C (GFP-C), implying that caldendrin binding to AKAP79 takes place via the AKAP79-B domain. (f) In a reciprocal experiment, GST-AKAP79-B (GST-B) pulls down caldendrin-full-legth-GFP from a HEK cell lysate. GFP alone does not bind to AKAP79-B. GST controls are negative. in – input; b – bound fraction. All blots are stained with anti-GFP and are representative for at least three independent experiments.

binding proteins present in HEK293 cells. To rule out this possibility, we used bacterially expressed and purified proteins in the following pull-down assays. The first 21 amino acids of caldendrin have a high score to be unstructured and introduce instability to the full-length protein (data not shown). Therefore, we generated a 6xHis-SUMO-caldendrin-Δ21 construct. The tag was cleaved before the experiment. As shown in Fig. 3a, purified caldendrin binds to the isolated AKAP79-B domain, thereby confirming that the interaction is indeed direct.



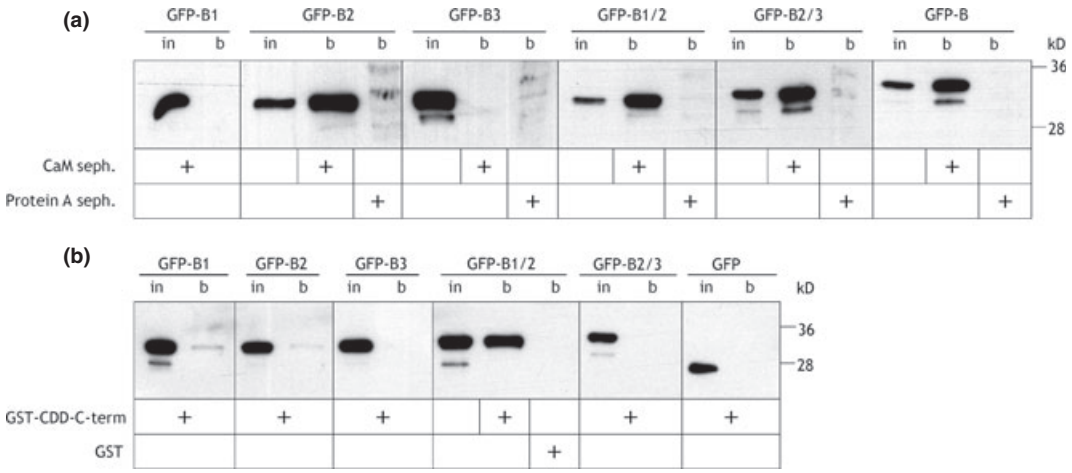


Fig. 2 Identification and comparison of the Calmodulin (CaM) and caldendrin-binding sites in A kinase-anchoring protein AKAP79. (a) CaM sepharose pulls down any GFP construct containing the predicted CaM-binding site B2 of AKAP79. The GFP-B1, -B3, and GFP alone do not bind to CaM sepharose. (b) Only AKAP79-B1/2, a construct combining AKAP79-B1 and -B2, is pulled down by GST-caldendrin-C-term, but not by GST alone. All other B domain fragments do not bind to GST-caldendrin-C-term. in – input; b – bound fraction; all blots are stained with anti-GFP. These pull-down experiments were performed under high Ca^{2+} conditions and replicated more than three times.

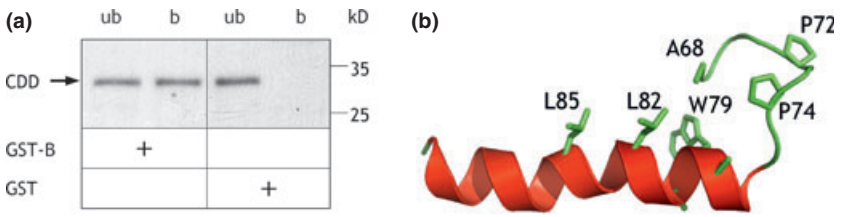


Fig. 3 Direct interaction between A kinase-anchoring protein AKAP79 and caldendrin. (a) Pull-down with purified proteins shows that untagged caldendrin (CDD-Δ21) is precipitated by GST-AKAP79-B under high Ca^{2+} conditions. ub – unbound fraction; b – bound fraction; blot is stained with anti-caldendrin. The pull-down was repeated five times. (b) Structure prediction of the AKAP79-B1/2 peptide using PEPFOLD. The helical portion shown in red corresponds to the region necessary and sufficient for Calmodulin binding. Caldendrin binding needs also the N-terminal loop structure (green) for binding. Residues L85, V86, L82, and W79 are the key residues for binding to Calmodulin or caldendrin.

CaM binds to AKAP79-B domain in a Ca^{2+} -dependent manner, whereas the interaction between AKAP79-B and caldendrin is less sensitive to Ca^{2+} conditions

Assuming that Ca^{2+} might play an essential role in the interaction between caldendrin and AKAP79, all experiments described so far were done under high Ca^{2+} concentrations (2 mM). To explore if the interactions of caldendrin and CaM with AKAP79-B are Ca^{2+} -dependent, we next compared pull-down experiments under high (2 mM Ca^{2+}) or low (2 mM EGTA) Ca^{2+} conditions.

The CaM binding to the AKAP79-B domain is completely dependent on the presence of Ca^{2+}, as under EGTA conditions no binding between both proteins occurs (Fig. 4a). Furthermore, the interaction between CaM and GFP-AKAP79-B is disturbed by the addition of EGTA, which elutes most GFP-AKAP79-B from CaM sepharose (Fig. 4b) or in the reciprocal experiment CaM from sepharose-bound GST-AKAP79-B (Fig. 4c).

For the AKAP-caldendrin interaction we obtained a very different picture: although there is a substantial decrease in binding in the absence of Ca^{2+}, the interaction is still clearly detectable in the presence of 2 mM of EGTA (Fig. 4d), suggesting that it is less sensitive to Ca^{2+} concentrations. Moreover, once the interaction was established in the presence of Ca^{2+}, it proved to be fully resistant to a decrease in free Ca^{2+} ions, as the addition of EGTA did not elute GFP-AKAP79-B from GST-caldendrin-C-term (Fig. 4e) or caldendrin from GST-AKAP79-B beads in the reciprocal experiment (Fig. 4f).

Caldendrin and CaM compete for binding to the AKAP79-B domain

Since the binding sites for caldendrin and CaM overlap within the AKAP79-B domain, it was tempting to speculate that both Ca^{2+} sensor proteins compete for binding to AKAP79. To test if competition occurs under high Ca^{2+}

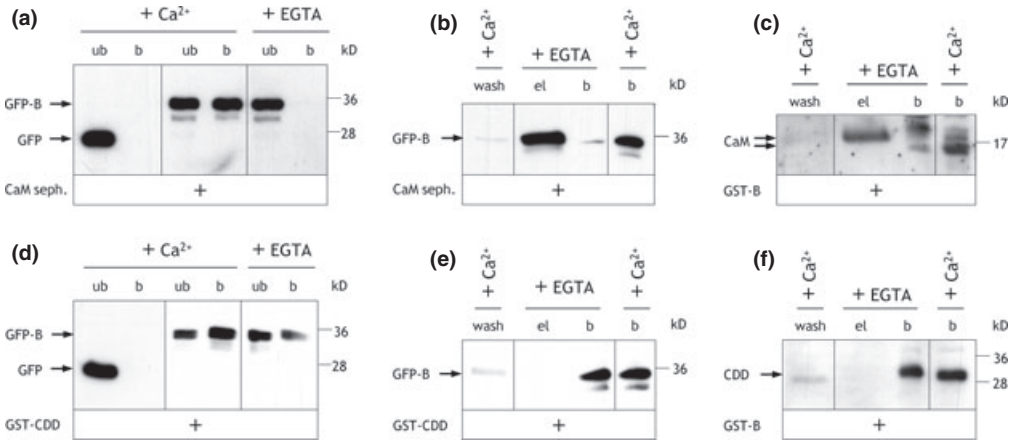


Fig. 4 Calmodulin (CaM) and caldendrin bind to A kinase-anchoring protein AKAP79-B under differential Ca^{2+} -conditions. (a) GFP-AKAP79-B (GFP-B) is pulled down by CaM sepharose only in the presence of Ca^{2+}. (b) 2 mM EGTA releases AKAP79-B previously bound to CaM sepharose under Ca^{2+} conditions into the eluate. The last wash fraction with Ca^{2+} buffer is almost devoid of AKAP-B immunoreactivity. Note that the AKAP-B portion retained at the beads is clearly diminished as compared with the bound fraction under Ca^{2+}. In the reciprocal experiment, CaM is eluted from GST-AKAP-B (GST-B) via EGTA as well (c). (d) GST-caldendrin-C-terminus (GST-CDD-C-term) pulls down GFP-AKAP79-B in both high Ca^{2+} and Ca^{2+} -free conditions, although binding in the absence of Ca^{2+} is diminished. After the establishment of binding between caldendrin and AKAP79-B Ca^{2+} depletion does neither release AKAP79-B (GFP-B; e) nor caldendrin (f) from the bound fraction into the eluate. ub – unbound fraction; b – bound fraction; wash – third wash after binding in the presence of Ca^{2+}; el – eluate; blots are stained with anti-GFP (a, b, d, e), anti-CaM (c), or anti-caldendrin (CDD; f). Blots are representative for at least three independent pull-downs.

conditions, equimolar amounts of CaM and caldendrin were incubated at the same time with GST-AKAP79-B. Both proteins were pulled down simultaneously by GST-AKAP79-B. This result indeed points to a competition, as a clear decrease in the amount of bound caldendrin in the presence of CaM could be observed as compared with CaM-free conditions (Fig. 5a).

Next we tested, whether CaM and caldendrin can displace each other in the interaction with AKAP79-B. Fig. 5b shows that caldendrin is able to elute GFP-AKAP79-B from a preformed CaM-AKAP complex. In contrast, CaM cannot disturb the established interaction between GST-caldendrin-C-term and GFP-AKAP79-B, even if present in fourfold or eightfold excess relative to caldendrin (CaM_4 and CaM_8 in Fig. 5c).

AKAP79-B has different interaction mechanism and binding kinetics with caldendrin and CaM

To confirm the direct interaction and further investigate the mechanism and kinetics of the interactions between AKAP79-B and caldendrin or CaM, SPR biosensor technology was used. We have previously employed this approach to identify and characterize neural protein-protein interactions between Piccolo/Aczonin, CAST1, and Munc13-1, proteins of the presynaptic active zone (Wang *et al.* 2009). For this study, several combinations of immobilized and interacting proteins in solution were initially tested. Immobilizing 6xHis-SUMO-AKAP79-B and studying the interactions with GST-caldendrin-C-terminus and CaM in solution provided highest data quality and was chosen for the SPR-based measurements.

The interaction between AKAP79-B domain and both Ca^{2+} sensor proteins was clearly detected (Fig. 6). Binding to CaM appeared to follow a simple reversible 1-step mechanism (Fig. 6a). The interaction between AKAP79-B domain and caldendrin differed qualitatively and was more complex (Fig. 6b).

AKAP79/150 co-immunoprecipitates with caldendrin

The data presented so far show that AKAP79 and caldendrin interact directly *in vitro*. To confirm the interaction between endogenous proteins we used antibodies targeted against caldendrin to immunoprecipitate caldendrin from rat brain fractions and tested for AKAP150 co-immunoprecipitation.

In Ca^{2+} -deprived membrane fractions caldendrin co-precipitated AKAP150 (Fig. 7), suggesting that both proteins associate at neuronal membranes under low Ca^{2+} conditions. However, no co-immunoprecipitation of AKAP150 was found under high Ca^{2+}, indicating that under these conditions other endogenous binding partners using the same AKAP79/150 interaction site might outcompete caldendrin.

Although caldendrin as well as AKAP150 immunoreactivity were found in cytosolic fractions, no clear co-immunoprecipitation result could be achieved, since the bound control IgG fraction also contained small amounts of AKAP150 (data not shown).

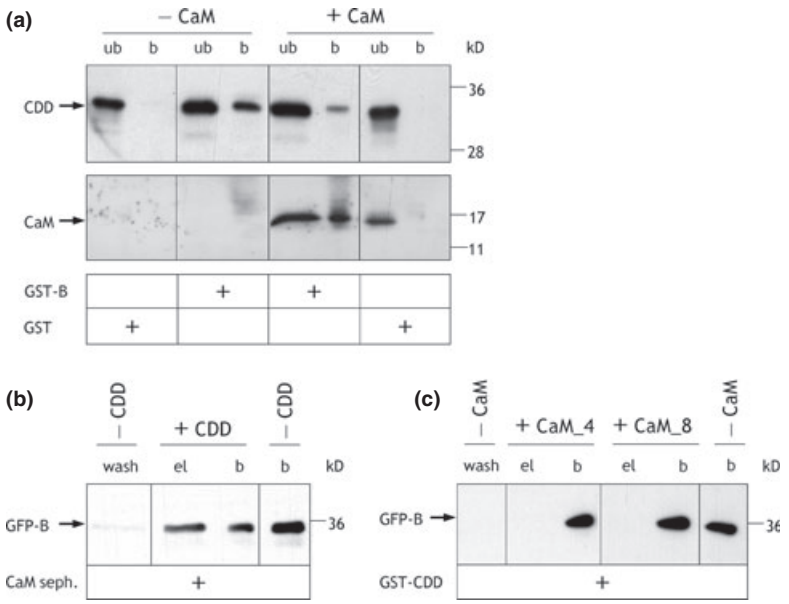


Fig. 5 Caldendrin and Calmodulin (CaM) compete for binding to A kinase-anchoring protein AKAP79-B in the presence of Ca^{2+}. (a) Competition between caldendrin and CaM for AKAP binding. In the presence of equimolar amounts of CaM, caldendrin binding to GST-AKAP79-B (GST-B) is clearly reduced, while CaM appears in the bound fraction. The blot is stained with anti-caldendrin (CDD) and subsequently with anti-CaM. Control experiments with GST do not show any binding of either caldendrin or CaM. (b) Caldendrin is able to elute a significant amount of AKAP79-B from CaM sepharose. After GFP-AKAP79-B binding to CaM sepharose, addition of caldendrin releases AKAP79-B from CaM. Only faint amounts of AKAP79-B are found in the caldendrin-free wash fraction. (c) No elution of AKAP79-B from GST-caldendrin-C-term (GST-CDD-C-term) by addition of excess amounts of CaM is detectable. Binding of GFP-AKAP79-B to GST-Caldendrin-C-term cannot be disturbed by the addition of a fourfold (CaM_4) or even eightfold (CaM_8) excess of soluble CaM in relation to GST-caldendrin-C-term. ub – unbound fraction; b – bound fraction; wash –third wash after binding in the presence of Ca^{2+}; el – eluate; blots are stained with anti-caldendrin (CDD; upper blot in a), anti-CaM (lower blot in a), or anti-GFP (b, c). Experiments were repeated not less than three times.

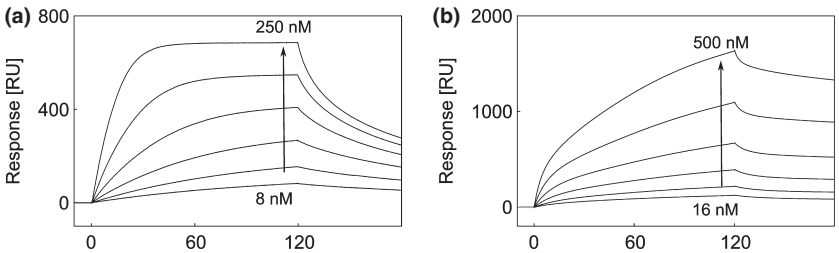


Fig. 6 Surface plasmon resonance (SPR) biosensor measurements showing the interaction between Calmodulin and caldendrin with SUMO-AKAP-B (a) Calmodulin was injected in twofold diluted concentration series (250–8 nM) over immobilized 6xHis-SUMO-AKAP79-B in the presence of 2 mM Ca^{2+}. (b) Caldendrin was injected as GST-caldendrin-C-term fusion protein (500–16 nM) under the same conditions as in (a). The experiment was performed in triplicate and the data were double referenced (blank data from two control experiments, either without analyte or without ligand, were subtracted from raw data). Note the different scales for the Response Units (RU) in the sensorgrams in (a) and (b) indicating different binding intensities. *X*-axis: time (sec).

AKAP150 and caldendrin co-localize in primary hippocampal neurons

To prove *in vivo* relevance of our biochemical findings, we assessed a possible co-expression of both proteins in neurons. To this end, rat hippocampal neurons at DIV21 were immunostained with AKAP150, caldendrin and PSD-95 antibodies as marker for postsynaptic densities to study co-distribution or co-localization of both proteins at postsynaptic sites. As assessed by confocal microscopy, immunostaining of AKAP150 and caldendrin shows a co-localization in a subset of puncta (Fig. 8). Some of these puncta overlap with PSD-95 immunofluorescence, pointing to a co-localization of AKAP150 and caldendrin at the same postsynaptic structures. Besides the postsynaptic enrichment, the expres-

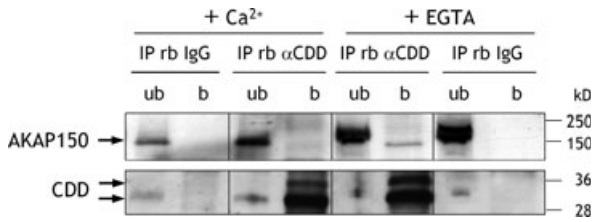


Fig. 7 Endogenous A kinase-anchoring protein AKAP150 and caldendrin associate under low Ca^{2+} conditions as shown by co-immunoprecipitation. Rabbit anti-caldendrin-C-term (rb αCDD) antibodies co-immunoprecipitate AKAP150 from membrane fractions of rat brain under Ca^{2+} -free conditions, but not in the presence of Ca^{2+}. Note the Ca^{2+} -induced migration shift of caldendrin. The blot is representative for four independent experiments. IP – immunoprecipitation; ub – unbound fraction; b – bound fraction; blots are stained with anti-AKAP150 (upper blot) and subsequently with anti-caldendrin (lower blot).

sion of both proteins was relatively wide-spread in the soma and dendrites of neurons. Changes in intracellular Ca^{2+} (ionomycin or BAPTA treatment prior to immunostaining; data not shown) had no obvious effect on the degree of co-localization and therefore were not followed up in detail.

Discussion

AKAP79/150 and its associated proteins constitute a signalosome-like protein assembly in the PSD. This complex alliance couples Ca^{2+} channels, glutamate receptors and adrenergic receptors, which transduce postsynaptic signals, either directly or via scaffolding proteins, to effector molecules, most prominently kinases and phosphatases. These enzymes functionally modulate the channels and receptors in correspondence to the respective signaling events important for excitatory synaptic plasticity [for review see (Sanderson and Dell'Acqua 2011)]. In these processes, like long-term potentiation (LTP) or long-term depression (LTD), the level, gradient, and duration of Ca^{2+} fluxes represent key signals.

Here, we characterized the interaction between AKAP79/150 and two important Ca^{2+} -binding proteins. We confirmed the second predicted CaM-binding site in AKAP79 and identified caldendrin as a novel neuronal AKAP79/150 binding partner. Using immunocytochemistry, we demonstrated co-localization of AKAP150 and caldendrin in the same synapses of mature primary neurons. Furthermore, we could show that both EF-hand proteins bind to AKAP79 at an overlapping binding site, and our data point to a competitive mechanism within the AKAP signalosome.

Two partners, one binding site, but different binding properties

Although caldendrin binding is also dependent on the presence of the sequence essential for CaM binding, caldendrin needs a longer binding interface, encompassing

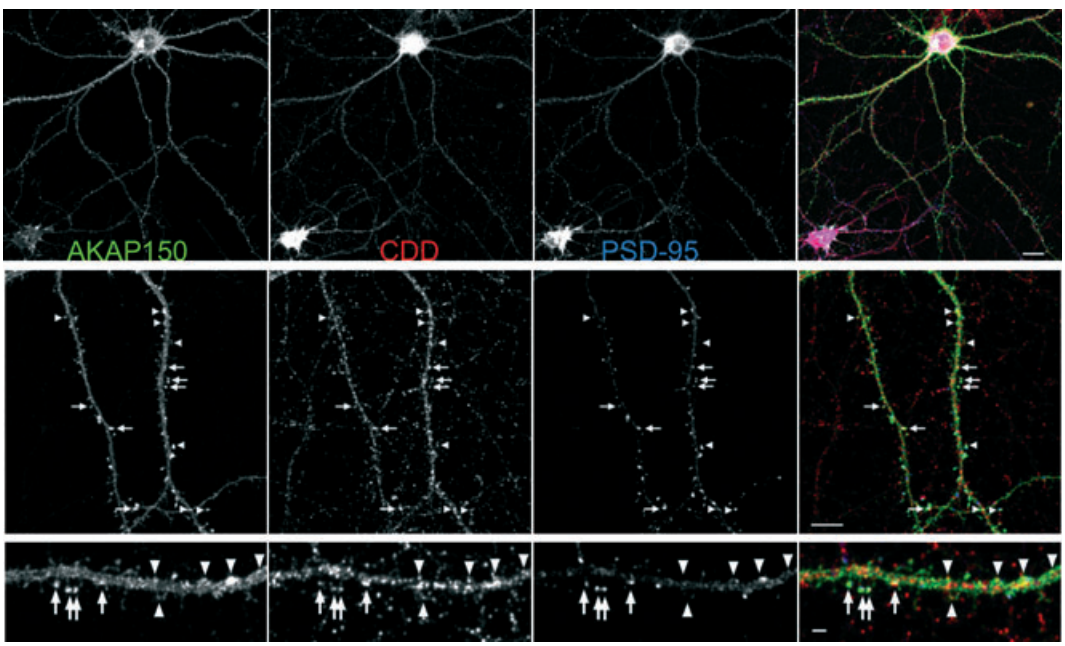


Fig. 8 Confocal microscopy shows that A kinase-anchoring protein AKAP150 and caldendrin are co-expressed in primary hippocampal neurons at DIV21 and co-localize in dendrites and in a subset of spines. Arrows point to puncta of co-localization of AKAP150 (green in merged picture), caldendrin (CDD; red in merged picture) and Post-synaptic density PSD-95 (blue in merged picture), suggesting a co-localization in postsynaptic densities, whereas arrowheads highlight locations of overlapping AKAP150 and caldendrin staining at PSD-95 negative sites. Scale bars: upper panel 20 μm, middle panel 10 μm, lower panel 2 μm. Stainings were repeated at least four times.

amino acids 61–97 of AKAP79 (Fig. 1a, AKAP79-B1/2). We speculate that the extended peptide binds to amino acid residues unique to caldendrin and facilitates the formation of a stably bound complex of both proteins (see scheme in Fig. 9).

Caldendrin and CaM bind to their interaction partners in either a Ca^{2+} -dependent or -independent manner (Mikhaylova *et al.* 2011). Pull-down experiments showed that the binding of CaM to AKAP79-B is clearly dependent on the presence of Ca^{2+} and becomes completely abolished when Ca^{2+} is missing. The interaction of caldendrin with AKAP79-B is supported by the presence of Ca^{2+} but also occurs in absence of Ca^{2+}. The SPR biosensor analyses fully support these results; under low Ca^{2+} conditions the binding mechanism is conserved but signal intensities are reduced (Seeger *et al.*, unpblished data).

As only the C-terminal EF hand pair of caldendrin/CaBP1 performs a conformational change upon Ca^{2+} binding, it was speculated that this part might be responsible for establishing Ca^{2+} dependent contacts with target proteins, whereas the N-terminal EF hand pair confers the ability of CaBP1 to constitutively bind to its targets in the absence of Ca^{2+} (Wingard *et al.* 2005). This might also be the case for the interaction between caldendrin and AKAP79/150 and might explain the differences regarding the interaction between caldendrin and AKAP79-B in the presence and absence of Ca^{2+}.

A model for the triangular liaison of caldendrin and CaM with AKAP

In our pull-down competition experiments caldendrin and CaM compete for binding to AKAP79-B in the presence of Ca^{2+}. This is supported by the SPR biosensor-based interaction experiments (Seeger *et al.*, unpublished data). The

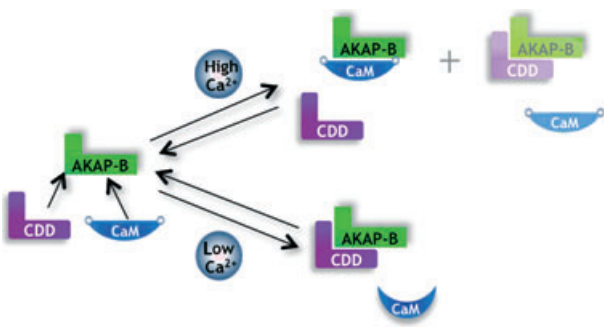


Fig. 9 Model of interactions between A kinase-anchoring protein AKAP79-B and the Ca^{2+} sensors Calmodulin (CaM) and caldendrin under different Ca^{2+} conditions. CaM and caldendrin (CDD) compete for binding to AKAP79-B (AKAP-B). As CaM is highly abundant in neurons, we speculate that it outcompetes caldendrin under high Ca^{2+} conditions (the AKAP-B-CDD complex is therefore shown in pale colors). Under resting conditions, when Ca^{2+} concentrations in the neuron are low, caldendrin but not CaM is able to bind AKAP79.

Ca^{2+}concentrations in dendrites vary vastly in time and space. These phenomena are referred to as Ca^{2+}waves, spikes, transients, and puffs (Berridge 1998; Mikhaylova *et al.* 2011). In micro- or even nanodomains in the direct vicinity of Ca^{2+} channels, Ca^{2+} concentrations can reach 6–16 μM within milliseconds and go back to resting levels in the nanomolar range (50–100 nM) within seconds (Kubota and Waxham 2010). As the Ca^{2+} affinity of caldendrin C-terminus and the shorter isoform s-CaBP1 is higher than that of CaM (2.5 μM and 5–10 μM, respectively (Mikhaylova *et al.* 2011), we assume that at *in vivo* Ca^{2+} concentrations below the Ca^{2+} affinity of CaM, caldendrin will be the preferential binding partner for AKAP79 and establish a tight interaction, either in a Ca^{2+} -bound or -unbound state (Fig. 9).

The assumption that caldendrin preferentially binds to AKAP79/150 under low Ca^{2+} conditions *in vivo* is further supported by our co-immunoprecipitation results, that also point to an association of caldendrin and AKAP79/150 at neuronal membranes under Ca^{2+} -free conditions. At this point, further experiments need to be done to verify this hypothesis.

In contrast to CaM, caldendrin/CaBP1 tends to dimerize via its C-terminal part (Wingard *et al.* 2005; Fries *et al.* 2010). This might account for structural heterogeneity of caldendrin, but not CaM. It is not known, whether these homodimers are capable of simultaneously binding to AKAP79/150 or if homodimerization excludes further interaction with AKAP79/150 (Fries *et al.* 2010; Mikhaylova *et al.* 2011). Recently it was shown that AKAP79 forms homo-oligomers (Gao *et al.* 2011), and that one AKAP79 homodimer can cluster two CaM molecules, two PKA-RII homodimers, and four PP2B heterodimers (Gold *et al.* 2011). Therefore, we speculate that the *in vivo* complex assembled by an AKAP79 homodimer could, under certain Ca^{2+} conditions, contain a caldendrin homodimer instead of two CaM proteins or even both calcium sensors at the same time, as CaM can also bind to the more N-terminally located binding site in the AKAP79 subdomain A (Faux and Scott 1997).

Cell-biological context of the AKAP79/150 interactions with both Ca^{2+} sensors

In the model shown in Fig. 9 we propose that under resting conditions caldendrin will bind to AKAP79. Competition with CaM can be expected to occur only under high Ca^{2+} conditions. The binding of either calcium sensor might have different consequences for the conformation of the scaffolder like it was proposed for instance for CaM binding to MaGUKs (Paarmann *et al.* 2008).

Most interestingly, caldendrin, CaM, and AKAP79/150 have one common binding partner, the L-type voltage-gated Ca^{2+} channel $Ca_V1.2$. Both Ca^{2+} sensors, caldendrin/CaBP1 and CaM, bind to a C-terminal IQ-domain in the $\alpha_1$1.2 -

subunit of the channel. Via binding they regulate channel properties differently, with CaM promoting a negative feedback mechanism called Ca^{2+} -dependent inactivation of the channel, while caldendrin/CaBP1 opposes this mechanism and stabilizes channel opening (Zhou *et al.* 2004, 2005; Tippens and Lee 2007).

AKAP79/150 and the Ca^{2+} channel $Ca_V1.2$ bind directly to each other via modified leucine zipper motifs in both proteins (Oliveria *et al.* 2007). Via this mechanism AKAP79/150-bound CaN/PP2B is linked to the channel. Upon Ca^{2+} influx through the channel, CaN/PP2B becomes activated and dephosphorylates the transcription factor NFATc4, which subsequently translocates to the nucleus (Oliveria *et al.* 2007). It is therefore conceivable that a CaM/caldendrin Ca^{2+} -relay module might be involved in fine-tuning of this process.

The AKAP79/150 N-terminal region encompassing the caldendrin/CaM-binding interface is furthermore implicated in the association with membrane phospholipids, the actin cytoskeleton, cadherins, the beta-2-adrenergic receptor, and M-type KCNQ2/3 K^+ channels (Li *et al.* 1996; Dell'Acqua *et al.* 1998; Fraser *et al.* 2000; Gomez *et al.* 2002; Gorski *et al.* 2005; Bal *et al.* 2010; Delint-Ramirez *et al.* 2011; Mikhaylova *et al.* 2011).

Gomez *et al.* observed that binding of CaM to the N-terminal part of AKAP79 (aa 1-153) prevents F-actin binding and subsequent co-sedimentation (Gomez *et al.* 2002). On the other hand, pre-incubation of the N-terminal AKAP79 fragment with F-actin significantly attenuates the ability of CaM to release the AKAP fragment from F-actin (Gomez *et al.* 2002). In our experiments, caldendrin released AKAP79-B from a pre-formed CaM-AKAP complex. Moreover, CaM was not able to release AKAP79-B bound to caldendrin. This points to a rather stable interaction between AKAP79 and caldendrin, as compared to CaM-AKAP79 complexes, which seem to have higher on/off rates, thus enabling caldendrin to compete.

In AKAP79/150 the binding sites of the KCNQ2 channel and CaM overlap. The same is true for the binding sites of AKAP79/150 and CaM in KCNQ2. Co-expression of all three proteins disrupted the association between AKAP79 and KCNQ2. Bal and colleagues found that this disruption was because of competition of AKAP79 and CaM for binding to KCNQ2 (Bal *et al.* 2010). It will be interesting to test if association with these components might interfere with caldendrin binding or if caldendrin has any impact on the binding of AKAP79/150 to these other partners.

Binding of F-actin to AKAP79 is regulated by PKC phosphorylation of the AKAP79 N-terminal part. CaM binding to AKAP79 (1–153) can prevent its phosphorylation by PKC (Gomez *et al.* 2002). We cannot exclude that phosphorylation might also play a critical regulatory role in the interaction between AKAP79 and caldendrin, but so far we have no evidence for phosphorylation dependence in our experiments. Nevertheless, this interesting point should be explored in future experiments.

Conclusion

Our study shows that the postsynaptic scaffold molecule AKAP79, a key regulator of postsynaptic signaling events via regulating the phosphorylation or dephosphorylation of receptors, can interact with caldendrin, a neuronal calcium sensor implicated in regulation of Ca^{2+} -influx and release. In competition with calmodulin it is an interesting novel player in the signalosome assembled by AKAP79/150 at postsynaptic sites.

Acknowledgements

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