Neuronal Ca\(^{2+}\) signaling via caldendrin and calneurons

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Abstract

The calcium sensor protein caldendrin is abundantly expressed in neurons and is thought to play an important role in different aspects of synapto-dendritic Ca\(^{2+}\) signaling. Caldendrin is highly abundant in the postsynaptic density of a subset of excitatory synapses in brain and its distinct localization raises several decisive questions about its function. Previous work suggests that caldendrin is tightly associated with Ca\(^{2+}\) - and Ca\(^{2+}\) release channels and might be involved in different aspects of the organization of the postsynaptic scaffold as well as with synapse-to-nucleus communication. In this report we introduce two new EF-hand calcium sensor proteins termed calneurons that apart from calmodulin represent the closest homologues of caldendrin in brain. Calneurons have a different EF-hand organization than other calcium sensor proteins, are prominently expressed in neurons and will presumably bind Ca\(^{2+}\) with higher affinity than caldendrin. Despite some significant structural differences it is conceivable that they are involved in similar Ca\(^{2+}\) regulated processes like caldendrin and neuronal calcium sensor proteins.

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1. Introduction

The postsynaptic density (PSD) of spinous excitatory synapses is characterized by an electron-dense filamentous meshwork of cytoskeletal proteins that are thought to be crucially involved in the topological organization of synaptic signaling pathways [1]. In a search for protein components of the PSD we identified a calcium sensor protein subsequently called caldendrin that was highly abundant in the somato-dendritic compartment of mainly principal neurons in brain regions with a laminar organization like cortex or hippocampus [2]. Caldendrin harbors a bipartite structure with an N-terminus not related to entries in public databases that is highly basic and a C-terminal part that resembles the EF-hand-structure of calmodulin with four EF-hands from which the second one is cryptic [2,3] (Fig. 1A and B). With respect to its C-terminal part caldendrin is the closest homologue of calmodulin (CaM) expressed in brain, and it turned out to be the founding member of an entire gene family termed CaBPs by Haeseleer and colleagues [4,5]. The family derives from five different genes that give rise to a number of differently spliced isoforms. In case of caldendrin two shorter isoforms were identified termed caldendrin S1 and S2 [3] or L-CaBP1 or S-CaBP1 [4]. These isoforms arise by usage of an alternative exon downstream of exon 1 of the caldendrin gene, which harbors an alternative start codon and thereby generates an N-terminus that is shorter than that of the long form [3]. Another splice variant derives from the insertion of a third exon located further downstream of exon 2 [3]. All

\textit{Abbreviations: ANS, 8-Anilino-1-naphthalene sulfonic acid; Ca\(^{2+}\), Calcium; CaM, Calmodulin; ITC, Isothermal titration calorimetry; Mg\(^{2+}\), Magnesium; NCS, neuronal calcium sensor}

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caldendrin promoter is unique within the gene family driving expression in mainly principal neurons like pyramidal cells. During the primary characterization of the protein a number of other interesting features could be revealed. In brain, caldendrin is highly enriched in the PSD fraction [2] and immunolocalized to dendritic spines and the dendritic shaft [2,3,8,9]. The protein occurs as two protein isoforms of 33 kDa and 36 kDa, which derive from the same primary translation product [2]. Upon subcellular fractionation of rat brain proteins, a significant amount of the 33 kDa isoform is found in both the soluble as well as the detergent-insoluble particulate fraction. In contrast the 36 kDa protein isoform is tightly associated with the cortical cytoskeleton and extraction conditions for the 36 kDa isoform from the particulate fraction are similar to those of core components of the PSD [2,3]. Both its unique bipartite structure and the differential association with subcellular compartments therefore suggest that caldendrin serves functions different from those of known neural Ca2+ sensor proteins. Previous work suggests that this function might be related to the organization of the postsynaptic scaffold [2,3,10,11] as well as the control of NMDA-receptor activated morphogenetic signaling to the nucleus [Dieterich et al., unpublished results]. Of particular interest is also the observation that caldendrin’s association with the PSD is stimulus-dependent and that enhanced synaptic activity leads to higher caldendrin-levels in the PSD and a recruitment to the postsynapse in vivo and in vitro [10,12,13]. During the course of its functional characterization a number of potential interaction partners for caldendrin and its shorter splice isoforms were identified that constitute Ca2+-channels and it has been therefore hypothesized that caldendrin might be a multifunctional regulator of intracellular Ca2+ levels [14]. Interactions of caldendrin/CaBP1 have been reported with TRPC [15], CaV2.1 [16], CaV1.2 Ca2+ channels [17,18] as well as with the inositol trisphosphate (InsP3) receptor (InsP3Rs) [19,20]. The functional consequences of caldendrin binding to these channels range from a facilitation of presumably postsynaptic L-type-CaV1.2 Ca2+ channels [17] and thereby increased synaptic Ca2+ influx to an inhibition of InsP3-induced Ca2+ release through InsP3Rs [19,20]. Interestingly the interaction of caldendrin with Ca2+-channels is in most cases Ca2+ independent (i.e. InsP3Rs, CaV2.1 and presumably CaV1.2 Ca2+ channels). Based on these findings, we propose that caldendrin depending upon its activity-dependent localization to different subcellular microdomains like the synapse might trigger different target interactions. Binding to L-type voltage-dependent CaV1.2 Ca2+ channels at the synapse will probably lead to increased Ca2+ currents following synaptic activation and thereby can indirectly promote caldendrin’s association with the PSD. In contrast, low synaptic activity and in consequence low dendritic Ca2+ levels might shift the balance of caldendrin binding to a preferential interaction with the InsP3R, which is more prominently localized to the smooth ER in dendrites than in spine synapses. Binding to the InsP3R will thereafter further reduce intracellular Ca2+ levels. Thus, caldendrin will potentially trigger different target interactions in a manner that is controlled by synaptic activity and its subcellular localization and in consequence could even amplify existing differences in synapto-dendritic Ca2+ signaling within a

![Fig. 1. (A) Alignment of the amino acid sequence of rat caldendrin (only the C-terminal half) with rat calneuron I and II. Sequences were aligned using the Multalin program (http://prodes.toulouse.inra.fr/multalin). Putative EF-hand motifs (EF1–EF4) are represented as boxes. Amino acids in red are identical in all three proteins, amino acids in blue only in calneuron I and II. (B) Schematic diagram depicting the EF-hand organization of different calcium sensor proteins that are abundantly expressed in brain and retina. N-terminal myristoylation is depicted as a round circle, non-functional EF-hand motif as a filled box, and N- or C-terminal extensions are represented in the appropriate proteins. CaM, GCAPs and NCS-1 represent for calmodulin, Guanylyl cyclase-activating protein and neuronal calcium sensor-1 respectively.](image)

Caldendrin/CaBP1 family members share the same EF-hand organization while their N-termini vary considerably in length and structure (Fig. 1B). The functional implication of this variability is at present unclear but it is conceivable that the different N-termini might be important for the subcellular distribution of the proteins. It should also be noted in this regard that CaBPs but not caldendrin can be N-terminal myristoylated, which could provide a lipid anchor to cellular membranes (Fig. 1B).

Surprisingly, apart from caldendrin all other family members are very low abundant in brain although they could be localized to neuronal cells in retina [4–7]. Thus, it seems that the caldendrin promoter is unique within the gene family driving expression in mainly principal neurons like pyramidal cells. During the primary characterization of the protein a number of other interesting features could be revealed. In brain, caldendrin is highly enriched in the PSD fraction [2] and immunolocalized to dendritic spines and the dendritic shaft [2,3,8,9]. The protein occurs as two protein isoforms of 33 kDa and 36 kDa, which derive from the same primary translation product [2]. Upon subcellular fractionation of rat brain proteins, a significant amount of the 33 kDa isoform is found in both the soluble as well as the detergent-insoluble particulate fraction. In contrast the 36 kDa protein isoform is tightly associated with the cortical cytoskeleton and extraction conditions for the 36 kDa isoform from the particulate fraction are similar to those of core components of the PSD [2,3]. Both its unique bipartite structure and the differential association with subcellular compartments therefore suggest that caldendrin serves functions different from those of known neural Ca2+ sensor proteins. Previous work suggests that this function might be related to the organization of the postsynaptic scaffold [2,3,10,11] as well as the control of NMDA-receptor activated morphogenetic signaling to the nucleus [Dieterich et al., unpublished results]. Of particular interest is also the observation that caldendrin’s association with the PSD is stimulus-dependent and that enhanced synaptic activity leads to higher caldendrin-levels in the PSD and a recruitment to the postsynapse in vivo and in vitro [10,12,13]. During the course of its functional characterization a number of potential interaction partners for caldendrin and its shorter splice isoforms were identified that constitute Ca2+-channels and it has been therefore hypothesized that caldendrin might be a multifunctional regulator of intracellular Ca2+ levels [14]. Interactions of caldendrin/CaBP1 have been reported with TRPC [15], CaV2.1 [16], CaV1.2 Ca2+ channels [17,18] as well as with the inositol trisphosphate (InsP3) receptor (InsP3Rs) [19,20]. The functional consequences of caldendrin binding to these channels range from a facilitation of presumably postsynaptic L-type-CaV1.2 Ca2+ channels [17] and thereby increased synaptic Ca2+ influx to an inhibition of InsP3-induced Ca2+ release through InsP3Rs [19,20]. Interestingly the interaction of caldendrin with Ca2+-channels is in most cases Ca2+ independent (i.e. InsP3Rs, CaV2.1 and presumably CaV1.2 Ca2+ channels). Based on these findings, we propose that caldendrin depending upon its activity-dependent localization to different subcellular microdomains like the synapse might trigger different target interactions. Binding to L-type voltage-dependent CaV1.2 Ca2+ channels at the synapse will probably lead to increased Ca2+ currents following synaptic activation and thereby can indirectly promote caldendrin’s association with the PSD. In contrast, low synaptic activity and in consequence low dendritic Ca2+ levels might shift the balance of caldendrin binding to a preferential interaction with the InsP3R, which is more prominently localized to the smooth ER in dendrites than in spine synapses. Binding to the InsP3R will thereafter further reduce intracellular Ca2+ levels. Thus, caldendrin will potentially trigger different target interactions in a manner that is controlled by synaptic activity and its subcellular localization and in consequence could even amplify existing differences in synapto-dendritic Ca2+ signaling within a
neuron. Interestingly, this in turn could possibly also feed back to caldendrin’s own Ca\(^{2+}\) dependent target interactions in the synapse and to its suggested role in synapse-to-nucleus communication.

Taken together the evidence so far points to an important role of the protein in many aspects of neuronal Ca\(^{2+}\) signaling and it was therefore rather surprising that the shorter caldendrin splice isoforms and the other CaBP family members that share the same EF-hand organization and show a high degree of homology are only prominent in retina. A search in public databases, however, revealed several EST- and cDNA clones from brain tissue that show significant similarity to the first two EF-hands of caldendrin/CaBPs and CaM.

2. The primary structure of caldendrin and calneurons

Further analysis of these clones disclosed the existence of two highly homologues proteins from which the sequence of one of them has been previously published under the name of calneuron [21], whereas the other was termed CaBP7 [5]. Based on our initial characterization (see below) we decided to introduce the name Calneuron for both proteins to indicate that they are Ca\(^{2+}\) binding proteins prominently present in neurons and constitute a new subfamily of CaM-like calcium sensors closely related to but distant from caldendrin/CaBPs. We cloned calneuron I (Accession number: XM344102) and II (Accession number: AY841152) using RT-PCR from rat brain and a subsequent alignment of their amino acid sequence revealed that they are highly homologous to each other (Fig. 1A). Calneuron I encompasses an open reading frame of 219 aa and calneuron II of 215 aa with an overall identity of 63% between both of them (Fig. 1A). They have a short N-terminal region flanking the EF-hands but interestingly also a 38 amino acid long extension of its C-terminus as compared to caldendrin (Fig. 1A). This extension is uncommon in neuronal calcium sensor proteins and a unique feature of the calneuron structure [22]. CaM, the archetypical calcium sensor, consists of two canonical EF-hand domains tethered by a flexible linker. As depicted in Fig. 1B, caldendrin has only three functional EF-hands, i.e. 1, 3 and 4, whereas EF-hand 2 does not bind Ca\(^{2+}\). In calneuron I and II only the first two EF-hands fulfill the criteria for a canonical Ca\(^{2+}\) binding EF-hand. Based on sequence comparison, it appears that there is a deletion of three residues in EF-hand 3 of Calneurons, and Asp is replaced by Ser at the \(\pm x\) coordinate position (first coordinating residue of the loop) in EF-hand 4 which makes both of them most likely incapable to chelate Ca\(^{2+}\) or other cations. Interestingly, the presence of an Asp at the \(\pm z\) coordinating position in EF-hand 1 (12th residue of the EF-hand loop) of caldendrin decreases its specificity for Ca\(^{2+}\) and it has been shown that it most likely exists in a constitutively Mg\(^{2+}\) bound form [23]. This is at variance with calneurons that have a Glu at this position and it can therefore be expected that their two functional EF-hands are high-affinity Ca\(^{2+}\) binding sites.

Moreover, the organization of functional EF-hands is unique for calneurons and does not match those of other neural calcium sensor proteins (Fig. 1B) [22]. The presence of non-functional EF-hands is a common feature of neuronal calcium sensor proteins (Fig. 1B) and it is thought to play an important structural role for the dynamics and specificity of their target interactions. It can be therefore concluded that the C-terminal non-functional EF-hand like structures might be significant for their cellular function. Interestingly, databank entries show that the amino acid sequence of calneuron I and II is 100% identical between human, rat and mouse. This high degree of conservation clearly supports the idea that also the c-terminal half of calneurons has some functional significance related to its structure. Of particular interest is the observation that in contrast to all CaBP family members as well as all to the closely related neuronal calcium sensor (NCS) proteins, caldendrin and calneurons lack a N-myristoylation motif that can provide a lipid anchor for membrane attachment (Fig. 1B). In conjunction with a bootstrap analysis of neighbor joining distances (data not shown), which showed that although caldendrin, CaBPs and calneurons are closely related the phylogenetic tree also indicates that Calneurons constitute a new subfamily of CaM-related EF-hand calcium sensors with a unique EF-hand organization.

3. Calneurons are in contrast to CaBP\(s\) abundant in brain

To initially address the important question whether calneurons are like caldendrin prominently expressed in brain we performed \textit{in situ} hybridization studies to localize calneuron transcripts in rat brain sections. These studies revealed that calneuron I and II mRNA are indeed present in rat brain with an expression pattern that exhibits partial overlap with those of caldendrin. Calneuron I transcripts have a widespread distribution with intense hybridization signals in the cerebellum but is also present in many other brain regions like cortex and hippocampus where high levels of caldendrin transcripts are found (Fig. 2A). This is in contrast to the expression of calneuron II, which is restricted to the CA3 region of the hippocampus, entorhinal cortex, the antero-dorsal and antero-ventral thalamus as well as the inferior and superior colliculus (Fig. 2A). We next generated antibodies to calneuron I and II to perform immunoblots and immunocytochemical stainings of hippocampal primary neurons. Subcellular fractionation studies of rat brain homogenates revealed expectedly that calneurons are exclusively present in soluble fractions with no particular enrichment in any other insoluble subcellular compartment like the PSD (Fig. 2B). Subsequent double immunofluorescence stainings of hippocampal primary neurons with the neuronal marker protein MAP2 confirmed a neuronal localization for both calneurons (Fig. 2C). Thus, calneurons are abundantly expressed in neurons of rat brain, show a partial overlap with the expression of caldendrin but are in contrast to the latter not prominently associated with particulate fractions and the PSD.

4. Ca\(^{2+}\) binding and Ca\(^{2+}\) induced conformational changes of caldendrin and calneuron 1

We next generated recombinant caldendrin and calneuron I to determine and compare their Ca\(^{2+}\) binding affinity by isothermal titration calorimetry (ITC). The binding isotherms
of Ca\(^{2+}\) binding to caldendrin and calneuron I are depicted in Fig. 3A and B. The resulting calorimetric isotherms were deconvoluted for the best-fit model using the Origin software package supplied by Microcal and the thermodynamic parameters for binding reactions obtained under our experimental conditions are listed in Table 1. Ca\(^{2+}\) binding to caldendrin as revealed by ITC is an exothermic reaction and follows a two-site model with dissociation constants of about 2 \(\mu\)M (for high affinity sites) and 40 \(\mu\)M (for a low affinity site) calculated at 30 °C (Table 1). Wingard et al. [23] reported a dissociation constant of CaBP1 for Ca\(^{2+}\) of about 2.5 \(\mu\)M (at 25 °C) which is consistent with our data for caldendrin. They also suggested that three Ca\(^{2+}\) binding sites exist in caldendrin/CalBP1 where EF-hand 3 and 4 are the high affinity sites, while EF-hand 1 as predicted from the sequence binds Ca\(^{2+}\) only with very low affinity [24].

Ca\(^{2+}\) binding to calneuron I by ITC follows a one set of site model with a dissociation constant in the range of 0.2–0.5 \(\mu\)M (Fig. 3B) which is significantly higher than the affinity of caldendrin but in the range of Ca\(^{2+}\) binding affinities reported for NCS proteins like NCS-1 [25]. The binding isotherms indicate an exothermic nature, implying the release of energy upon Ca\(^{2+}\) binding to the protein. The thermodynamic parameters obtained for Ca\(^{2+}\) binding are shown in Table 1. Since the amino acid sequence of the first two EF-hands are largely identical in both calneurons, we assume that the Ca\(^{2+}\) binding to calneuron I is an exothermic reaction and follows a two-site model with dissociation constants of about 2 \(\mu\)M (for high affinity sites) and 40 \(\mu\)M (for a low affinity site) calculated at 30 °C (Table 1).
binding affinity of calneuron II will be similar to those of calneuron I.

We next investigated Ca^{2+} binding induced conformational changes in caldendrin and calneuron I. Since caldendrin has no Trp, it is not possible to monitor the changes in protein conformation using intrinsic fluorescence. We have therefore used 8-Anilino-1-naphthalene sulfonic acid (ANS) fluorescence to assess the changes in protein surface hydrophobicity upon Ca^{2+} binding. As shown in Fig. 4A, ANS binds caldendrin and exhibits a quite unusual broad emission at about 480–495 nm. The broad emission peaks that start at about 480 nm suggest ANS binds at high hydrophobic sites, whereas those at 495 nm suggest binding at moderately hydrophobic site. Addition of Ca^{2+} to the caldendrin-ANS complex decreased the fluorescence intensity marginally, suggesting that only minor changes in protein surface hydrophobicity occur upon Ca^{2+} binding (Fig. 4A). This is surprising and interesting for caldendrins interaction with other proteins since many Ca^{2+} dependent interactions of EF-hand proteins are supposed to be modulated by increased surface hydrophobicity upon Ca^{2+} binding. The relatively modest increase of surface hydrophobicity in caldendrin might be one reason why interactions of caldendrin to several proteins are Ca^{2+} independent and it will be

Fig. 3. (A) Calorimetric titration of caldendrin with Ca^{2+}. Protein concentration used was 30 μM and titrant concentration was 2 mM. The data fitting was performed using two sets of site model. (B) Calorimetric titrations of calneuron I with Ca^{2+}. Protein concentration was 25 μM and titrant concentration was 1 mM. Fitting was performed using one set of site model after subtracting the titration curve for buffer. All titrations were carried out at 30 °C in 50 mM Tris buffer, pH 7.2 containing 100 mM KCl.

Table 1
Thermodynamic parameters of Ca^{2+} binding to caldendrin and calneuron I by ITC. The binding experiments were performed in 50 mM Tris buffer, pH 7.2, containing 100 mM KCl at 30 °C.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Fitting model</th>
<th>Macroscopic binding constants (M^{-1})</th>
<th>Enthalpy (ΔH) (J/mole)</th>
<th>Entropy (ΔS) (kJ/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caldendrin</td>
<td>Two sites model</td>
<td>2.51E4±2.10 E4; 5.55 E6±4.20E4</td>
<td>−4.413E3±619; 5.58; −8.57</td>
<td></td>
</tr>
<tr>
<td>Calneuron I</td>
<td>One set of sites model</td>
<td>4.77 E6±2.16E6</td>
<td>−6694±321</td>
<td>8.48</td>
</tr>
</tbody>
</table>

Fig. 4. (A) Change in the fluorescence of the ANS/caldendrin complex upon addition of Ca^{2+}. ANS concentration was 100 μM; protein concentration 0.1 mg/ml. Ca^{2+} added was: 1–200 μM. The mixture was excited at 365 nm. (B) Steady state fluorescence of calneuron I at 295 nm excitation in 50 mM Tris buffer, pH 7.2, containing 100 mM KCl. Protein concentration was 80 μg/ml.
interesting to investigate how high affinity and Ca\(^{2+}\) dependent interactions are mediated. It is also interesting to note that the protein is suspected to be in a molten globule like state in the apo form as suggested Wingard et al. [23] and therefore the minor changes in surface hydrophobicity upon Ca\(^{2+}\) binding are clearly not expected.

Calneuron I harbors only one Trp at 124th position, which is not in close proximity of any functional EF-hand. We have utilized the Trp fluorescence to determine the changes in calneuron I conformation upon binding calcium. As shown in Fig. 4B, the emission maximum obtained for protein is about 340 nm suggesting that Trp is not deeply buried in the hydrophobic region. The fluorescence intensity decreases more substantially than in caldendrin upon addition of Ca\(^{2+}\) suggesting that Ca\(^{2+}\) binding imparts moderate changes in protein conformation.

5. Comparative modeling of caldendrin and calneurons EF-hand structures

We therefore looked more closely into the 3D structure of calneurons in comparison to those of caldendrin. The 3D structure of caldendrin is not yet known though the resonance assignments of apo, Mg\(^{2+}\) and Ca\(^{2+}\) bound forms of CaBP1 by NMR have been completed [23]. Using the Swiss Modeller server [25], we first generated a structural model of caldendrin (Fig. 5A). The topology of caldendrin appears different than the CaM structure since it does not show the typical CaM-like dumb-bell shaped topology, though it has a two-domain structure. Unlike CaM, which in the region joining both domains forms a long central helix, caldendrin appears to be more in a coiled-coil conformation in this region (Fig. 5A). Calneuron I is predicted to have a more CaM like topology (Fig. 5B). Interestingly, however, also in calneuron I the CaM-like central linker region seem to be different and might exhibit a more coiled-coil structure. As mentioned above, calneuron I has two functional EF-hands (EF-hand 1 and 2) located in the N-terminal domain whereas both non-functional EF-hands (3 and 4) are located at the C-terminal domain. Though non-functional, both of these EF-hands have typical helix–loop–helix conformations, which will, however, not bind Ca\(^{2+}\).

The intrinsic specificity of the CaM-fold is determined by each of the dumb-bell shaped halves and the various arrangements of the two halves upon target peptide binding. Ca\(^{2+}\) free EF hands sterically allow the complete closure of the concave hydrophobic target peptide binding pocket build by one double EF hand domain. The saturation of Ca\(^{2+}\) ions fixes the flexibility of both EF hand loops and shifts the equilibrium towards the open state. Mutated EF hand loops not capable to bind Ca\(^{2+}\) may exhibit either all time flexibility or stiffness depending on the type of residue mutation or deletion. Interestingly, caldendrin and calneurons show both types of mutated EF hands. In caldendrin the flexibility of the mutated second EF hand is increased by a mutation of a Ca\(^{2+}\) binding aspartate to glycine. The third EF hand in calneurons is in part deleted and mutated with hydrophobic side chains to make a short stiff conformation of the helix–loop–helix motif. The fourth EF hand remains flexible, which may be moderated by Ca\(^{2+}\) ions as three glutamic acids and in calneuron I an additional aspartate are located within this loop. In consequence, both double EF hand domains of caldendrin and calneurons may bind to target peptides, but using different protein dynamics. The first domains of calneurons and the second domain of caldendrin exhibit similar Ca\(^{2+}\) dependent binding properties as CaM, while the remaining domains require a high-affinity binding peptide, which forces the domain in an open conformation.
Interestingly, while looking more closely at the EF-hand structure, it appears that the calcium binding pockets in calneurons differ in cavity sizes thereby influencing the affinity for Ca\(^{2+}\) (Fig. 6). Leu48 (calneuron I) at the beginning of the EF-hand 1 loop is replaced by Phe45 (calneuron II), which fills up the cavity due to its large side chain (Fig. 6). Therefore calneuron II may preferentially bind to helices having a shorter hydrophobic side chain anchor (Fig. 6). The surface charge distribution is comparable in all three proteins. Notable differences are seen at the tip of the linking loop between the two EF-hands. Ser73 and Ala78 of calneuron I are replaced by Asn70 and Glu75 in calneuron II, respectively. Larger sequence deviations are also on the concave surface and as mentioned the helix, which joins the second double EF hand domain. In calneurons and CaM, the second EF-hand is more dependent on pH and it is likely, that the second EF-hand undergoes a larger conformational change at low Ca\(^{2+}\) concentrations. Interestingly, there is a tyrosine residue at the tip of the linking loop between EF-hand 1 and EF-hand 2 (calneuron I: Tyr70, calneuron II: Tyr67) and a corresponding Tyr was also noted for caldendrin [13]. The influence of this residue on the binding specificity for target interactions is unknown.

6. Conclusions and future perspectives

The primary characterization of calneurons raises several important issues concerning their diverse structure as compared to other calcium sensor proteins, their Ca\(^{2+}\) binding properties, their neuronal binding partners and their putative function for neuronal Ca\(^{2+}\) signaling. From the available data some interesting notions can be already deduced. In situ hybridization studies suggest that in many cases caldendrin and calneurons will not be expressed in the same neurons. Notable exceptions are the hippocampus where all three transcripts are abundantly present. Moreover, the expression of calneuron II is strikingly restricted which suggests rather specific functions in a subset of neurons. Calneuron I exhibits a more widespread distribution and it is therefore plausible that it will be co-expressed with caldendrin in the same cells. Since both calneurons are almost completely found in soluble protein fractions it can also already be predicted that they will in contrast to caldendrin predominantly target binding partners in the cytoplasm. Accordingly, the four to five times higher Ca\(^{2+}\) binding affinity of calneuron I as compared to caldendrin might reflect that a number of Ca\(^{2+}\) dependent interactions will occur already at much lower free Ca\(^{2+}\) concentrations than the Ca\(^{2+}\) dependent interactions of caldendrin that might happen in cell compartments like synapses which have very high Ca\(^{2+}\) transients. A decisive question to be addressed is whether caldendrin and calneurons share a similar set of binding partners and whether they can substitute for each other. A closer examination of known caldendrin/CaBP1 target structures reveals that they have no clear preference for one specific type of CaM binding sites. Comparative studies with calneurons might therefore help to learn more about the structural requirements for binding of both families, which will lead to a deeper appreciation of the molecular mechanisms. Another important step in the search of calneurons cellular function will be to understand the role of the long C-terminal extension in both proteins. Albeit EF-hands 3 and 4 are cryptic they seem to have a helix–loop–helix like structure and their amino acid sequence is highly conserved between different species. In conjunction with the 38 amino acid extension as compared to caldendrin/CaBPs and NCS proteins this part of calneuron might contribute to Ca\(^{2+}\) independent interactions via their helix–loop–helix structures or provide together with the first two EF-hands target specificity for certain binding partners.

7. Experimental procedures

7.1. Structural modeling

The 3D structures were generated using automated homology modeling on Swiss Modeller Server (http://swissmodel.expasy.org/SWISS-MODEL.html) with a high confidence score. The model generated was visualized and corrected using the SETOR program [27] on a Silicon Graphics machine. The structure is generated with suitable targets and energy minimization was performed on the developed model. The quality of the model and robustness was evaluated by performing molecular dynamics using the InsightII program. EF-hand structures of calneurons and caldendrin have been modeled using coordinates from the calcium saturated structure of human calmodulin (protein data bank, pdb entry: 1cll [28] and the Ca\(^{2+}\) free calmodulin-like skin protein CLSP [29].
7.2. Protein preparation

Recombinant caldendrin and calneuron were prepared as MBP-tagged proteins and the tag was removed by enzyme digestion. Calcium-free-protein was prepared using EDTA treatment followed by Chelex-100 treatment [24].

7.3. Steady-state fluorescence

Fluorescence emission spectra were recorded in the correct spectrum mode on a F-4500 Hitachi Fluorescence spectrophotometer at excitation wavelength of 295 nm as described previously [29]. Buffer solution was used was 50 mM Tris–HCl, pH 7.2 containing 100 mM KCl. The excitation and emission band passes were set at 5 nm.

7.4. 8-Anilino-1-naphthalene sulfonic acid (ANS) binding

ANS-binding experiments were performed by mixing the protein solution with ANS (100 μM). Fluorescence spectra were recorded by excitation at 365 nm in the correct spectrum mode on a fluorescence spectrofluorometer (model F-4500, Hitachi). Ca2+ was successively added in the protein ANS complex and the fluorescence recorded as described previously [24]. The spectra were corrected for ANS fluorescence in buffer without protein.

7.5. Ca2+ binding by isothermal titration calorimetry (ITC)

ITC measurements with caldendrin and calneuron I were performed using a Microcal Omega Titration Calorimeter (VP-ITC). Samples were centrifuged and degassed prior to titration. In case of titration of proteins with Ca2+, the protein samples were titrated until it reached saturation. A typical titration consisted of injecting 1.5 μl aliquots of 2 mM CaCl2 solution into 1.45 ml of the protein solution at a concentration of 20–50 μM. In a separate run, aliquots of ligand solution were injected into the buffer solution (without the protein), in order to subtract the heat of dilution. All titrations were carried out at 30 °C in 50 mM Tris–HCl buffer, pH 7.2 containing 100 mM KCl. All experiments were repeated twice or three times and the ITC data were analyzed using Origin (supplied with Omega Micro Calorimeter).

7.6. In situ hybridization, antibody production, immunostainings and immunoblotting

In situ hybridization was performed exactly as described previously [3]. Oligonucleotide sequences for Calneurons were as follows: calneuron I [antisense: 5′-gcg ctg ctc cga cag cag gtt ggg cac ggt gta ga-3′; sense control: 5′-gcg ctg ctc cga cag gtt ggg cac ggt gta ga-3′]; calneuron II: [antisense: 5′-gcg ctg ctc cga cag gtt ggg cac ggt gta ga-3′; sense control: 5′-tct aca ccc tgc tca ccc tgt cgg age ge-3′]. Sections treated with RNase H or hybridized with sense controls showed no signal above background. Antibodies directed against calneuron I and II were generated in rabbits using recombinant protein (Biogenes, Berlin, Germany). Antibodies were affinity-purified and their specificity was checked using standard procedures. Immunoblotting, multiple tissue western blots and subcellular fractionation experiments were done in accord to published protocols [2,3]. Double immunofluorescence stainings of hippocampal primary neurons were done as published previously [11] with an antibody dilution for calneuron I and II of 1:200.

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