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Association of Caldendrin splice isoforms with secretory vesicles in neurohypophyseal axons and the pituitary

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Abstract Caldendrin is a neuronal calcium-binding protein, which is highly enriched in the postsynaptic density fraction and exhibits a prominent somato-dendritic distribution in brain. Two additional splice variants derive from the caldendrin gene, which have unrelated N-termini and were previously only detected in the retina. We now show that these isoforms are present in neurohypophyseal axons and on secretory granules of endocrine cells. In light of the described interaction of the Caldendrin C-terminus with Q-type $Ca_v2.1$ calcium channels these data suggest that this interaction takes place in neurohypophyseal axons and pituitary cells indicating functions of the short splice variants in triggering Ca^{2+} transients to a vesicular target interaction.

Key words: EF-hand; Immunogold electron microscopy; Alternative splicing; Hormone release; Meuronal calcium sensor

1. Introduction

Caldendrin is a calcium-binding protein (CaBP) associated with the somato-dendritic cytoskeleton of mature neurons that is particularly enriched in the postsynaptic density (PSD)[1,2]. Like its closest relative Calmodulin (CaM) Caldendrin contains four EF-hand motifs as high affinity Ca^{2+}-binding sites, however, modeling of the EF-hand structure suggests that Caldendrin's surface charges exhibit an altered distribution as compared to CaM [3]. The N-terminal half of Caldendrin exhibits no similarity to other known proteins [1]. Because of its unique structure, the altered distribution of surface charges, its tight association with the cortical cytoskeleton and its enrichment in the PSD, it is assumed that Caldendrin fulfills functions different from those of hitherto characterized neuronal Ca^{2+} sensors [1–4].

Interestingly two alternatively spliced isoforms of Caldendrin also named CaBP1a+b have been identified recently [2,5]. These isoforms arise by usage of an alternative start codon on an exon downstream of exon 1 [2] and the insertion of another alternatively spliced exon downstream of exon 2 (Fig. 1). This results in the generation of two proteins with N-termini unrelated to Caldendrin, both harboring an *N*-myristoylation motif, fused to the C-terminus containing the EF-hand motifs (Fig. 1). To date, both splice isoforms were detected only at low levels in retina and sparsely in cerebellum [3,4], questioning a major physiological significance of this splicing event in brain. In a recent study in human brain we surprisingly found a prominent granular staining of hypothalamic axons [6]. In parallel two recent studies suggested that the C-terminal half of Caldendrin interacts with essential protein components of Ca^{2+}-dependent neuronal exocytosis, i.e. the $Ca_v2.1$ calcium channel and the inositol 1,4,5-triphosphate ($InsP_3$) receptor ($InsP_3R$) [7,8]. At present, however, the interaction with the P/Q-type calcium channel $Ca_v2.1$ is difficult to reconcile with the prominent somato-dendritic localization of Caldendrin and its clear absence from presynaptic structures in brain [1,2]. Therefore this unexpected observation prompted us to investigate the expression of Caldendrin splice variants in rat hypothalamus and pituitary in more detail.

2. Materials and methods

Adult Wistar and Lewis rats of both sexes, kept under normal laboratory conditions, were used for the investigation. Dissected tissue specimens from rat pituitary were quenched in isopentane precooled with liquid nitrogen, freeze-dried for 48 h and fixed by vapor-phase formaldehyde. Following embedding of the specimens in epoxy resin (Araldite), serial semithin sections were cut and processed for immunohistochemical staining. Sections were incubated with caldendrin-antibody [1,2] (at dilutions between 1:300 and 1:1000) in phosphate-buffered saline containing 0.1% Triton X-100 for 24 h at 4°C, followed by incubation for 30 min at room temperature with the second antibody, biotin-labeled goat anti-rabbit IgG (Jackson Immuno Research, West Grove, USA). The sections were then incubated for 30 min with a preformed complex of biotin-peroxidase/streptavidin (Jackson Immuno Research), and peroxidase activity was revealed using 0.02% diaminobenzidine hydrochloride with 0.3% nickel sulfate as chromogen. Embedding of tissue specimens was performed as outlined elsewhere [9,10].

Reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis and hypothalamic immunostainings were done as described previously [1,2]. Immunocytochemical procedures were performed according to the standard DAB/Ni immunoperoxidase technique [2]. Double immunolabeling for Caldendrin and phosphorylated neurofilament as an axonal marker was done with a monoclonal antibody (SMI312, Sternberger, 1:4000).

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For fractionation experiments pituitary glands of 10 animals (week 7–16) were dissected and stored at −80°C. Tissue homogenization was done in 0.32 M Sucrose, 5 mM HEPES pH 7.4, and proteinase inhibitor cocktail (Complete/Roche). After removal of nuclei and cell debris P2 (crude membranes) was prepared (15 min 12 000×*g*). P2 pellets were extracted with 0.1% Triton X-100 in 20 mM HEPES pH 7.4 for 30 min on ice. Centrifugation for 30 min at 12 000×*g* resulted in a Triton-soluble and -insoluble fraction.

3. Results

Utilizing an antibody directed against the common C-terminal part of Caldendrin, detecting all splice variants [2], we could identify two immunoreactive bands in hypothalamic protein homogenates (Fig. 2a). The band at 28 kDa most likely represents the Caldendrin splice isoform Caldendrin-S2 [2], whereas the doublet at 33/36 kDa corresponds to the two protein products of the long Caldendrin isoform (Caldendrin-L) that most likely differ in their phosphorylation status [1]. In comparison to protein homogenates from cortex Caldendrin immunoreactivity is clearly less prominent in the hypothalamus and the ratio of bands shows a relatively higher abundance of the 28 kDa isoform (Fig. 2a). Interestingly, in pituitary tissue we found almost exclusively immunoreactivity of Caldendrin-S1, the 25 kDa isoform, and to a lesser extent of Caldendrin-S2, the 28 kDa isoform (Fig. 2a). Dissection of the pituitary into the anterior/intermediate and the neural lobe revealed the presence of Caldendrin-S1 and -S2 in neural lobe protein homogenates at similar levels, indicating that alternative splicing of the Caldendrin RNA is uniquely regulated in the hypothalamic-hypophyseal system. Thus, expression of the long isoform seems to be restricted to neurons, whereas Caldendrin-S1 and -S2 in addition are found in endocrine tissue and Caldendrin-S1 seems to be the major isoform expressed in endocrine cells of the adenohypophysis (Fig. 2a).

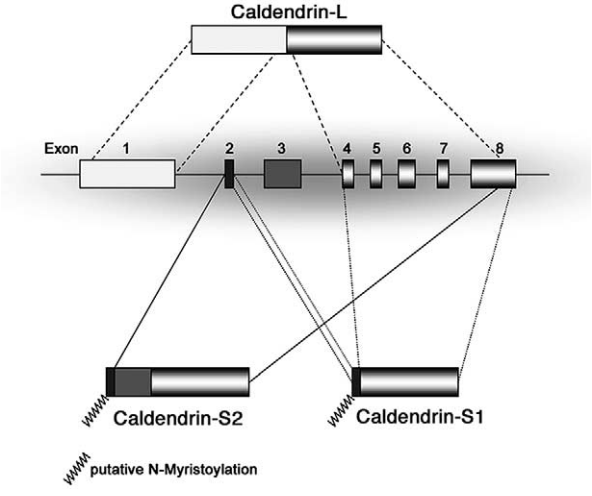

Fig. 1. Alternative splicing generates three Caldendrin splice isoforms differing in their N-terminal sequence. Depicted is a schematic representation of the three Caldendrin splice isoforms. Exons are depicted as numbered boxes. In contrast to the long isoform, Caldendrin-S1 and -S2 arise from two separate exons downstream of exon 1. Exon 2 harbors an independent start codon, which can lead to the generation of two shorter isoforms containing or lacking exon 3. The N-terminus of the resulting proteins is unrelated to Caldendrin and shows a putative *N*-myristoylation site. Exon 4 to exon 8 are common for all Caldendrin isoforms and encode the four Ca^{2+} binding EF-hands starting with EF-hand No. 1 on exon 5, No. 2 on exon 6 and No. 3 and No. 4 on exon 7.

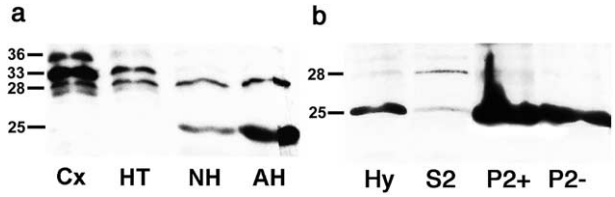

Fig. 2. Western Blot analysis of pituitary, hypothalamus and cortex. Protein content was determined and same amounts loaded per lane (30μg). a: Protein homogenates from cortex (Cx), hypothalamus (Ht), adenohypophysis (Ah) and neurohypophysis (Nh) exhibit a different pattern of immunoreactive bands. Whereas the 33/36 kDa doublet (Caldendrin-L) is prominent in cortex, the ratio of the 28 kDa isoform (Caldendrin-S2) relative to the abundance of the larger bands is increased. In pituitary the 33/36 kDa bands are virtually absent but the 28 kDa and especially the 25 kDa isoform (Caldendrin-S1) are prominently present. b: Whereas the 28 kDa immunoreactive band is almost exclusively in the soluble fraction (S2) after subcellular fractionation of pituitary protein homogenates, the 25 kDa isoform is associated with the particulate fraction (P2−). Interestingly a large proportion of this immunoreactivity is releasable in the presence of 0.1% Triton X-100 (P2+), indicating an association with membraneous protein compartments.

As previously reported in humans [6] subsequent immunostainings of rat hypothalamic tissue sections revealed a somatodendritic (not shown) as well as an axonal localization of Caldendrin throughout the hypothalamus (Fig. 3a). Strong immunolabeling on axons, which had frequently the appearance of Hering-bodies, vesicular structures known to contain hypothalamic releasing hormones, was observed in the lateral hypothalamus (Fig. 3a,b). Immunolabel of the median eminence was very faint (not shown), suggesting that mainly axons descending from the supraoptic and paraventricular nucleus to the neurohypophysis are Caldendrin immunopositive.

The antibody used in this study does not allow differentiation between splice isoforms in imunostained sections. We therefore determined by transcript analysis whether the axonal and putative vesicular immunolabel is due to an association of specific Caldendrin isoforms with these structures. RT-PCR with hypothalamic and pituitary tissue revealed a differential expression of mRNA for the three splice isoforms (Fig. 4). In accordance with the immunoblot data, Caldendrin-L and -S2 transcripts were found to be present in the hypothalamus (Fig. 4), whereas Caldendrin-S1 was only detected at low levels (Fig. 4). No PCR product for Caldendrin-L, however, was present after reverse transcription of pituitary RNA, whereas intense bands were seen for Caldendrin-S1 and -S2 (Fig. 4). These results suggest the localization of Caldendrin-S1 and -S2 in axons most likely descending from the hypothalamus. Accordingly, we observed in the pituitary punctate immunoreactivity in nerve terminals of the neural lobe (Fig. 5). In addition, strong immunoreactivity was detectable in all endocrine cells of the intermediate (Fig. 5) and many Caldendrin-immunoreactive cells were identified throughout the anterior lobe (Fig. 5). Colocalization studies of serial semithin sections demonstrated that from all endocrine cell types somatotrophs were most densely stained (Fig. 5), whereas lactotrophs and thyrotrophs were only faintly immunopositive or entirely nonreactive. Caldendrin immunostaining in endocrine cells was mainly cytoplasmic and obviously paralleled the distribution of secretory granules (Fig. 5). Moreover, immunogold electron microscopy (EM) provided direct evidence that gold particles predominantly labeled secretory granules in hypophyseal

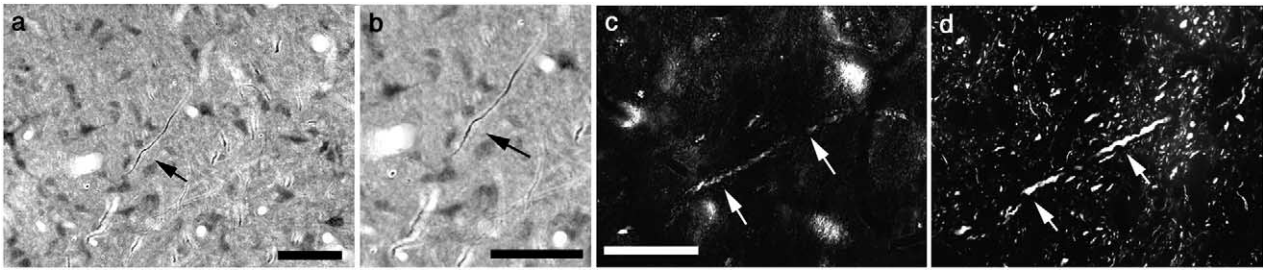

Fig. 3. Frontal sections depicting Caldendrin immunoreactivity in the rat hypothalamus. a,b: Caldendrin-immunopositive axons could be detected in the lateral hypothalamic area (see arrows). c,d: Double immunolabelling with an antibody directed against phosphorylated neurofilament (d) showed that Caldendrin-immunopositive (c) fibers are axons. These axons frequently exhibited Hering-body-like blebs which are known to consist of large dense core vesicles containing polypeptide-releasing hormones (see arrows). Interestingly, these blebs were frequently colocalized with Caldendrin immunoreactivity. Scale bar is 50 μm.

endocrine cells in the adenohypophysis and to a markedly lesser extent neurosecretory granules in nerve terminals of the neural lobe (Fig. 5). Unfortunately, immunogold EM of hypothalamic tissue did not lead to conclusive results.

To further substantiate a vesicular association of the short Caldendrin isoforms we analyzed their behavior in comparison to the long isoform after subcellular fractionation and subsequent Triton X-100 extraction of P2 pituitary tissue homogenates. Previous work in brain has shown that Caldendrin-L is only extractable from the insoluble P2 protein fraction under very harsh conditions, suggesting a very tight association with cytoskeletal but not membraneous brain protein components [1,2]. In contrast Caldendrin-S1 is mainly present in the insoluble P2 fraction and Triton X-100 extraction of this protein preparation leads to a substantial release of this isoform (Fig. 2b), suggestive of an association with membraneous but not cytoskeletal elements. Similarly, Caldendrin-S2, which is less abundant in the pituitary and seems to be more soluble than Caldendrin-S1, could be released from the insoluble P2 protein pool among detergent treatment (Fig. 2b).

4. Discussion

Despite close sequence similarities neuronal calcium sensor (NCS) proteins are suggested to have different functions, which include roles in the modulation of neurotransmitter release and the direct regulation of ion channels [11,12]. In the present study compelling evidence was provided that the Caldendrin low molecular weight isoforms are associated with vesicles and secretory granules and are present in axons of hypothalamic neuroendocrine cells. Previous studies have shown that somato-dendritic Caldendrin-L immunoreactivity is prominent in brain regions with a laminar organization [2], indicating a striking dichotomy in the cellular localization of the short and long Caldendrin splice variants. This suggests that either a different promoter or a yet unknown differentially expressed splicing factor might regulate the splicing event in the corresponding cell types. It should be noted, however, that Caldendrin-L is also expressed in testicular tissue where it is localized to the specialized acrosomal cytoskeleton [13].

The function of the short Caldendrin isoforms at secretory granules is at present not yet clear. Interestingly, however, secretory granules in endocrine cells are considered as $InsP_3$-sensitive Ca^{2+} stores [14] and Caldendrin/CaBP1 has recently been shown to act as a ligand of $InsP_3$ receptors [8] in a Ca^{2+}/CaM-independent manner. Notably, a prominent component of secretory granules, chromogranin A, has been shown to modulate $InsP_3$ receptor channel activity of the granule membrane and may thus be involved in the control of intracellular Ca^{2+} concentration [15]. Moreover, the short splice isoforms harbor a *N*-myristoylation motif, which could provide a means by which they can be anchored to vesicular membranes. Additionally, it was recently reported that Caldendrin/CaBP1 interacts with $Ca_v2.1$ channels, a group of P/Q-type voltage-dependent Ca^{2+} channels which are presumably localized presynaptically and involved in neurotransmitter release [7]. As already mentioned above this is at variance with the somato-dendritic localization of the long Caldendrin isoform and the very low abundance of the shorter splice variants in brain. Previous studies on acutely dissociated nerve endings of rat neurohypophyses, however, have shown that P/Q-type Ca^{2+} channels are present on a subset of neurohypophysial terminals where, in combination with N- and L-channels, they control arginine vasopressin but not oxytocin peptide neurosecretion [16]. Furthermore it was demonstrated that peptide hormone secretion from neurohypophyseal nerve endings requires activation of L-type Ca^{2+} channels [16,17]. This is quite different from the role of Ca^{2+} channels in classical neurotransmission, in which the N- and P/Q-type, rather than L-type Ca^{2+}-channel currents dominate the control of neurotransmitter release [18]. It is therefore an attractive hypothesis resulting from our findings that the short Caldendrin splice isoforms might be selectively involved in the regulation of arginine-vasopressin release in the neural lobe, which seems to require Q-type Ca^{2+} channels. Thus the secretion of peptide hormones in the neuroendocrine system might have Ca^{2+}-signaling requirements different from those of synaptic neurotransmitter release, which might explain the vesic-

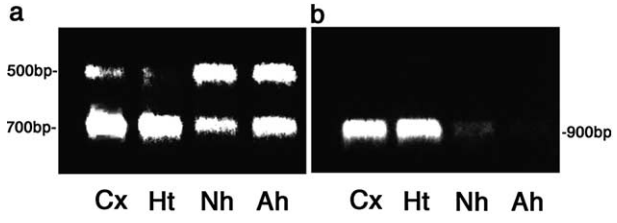

Fig. 4. RT-PCR from mRNA extracted from cortex (Cx), hypothalamus (Ht), adeno- (Ah) and neurohypophysis (Nh). Whereas transcript of Caldendrin-L (b; 900 bp) is largely restricted to neural tissue, Caldendrin-S2 transcripts (a; 700 bp) are found in neural as well as endocrine tissue. Caldendrin-S1 mRNA (a; 500 bp), however, is particularly abundant in the adeno- and neurohypophysis.

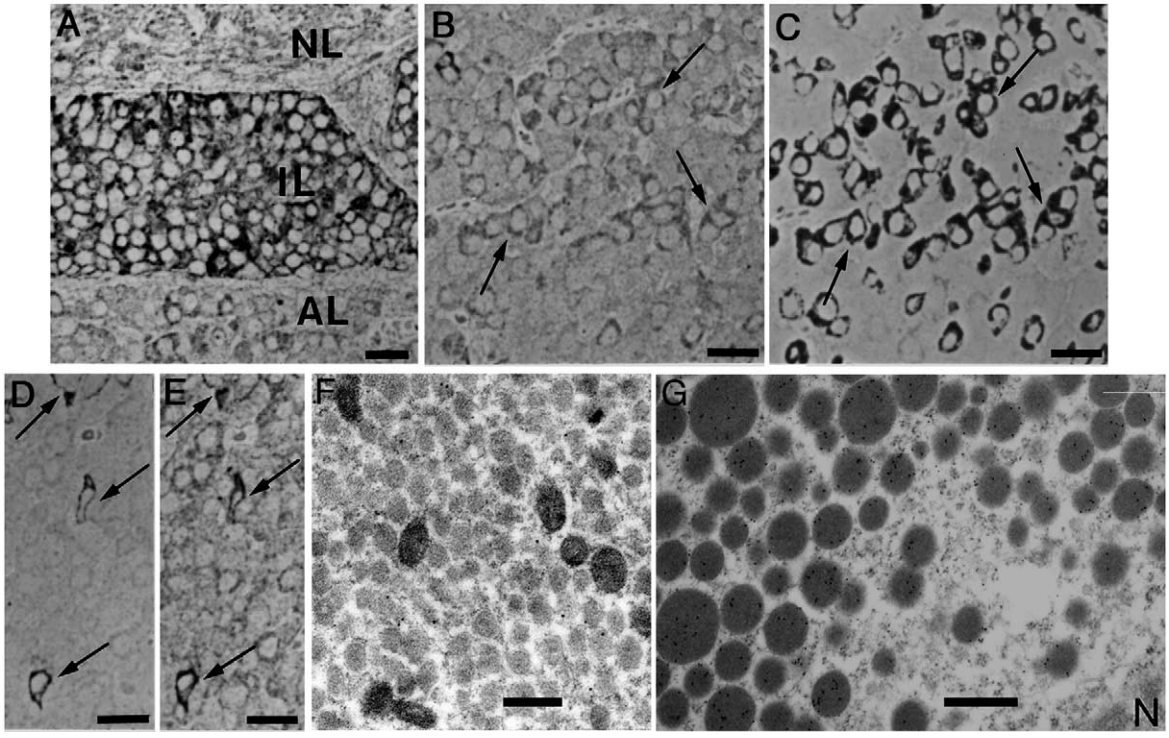

Fig. 5. Immunohistochemical localization of Caldendrin in the pituitary. Semithin sections of the pituitary gland (A) show punctate Caldendrin immunoreactivity in the neural lobe (NL), densely immunostained endocrine cells in the intermediate lobe (IL), and variable degrees of immunoreactivity in the anterior lobe (AL). B: Two adjacent sections of the anterior lobe reveal that Caldendrin-positive cells largely correspond to growth hormone-positive somatotrophs (C; arrows point to identical groups of cells in both sections). Subplasmalemmal immunoreactivity is evident in anterior lobe cells immunostained for either Caldendrin (D) or corticotropin (E; arrows point to identical cells in both sections). Immunogold labelling of hypophyseal neural lobe (F) and intermediate lobe (G). Gold particles indicating Caldendrin immunoreactivity are associated with secretory granules. N = nucleus. Bars = 20 μm in A–E and 200 nm in F–G.

ular localization of the short Caldendrin splice isoforms. Together our findings demonstrate that alternative splicing of Caldendrin can generate isoforms with a different cellular localization and most likely different functions in the transduction of Ca^{2+} signals. Interestingly, the primary structure of short Caldendrin isoforms is similar to those of other NCS proteins, in that they harbor a short N-terminal segment with a myristoylation motif in front of the four EF-hands, which might have arisen by duplication of the calmodulin gene [12]. We therefore speculate that in contrast to Caldendrin-S1 and -S2, Caldendrin-L has evolved with uncommon requirements for somato-dendritic Ca^{2+} signaling of mainly limbic and cortical neurons, whereas the short variants are more closely related to classical NCS proteins.

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