RESEARCH PAPER

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AKAP79 modulation of L-type channels involves disruption of intramolecular interactions in the $Ca_V1.2$ subunit

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Abbreviations: VGCC, voltage gated calcium channel; PKA, protein kinase A; AKAP, A-kinase anchoring protein; PP, poly-prolines; PRD, proline rich domain; PCR, polymerase chain reaction; bp, base pair; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum

L-type voltage gated calcium channels (VGCCs) interact with a variety of proteins that modulate both their function and localization. A-Kinase Anchoring Proteins (AKAPs) facilitate L-type calcium channel phosphorylation through β adrenergic stimulation. Our previous work indicated a role of neuronal AKAP79/150 in the membrane targeting of $Ca_V1.2$ L-type calcium channels, which involved a proline rich domain (PRD) in the intracellular II–III loop of the channel.[1] Here, we show that mutation of proline 857 to alanine (P857A) into the PRD does not disrupt the AKAP79-induced increase in $Ca_v1.2$ membrane expression. Furthermore, deletion of two other PRDs into the carboxy terminal domain of $Ca_V1.2$ did not alter the targeting role of AKAP79. In contrast, the distal carboxy terminus region of the channel directly interacts with AKAP79. This protein-protein interaction competes with a direct association of the channel II–III linker on the carboxy terminal tail and modulates membrane targeting of $Ca_V1.2$. Thus, our results suggest that the effects of AKAP79 occur through relief of an autoinhibitory mechanism mediated by intramolecular interactions of $Ca_v1.2$ intracellular regions.

Introduction

Calcium influx through voltage gated calcium channels (VGCCs) mediates a range of key physiological functions, such as enzyme activation, muscle contraction, neurotransmitter release and gene transcription.[2-4] Several different calcium channel subtypes have been identified and classified by their distinct electrophysiological and pharmacological properties into T-, N-, L-, Q-, P- and R-types. High-voltage activated calcium channels are transmembrane proteins with a pore-forming α_1 subunit and auxiliary α_2-δ, β and possibly γ-subunits.[5,6] Like many other membrane proteins, L-type calcium channels are subject to phosphorylation. The efficiency of second messenger regulation of voltage-gated calcium channels by cAMP/PKA signaling is enhanced by different A-Kinase Anchoring Proteins (AKAPs) which act like scaffolding proteins generating microsignaling domains.[7-14] By anchoring these enzymes close to the calcium channel substrate, they allow for tighter temporal and spatial control making them a very efficient regulatory signaling complex. The human brain isoform AKAP79 or its rodent ortholog AKAP150 is present in brain, skeletal and smooth muscle, and myocardium[7-10] and is involved in a range of modulatory roles of ion channels, such as the $GABA_A$ receptor,[15] glutamate receptor,[16] inward rectifying potassium channels,[17] and the M-current.[18] AKAP79 is found in the postsynaptic density (PSD) and thought to be a central regulator of synaptic strength. The $Ca_V1.2$ L-type calcium channels are clustered at postsynaptic sites and are substrates for PKA, PP2A, calcineurin and Calmodulin Kinase II (CamKII).[14,19] The assembly of this signaling complex provides a mechanism for efficient modulation of dendritic excitability by β-adrenergic receptors, but in addition, it may also facilitate channel biogenesis.[19,20] In cardiac myocytes, a similar regulation of L-type calcium channel by β-adrenergic receptor activation requires AKAP15/18 and involves a conserved leucine zipper motif in the C-terminal domain of the Ca_V1 calcium channel α_1 subunit.[8]

We previously described an AKAP79-mediated targeting effect on $Ca_V1.2$ that was independent of PKA, and which involved a Proline Rich Domain (PRD) contained within the II–III linker of the channel.[1] In this study we show that AKAP79 co-immunoprecipitates with $Ca_V1.2$. We identify the interacting motif of

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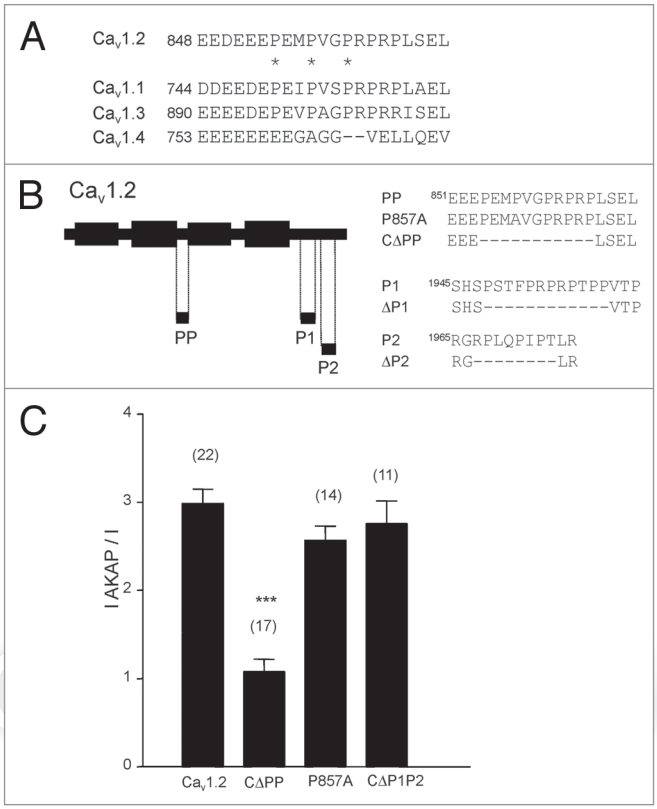

Figure 1. Substitution of Pro 857 by Ala in the Proline Rich Domain of the II-III loop and deletion of the two PRDs of the $Ca_v2.1$ C-terminus does not alter the AKAP79 modulation. (A) Conservation of the II-III-loop-PRD among the L type calcium channel family with the exception of $Ca_V1.4$ channels. (B) Schematic representation of the locations of PRDs along the $Ca_V1.2$ channel sequence, and amino acids sequence of the PRDs in the II-III linker and the C-terminus of $Ca_V1.2$. The top sequence is located in the domain II-III linker and starts at position 854. In the P875A mutant, Pro857 is replaced by Ala. The CΔPP mutant lacks the PEM PVG PRP RP stretch in the II-III linker. In the CΔP1P2 construct, the indicated proline rich regions at residues 1,948 and 1,967 are deleted. (C) Current amplitude ratio in the presence and absence of AKAP79 recorded for the different mutants. Current amplitude was taken at the peak of the I/V curve in each case and plotted as ratio between AKAP-injected and non-injected batches. Note that with the exception of the CΔPP mutant (without the poly-proline domain into the II-III loop), none of the other mutants are insensitive to AKAP79. Numbers in parenthesis indicate numbers of experiments.

$Ca_V1.2$ that is involved in the AKAP79 mediated upregulation or stabilization of channel surface expression. We report that the C-terminal domain of $Ca_V1.2$ interacts with both AKAP79, as well as with the PRD of the II-III loop of the channel. AKAP79 binding to the C-terminal distal region competitively inhibits the association between the II-III loop and the carboxy terminus of $Ca_V1.2$, and thereby promotes channel targeting to the membrane. Furthermore, we show that the AKAP18 isoform, which interacts with a leucine zipper motif within the distal $Ca_V1.2$ C-terminal region, is unable to promote $Ca_V1.2$ membrane trafficking but antagonizes the AKAP79 effects. By mutating the leucine zipper motif we show that AKAP79 is still able to promote channel trafficking. Altogether these data suggest that the $Ca_V1.2$ C-terminal region integrates a crosstalk regulation by distinct AKAP isoforms.

Results

A single PRD into the II-III loop determines AKAP79-mediated increase in $Ca_V1.2$ current amplitude. We previously reported the requirement of a Proline Rich Domain in the II-III loop of the $Ca_V1.2$ α_1 subunit in AKAP79 mediated L-type channel targeting.[1] This motif is conserved in $Ca_V1.1$ and $Ca_V1.3$ but not $Ca_V1.4$ (**Fig. 1A**). The C-terminal region of the channel contains two additional PRDs (see **Fig. 1B**), which have not previously been investigated. To elucidate the role of these regions with respect to the action of AKAP79, we replaced Pro 857 in the II-III linker with alanine (**Fig. 1B**) in order to perturb two potential tandem SH3 binding domains (PxxPxxP) that are contained in the poly proline (PP) motif. In addition, we deleted the entire poly proline region in the II-III linker (CΔPP) plus the additional PRDs (P1 and P2) in the C-terminal domain of $Ca_V1.2$.

Following co-expression of AKAP79 with wild type $Ca_V1.2$ in Xenopus oocytes, a robust enhancement of $Ca_V1.2$ current amplitude was observed with the wild type channel. Deletion of the PRD in the II-III loop made $Ca_V1.2$ surface expression insensitive to AKAP79 as described by us previously in reference 1 (**Fig. 1C**). Neither the disruption of the putative SH3 domains in the II-III linker by the P857A point mutation, nor the removal of the PRDs in the C-terminus significantly antagonized the action of AKAP79 ($Ca_V1.2$, IAKAP/I = 2.98 ± 0.16, n = 22; CΔPP, IAKAP/I = 1.08 ± 0.14, n = 17; P857A, IAKAP/I = 2.56 ± 0.16, n = 14; CΔP1P2, IAKAP/I = 2.75 ± 0.25, n = 11). These results indicate that only the PP motif in the II-III loop is required for the AKAP79 effect. Moreover, although the presence of the PRD of the II-III loop is essential, altering the SH3 consensus motif did not abolish AKAP79 mediated upregulation.

$Ca_V1.2$ channels and AKAPs form a signaling complex. To demonstrate a physical interaction between AKAPs and the $Ca_V1.2$ channel as shown in native hippocampus between the AKAP79 mouse ortholog (AKAP150) and $Ca_V1.2$,[12] we performed immunoprecipitation of the channel complex with myc-tagged AKAP79, HA-tagged $Ca_V1.2$ and β_3-flag in tsA-201 cells using the anti-Flag Sepharose beads. Co-purification of the channel was assessed by western blot (**Fig. 2**). AKAP79-myc (**Fig. 2A**) and $Ca_v1.2$-HA (**Fig. 2B**) were found to co-immunoprecipitate with β_3-flag subunit, thus corroborating the notion that L-type calcium channels and AKAP79 (or its ortholog) form a signaling complex.

The distal C-terminal domain of $Ca_V1.2$ directly interacts with AKAP79. Considering the critical importance of the II-III-linker PRD in the functional effects of AKAP79, we wanted to determine by using a yeast two-hybrid system whether AKAP79 could physically interact with this region. However, as shown in **Figure 3B**, we could not detect an interaction between the domain II-III linker and AKAP79. As it has been shown that the coiled-coil domain of AKAP15/18 interacts with a leucine zipper in $Ca_V1.1$ calcium channels, we examined the $Ca_V1.2$ sequence for a similar motif, and indeed, as illustrated in **Figure 3A**, the

C-terminus contains a leucine zipper like motif. Our initial findings showed that the AKAP79 trafficking effects do not rely on its coiled-coil domain.[1] When we performed a yeast two-hybrid assay using a 106-amino acid long distal $Ca_V1.2$ region (aa 1,997–2,106) containing the leucine zipper, and the full length AKAP79, we detected a positive interaction between AKAP79 and the distal C-terminus of $Ca_V1.2$ using this assay (**Fig. 3B**). To confirm this interaction, we performed an in vitro binding assay between immobilized GST-AKAP79 and 6xHis fusion proteins of both the $Ca_V1.2$ full length C-terminus (residues 1,526–2,143), and of the distal C-terminus (residues 1,997–2,106). As shown in **Figure 3C**, both the $Ca_V1.2$ full length C-terminus and the shorter distal fragment bind to recombinant AKAP79 in vitro. Hence, the distal C-terminus and not the PRD in the II-III linker is the primary binding target of AKAP79.

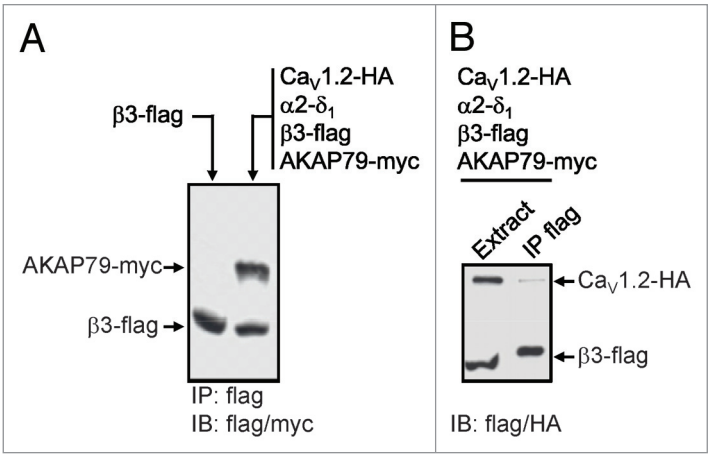

Figure 2. Immunoprecipitation of $Ca_V1.2$ and AKAP79. (A) western blots of co-immunoprecipitations of $Ca_V1.2$-HA, calcium channel β_3-flag and AKAP79-myc in 1% CHAPS using FLAG sepharose beads. The anti-flag antibody identifies the β_3-flag protein, while the AKAP79-myc protein is detected in the calcium channel complex indicating that AKAP79-myc binds to the calcium channel complex. (B) $Ca_V1.2$-HA is also immunoprecipitated by β_3-flag protein as expected.

AKAP79 antagonizes an intramolecular interaction between the $Ca_V1.2$ II-III linker and the C-terminus. How can the functional role of the II-III PRD be reconciled with the lack of biochemical interaction with AKAP79? As we reported in our previous study in reference 1, although deletion of the II-III linker PRD ablates the AKAP79 mediated upregulation, current densities were increased in the deletion mutant to the same levels as those observed with the wild type channel coexpressed with AKAP79, with no further effect of AKAP79. This might suggest the intact II-III linker is involved in an autoinhibition of channel expression that depends on a functionally intact PRD, which may perhaps be relieved in the presence of AKAP79. To observe this possibility, we examined whether AKAP79 might regulate the interaction of the II-III linker with another part off the channel, such as the C-terminus. As shown in **Figure 4A**, the II-III loop physically interacts with the C-terminus of the channel in vitro. Deletion of the PRD in the II-III linker virtually abolishes the interaction. However, no such interaction could be observed between the domain II-III linker and the leucine zipper region per se (not shown), indicating that a region distinct from the AKAP79 interaction site controls the intramolecular interaction between the C-terminus and the II-III loop. To determine whether AKAP79 could disrupt these intramolecular interactions within the channel protein, we incubated GST-fusion protein of the $Ca_V1.2$ C-terminus with increasing concentrations of recombinant AKAP79, and examined the consequences on 6xHis II-III linker binding. As shown in **Figure 4B**, AKAP79 competitively inhibited the II-III linker interactions with the C-terminus of the channel. These data therefore suggest that AKAP79 antagonizes an intrinsic interaction between the II-III linker and the C-terminus of the channel.

AKAP isoform dependence of the AKAP mediated effects on $Ca_V1.2$ membrane expression. To confirm that the distal C terminus region containing the leucine zipper motif was indeed important for AKAP79 regulation, we deleted this region from $Ca_V1.2$ channel. As shown in **Figure 5A**, this resulted in ablation of in vitro binding of AKAP79 to the $Ca_V1.2$ C-terminus region, as well as the suppression of the functional effects of AKAP79 on L-type current amplitudes. AKAP15/18 also interacts directly with the C-termini of $Ca_V1.1$ and $Ca_V1.2$.[8,21] To examine whether AKAP15/18 could mediate a similar functional effect as AKAP79, we determined whether AKAP15/18 coexpression could potentiate current amplitudes of wild type $Ca_V1.2$ channels. As shown in **Figure 5B**, AKAP15/18 failed to enhance $Ca_V1.2$ current amplitude. Interestingly, in the presence of AKAP15/18, the AKAP79 mediated enhancement of $Ca_V1.2$ expression was completely abolished (**Fig. 5B and I** ($Ca_V1.2$ + AKAP79) = 2.72 ± 0.11, n = 11; I ($Ca_V1.2$ + AKAP79 + AKAP15/18) = 0.32 ± 0.08, n = 16), indicating that AKAP18 mediates a dominant negative effect on AKAP79 action but is unable to disrupt the auto-inhibitory interactions between the II-III linker and the C-terminus of the channel leading to membrane expression. To ascertain that AKAP79 and AKAP18 share overlapping binding site on the channel, three key residues of the leucine zipper were mutated to alanine. **Figure 5C** shows that (I2046A, F2053A and I2060A) mutations were without effect on basal current amplitude of $Ca_v1.2$ mut LZ. Furthermore, these mutations did not affect AKAP79-mediated current increase, suggesting that AKAP79 binding site(s) on distal $Ca_V1.2$ C terminus do not exclusively depends on the leucine zipper motif.

Discussion

Our findings support the idea that AKAP79 enhances L-type $Ca_v1.2$ current density by disrupting an intramolecular inhibitory module acting on $Ca_v1.2$ channel. Here we show that this module requires intramolecular interaction within $Ca_v1.2$ intracellular linkers. This provides novel insights in to the molecular basis underlying the regulation of L-type calcium channels by the postsynaptic scaffolding protein AKAP79. As we showed previously in reference 1, AKAP79 coexpression with $Ca_V1.2$ channels promotes surface expression of the channel. Whereas

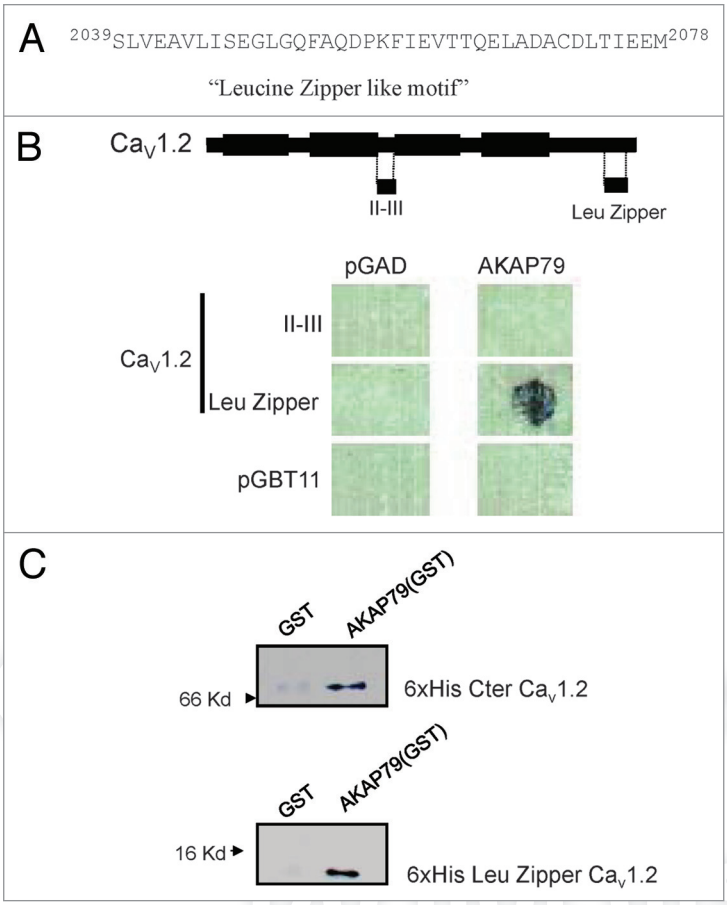

Figure 3. AKAP79 directly binds to the leucine zipper motif in the C-terminus of the L-type calcium channel $Ca_V1.2$. (A) Amino acid sequence of the leucine zipper like motif in the C-terminal domain of $Ca_V1.2$. (B) Top: Schematic representation of the location of the II-III loop and the leucine zipper motif along the $Ca_V1.2$ channel sequence. Bottom: Yeast-two hybrid assay between AKAP79 and either the domain II-III linker, or the C-terminal leucine zipper motif of $Ca_V1.2$. pGAD was used as a negative control. Note the yeast growth in the leucine zipper/AKAP79 transformation. (C) In vitro binding of 6xHis $Ca_V1.2$ C-terminal and 6xHis $Ca_V1.2$ leucine zipper to immobilized AKAP79 (GST). The western blot was probed using an anti-Xpress antibody (Invitrogen). Both the carboxy terminus and the Leucine Zipper motif bind to AKAP79.

AKAP15 directly interacts with L-type channels via the Leucine Zipper motif[21] our data suggest that AKAP79 directly binds to the $Ca_V1.2$ channel in the distal part of the C-terminus nearby the leucine zipper motif. Second, we present evidence that the II-III linker of the channel can interact with the C-terminus in vitro, and that this interaction is critically dependent on the presence of a proline rich domain in the II-III linker region, but does not involve the leucine zipper. Finally, we show that AKAP79 binding to the C-terminus competitively inhibits the II-III linker C-terminus interaction. Based on this collective evidence, and our previous work in reference 1, we propose the following model. In the absence of AKAP79, the II-III linker region and the C-terminus of the channel are bound to each other. In this conformation, the channel has a reduced likelihood of being transported to the plasma membrane. Disruption of the II-III linker interaction with the C-terminus via deletion of the II-III linker PRD results in an increased efficiency of membrane expression.[1] AKAP79, by associating nearby the leucine zipper motif in the C-terminus may sterically interfere with the II-III linker C-terminus interaction, thereby removing the autoinhibitory effect on membrane expression. These results are consistent with our previous observation that removal of the II-III linker PRD results in an upregulation of membrane expression with no additional effect of AKAP79. In contrast, AKAP18 did not mediate an increase in $Ca_V1.2$ current density despite being able to bind to the $Ca_V1.2$ C-terminus,[8] but acted in as a dominant negative inhibitor of the AKAP79 effects. This suggests that AKAP79 and AKAP15/18 compete for a close region of the channel, but that only AKAP79, is capable of disrupting the interaction between the C-terminus and the II-III linker region of the channel. Whether AKAP79 interactions with $Ca_v1.2$ can regulate the ubiquitination state of the channel, as reported recently for ancillary $Ca_v\beta$ subunits, remains to be determined.[22]

AKAP proteins are key regulators of various types of ion channel proteins.[14,23] Previous studies have established the interaction between mAKAP and ryanodine-sensitive calcium-release channels of the sarcoplasmic reticulum,[24] Yotiao (another member of AKAP family) and the I_{ks} channel subunit hKCNQ1.[25] These interactions appear to also involve a leucine zipper like motif. Similar interactions between AKAP15/18 and $Ca_V1.1$ in skeletal muscle,[21] $Ca_V1.2$ and AKAP15/18 or AKAP79 in cardiac myocytes and in neuronal tissues[8,12,13,20,26] or $Ca_V1.3$ and AKAP15/18 in brain have been reported in reference 13. Hence, there appear to be conserved motifs across multiple types of ion channels that regulate interactions with AKAPs and related proteins. In addition, AKAP79 interactions with cognate partners also involve non leucine zipper motifs as in TREK1 background potassium[27] or $K_v4.2$ channels.[28] Additional interacting motifs were also found in the N-terminus and the I-II linker of $Ca_v1.2$ calcium channel,[12,14] although in this latter case these sites seem to be secondary sites compared with the one located in the C-terminus.[13,20]

It is noteworthy that C-terminal fragments of $Ca_V1.2$ have been shown to be post-translationally cleaved,[29] but nonetheless remains localized near the plasma membrane.[30] These hydrophilic C-terminal fragments associate with and affect the PKA-mediated regulation of L-type $Ca_v1.2$ channels in particular in the heart.[11,31] It is not clear whether AKAP79 interacts with these cleaved fragments, or whether a tethered C-terminus is required. Moreover, it remains to be established as to whether proteolytically cleaved C-terminal fragments associate with the II-III linker region in vivo. However, the observation that splice isoforms of $Ca_V1.2$ with shorter C-termini[31] or artificially truncated $Ca_V1.2$ channels (Stotz and Zamponi, unpublished observations) produce drastically enhanced whole cell currents, is consistent with the removal of an autoinhibitory mechanism that is intrinsic to the $Ca_V1.2$ subunit and depends on the distal C-terminus domain.[11] Finally

these results have to be considered in the context of recent study showing that deletion of the $Ca_v1.2$ distal C-terminus in mice leads to reduced L-type channel functional activity in hippocampal and heart cells.[13,26] Therefore, further experiments will be needed to find a consensus about the role of C-terminus proteolytic fragments on the AKAP79-mediated regulation of $Ca_v1.2$ in vivo.

A physical interaction among cytoplasmic regions of calcium channels is not without precedent. For example, the I-II loop of $Ca_v2.1$ is able to directly associate with the C-terminal, N-terminal and III-IV loop of the channel.[32] The interaction between the domain I-II linker and the III-IV loop of that channel occurs only in the absence of the calcium channel β-subunits. The proximal and distal C-terminal fragments of $Ca_v1.2$ interact with each other and their association/dissociation influence $Ca_v1.2$ channel PKA-dependent modulation.[11] Hence, the intracellular side of the channel does not simply consist of loosely hanging tails and linkers, but may instead comprise a tightly packed web of various cytoplasmic regions, whose association may be regulated by proteins such as AKAPs and possibly accessory calcium channel subunits as well as calcium sensors such as calmodulin.[33] It is well established that L-type calcium channels mediate calcium-dependent gene transcription by activating calmodulin molecules that are preassociated with the C-terminus of the channel.[34] It is also known that L-type calcium channel localization and AKAP79-L-type channel interactions are critical for the regulation of gene transcription.[20,35,36] Moreover, AKAP79 is most definitely a calmodulin binding protein,[37] and activation of calmodulin induces a release of AKAP79 from the plasma membrane.[38-40] Hence, L-type channels appear to exist as a multiprotein signaling complex in the postsynaptic membrane, whose composition may be dynamically regulated by physiological stimuli. Indeed, NMDA receptor stimulation disrupts AKAP79 localization in dendritic spines, and results in a diffuse redistribution in the cell soma.[39-41] This suggests the possibility that under glutamate stimulation, AKAP79 could contribute to the import/export/movement of L-type channels between the soma and at more distal sites such as the dendritic spines. This dynamic process may also regulate gene transcription mediated by L-type calcium influx.[36] Thus, the picture emerges that AKAP79 may act as a key signaling molecule for regulation of L-type channel function.

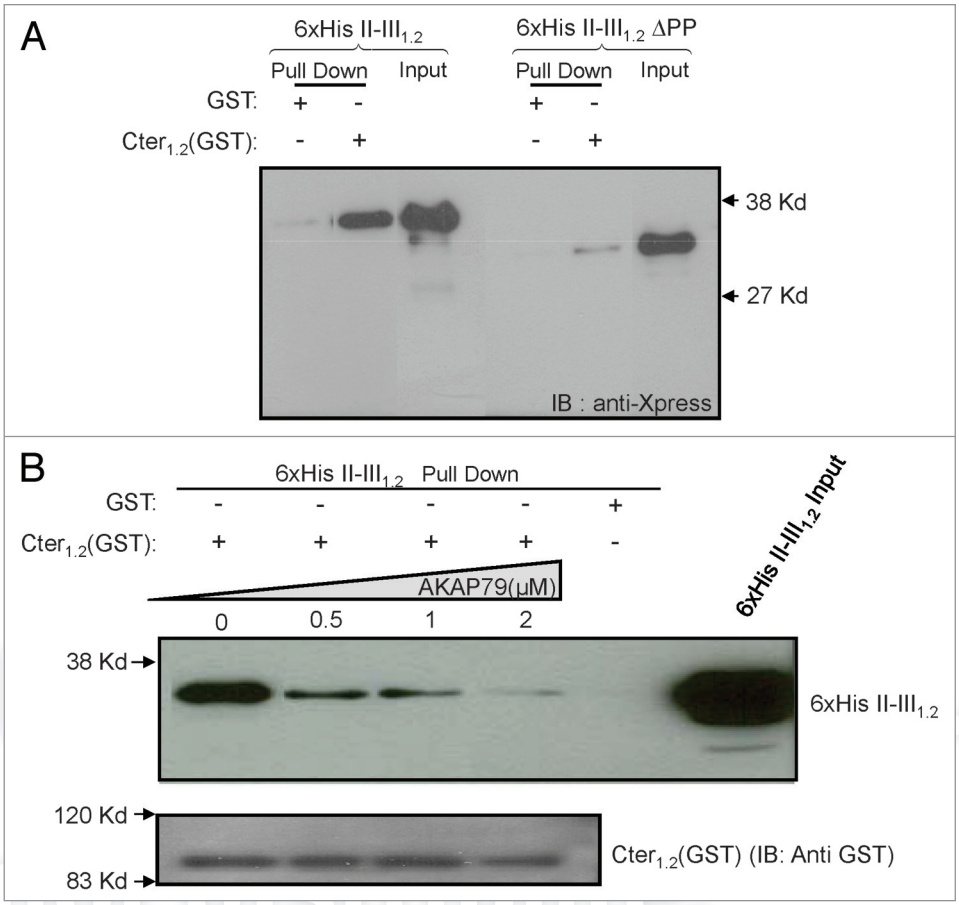

Figure 4. Interaction between the $Ca_V1.2$ C-terminal and II-III linker region. (A) In vitro binding of 6xHis II-III loop of $Ca_V1.2$ to immobilized $Ca_V1.2$ GST C-terminus. The western blot was probed using the anti-Xpress antibody. Grouping of images from different parts of the same gel have been made. (B) Binding between 6xHis II-III loop of $Ca_V1.2$ and $Ca_V1.2$ GST-C-terminal in the presence of increasing AKAP79 levels. Note that AKAP79 prevents the II-III linker C-terminus association. Bottom: Control blot to show that identical amounts of GST C-terminal fusion protein were used in the assay.

Materials and Methods

Molecular biology. Deletions of the PP motif in the $Ca_V1.2$ II-III loop (aa 854 to 864) were created by overlapping PCR using as template a wild type $Ca_V1.2$ cDNA engineered to contain two unique silent restriction sites (*MluI* and *SpeI*) flanking the II-III loop region. The amplified 780 bp *MluI-SpeI* fragment was reintroduced into the template DNA. Mutant P857A was created by site-directed mutagenesis within the *MluI-SpeI* fragment and reintroduced into template DNA. The Leucine Zipper ΔLZ mutant (I2046A, F2053A, I2060A) and deletions in the C-terminal domain of $Ca_V1.2$ were obtained by site directed mutagenesis and PCR overlap extension respectively, in the SpeI-BsrGI fragment then cloned into the $Ca_v1.2$ pMT2 plasmid backbone.

To create Yeast Two-hybrid assay constructs, the II-III loop, the C-terminal domain and the leucine zipper motif of $Ca_V1.2$ were subcloned in frame into the Gal4 DNA binding domain vector pGBT11. AKAP79 was subcloned into the Gal 4 activation domain vector pGAD.

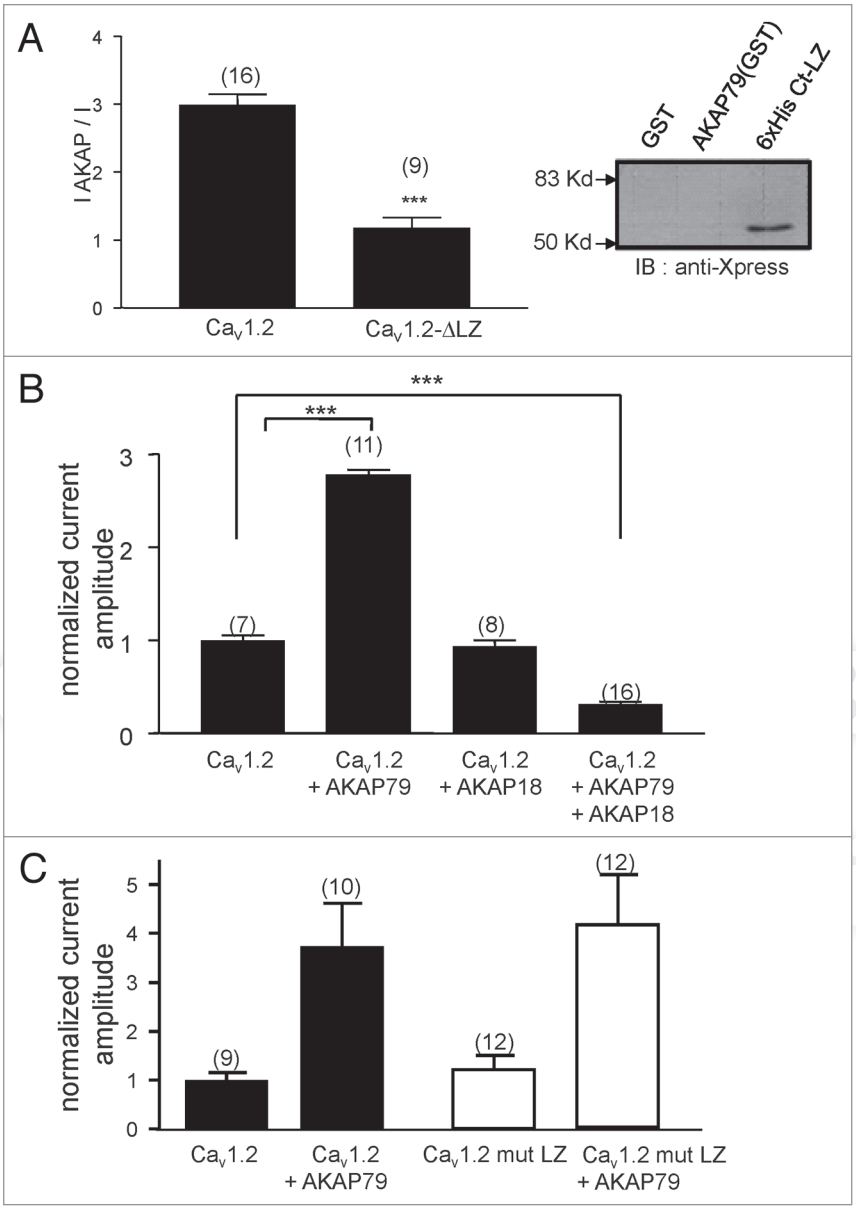

Figure 5. AKAP mediated effect is isoform dependent. (A) Mean AKAP79 effect on current amplitude recorded for $Ca_V1.2$ wt and a leucine zipper deletion mutant $Ca_V1.2$-LZ. Note that AKAP79 does not increase current amplitudes of $Ca_V1.2$ channels lacking the leucine zipper. Right part: Western blot of in vitro binding assay between AKAP79 and the C-terminal domain of $Ca_V1.2$ lacking the leucine zipper motif. Note that the $Ca_V1.2$-LZ C-terminus region cannot bind AKAP79 in vitro. (B) Effect of AKAP18 on the $Ca_V1.2$ current amplitude. AKAP18 does not mediate an increase in current amplitude, but acts as a dominant negative to suppress the AKAP79 mediated current enhancement.

For in vitro binding assays, AKAP79, the II-III loop and the C-terminal domain of $Ca_V1.2$ were subcloned into pGEX-4T-3 and pGEX-5T-1 respectively (Amersham Biosciences) to make Glutathione S-transferase (GST) fusion proteins. For 6xHis fusion proteins, the II-III loop, C-terminal domain, ΔPPII-III loop, Cter-LZ and Leu Zipper motif of $Ca_V1.2$ were subcloned into the pTrcHis vector (Invitrogen). AKAP79 tagged with the c-Myc epitope was created by PCR amplification and subcloning into c-Myc/pcDNA3. The HA-$Ca_V1.2$ construct has been previously described in reference 1, and the β_3-flag was generously provided by Dr. T.P. Snutch. Accuracy of the various constructs was analyzed by sequencing and restriction digests.

Yeast two-hybrid assay. Direct interaction between the $Ca_V1.2$ constructs and AKAP79 was analyzed by both co-transformation and mating using the Y190 yeast strain. Yeast was mixed with 500 µl PEG (50% w/v), 100 µl of 0.1 M lithium acetate pH 8, 2 µl of ssDNA and 1 µg of DNA construct. The mix was incubated at room temperature for 4 h then heat shocked at 42°C for 20 min. Yeast was pelleted at 2,000 tr/min and resuspended in 500 µl H_2O, pelleted again and resuspended in 100 µl H_2O and finally plated on appropriate YPD medium. Clones were selected in media without tryptophan, leucine and histidine supplemented with 0.04% X-gal and 20 mM 3-amino-1,2,4-triazole (3-AT) to reduce the background growth of the Y190 yeast strain. Mated yeast that had both plasmids grew on leu-, trp-plates, then, they were allowed to grow for approximately 3 d on leu-, trp-, his-plates.

Transient expression of recombinant calcium channels. The following cDNA sequences inserted in expression vectors were used (GenBank™ accession numbers are in parentheses): $Ca_V1.2$ (M67515), β_{1b} (NM017346), α_2-$\delta_{1\text{-}b}$ (AF286488), AKAP79 (NM004857), AKAP15/18 (NM004842). For transient expression in Xenopus oocytes, nuclear injection was performed as previously reported in reference 1. When AKAP79 was coexpressed with the Ca^{2+} channel subunits, we used a ratio of 1 (AKAP) to 3 (Ca^{2+} channel mix). As control, the empty vector was used to obtain the same dilution. Oocytes were then incubated at 18°C for 2 to 4 d in ND96 medium on rotating platform.

Electrophysiology. Macroscopic oocyte currents were recorded using two-electrode voltage-clamp as previously described in reference 1, using 5 mM Barium as charge carrier. pCLAMP7 software was used for data acquisition and analysis was performed with pCLAMP9, Excel and GraphPad Prism software. Results are presented as the mean ± SEM, and compared using Student's t-tests or ANOVAS for multiple comparisons.

Purification of fusion proteins. 6xHis constructs in pTrcHis vector were transformed in *Escherichia coli* TOP10 cells (Invitrogen). Constructs were grown in a 200 ml culture of SOB broth to A_{600} = 0.6, at which point protein expression was induced with 100 mM isopropyl-1-thio-β-D-galactopyranoside and the cultures were grown for 3.5 to 4 h. Cells were harvested, lysed by 3 cycles of sonication/freeze thaw in the presence of

100 μl of egg white lysozyme (Sigma) and 20 μl of protease inhibitor mixture containing 4-(2-aminoethyl) benzenesulfonyl fluoride, bestatin, pepstatin, E-64, phosphoramidon (Sigma, P-8849). The lysates were treated with RNase A (5 μg/ml), centrifuged to remove insoluble debris, filtered and used immediately or stored at -80°C. Batch purifications proceeded as follows: Ni_2-NTA beads (Qiagen) were buffer equilibrated with phosphate-buffered saline (20 mM Na_2HPO_4, pH 7.8, 500 mM NaCl) and made up to 50%. All incubations/washes were conducted at 4°C. Beads were incubated with 3 volumes of lysate in the presence of 12 mM imidazole, 10 mM β-mercaptoethanol, and 0.1% Triton X-100 for 30 min at 4°C, followed by a 10 min washing. The wash buffer consisted of 20 mM Na_2HPO_4, pH 6.0, 500 mM NaCl, 21 mM imidazole, 10 mM β-mercaptoethanol, 0.1% Triton X-100. The beads were then incubated a second time then washed with 2 x 30 bed volumes of wash buffer for 15 min each. For elution, proteins were incubated at 4°C for 30 min in 20 mM Na_2HPO_4, pH 6.0, 500 mM NaCl, 500 mM imidazole. Eluted proteins were dialyzed overnight in Slide-A-Lyzer cassettes (Pierce) at 4°C. 6xHis proteins were use immediately or stored at -80°C.

Constructs in pGEX vectors were transformed into BL21 cells. A 1-L culture at 37°C was grown to A_{600} = 0.5, at which point protein expression was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were grown for 4 h and harvested by centrifugation, resuspended in 35 ml of resuspension buffer (PBS supplemented with 0.1% Tween 20, 2 mM EDTA, 350 mM NaCl, 0.1% β-mercaptoethanol and 20 μl of protease inhibitor mixture P8849 (Sigma), and passed through a French press. Cellular debris was removed by centrifugation. Glutathione-Sepharose beads (Sigma) were equilibrated with PBST and made to yield a 50% slurry for batch purification. 50% beads were incubated 1:3 with the lysate for 1 h followed by a 20 bed volume wash with PBST and a second incubation with lysate. The final washes consisted of 20 bed volumes of PBST for 10 min, 20 bed volumes of MKM buffer (10 mM MOPS, pH 7.5, 150 mM KCl, 4.5 mM $Mg(CH_3COO)_2$, 0.2% Triton X-100) for 10 min, 20 bed volumes of PBST supplemented with 350 mM NaCl, 2 mM EDTA and 0.1% β-mercaptoethanol for 10 min, and finally 20 bed volumes of PBST for 10 min. After the final wash, the beads were resuspended to obtain a 50% slurry in PBST, which was subsequently used for binding assays.

In vitro binding assay. Beads-bound GST fusion proteins were incubated at 4°C with purified 6xHis fusion proteins for 2 h. For competition assay, GST-AKAP79 was purified on Glutathione-Sepharose beads and then cleaved from GST using Thrombin. Each reaction was washed at least 30 min with PBST before proteins were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). Membranes were probed with an anti-Xpress antibody to detect polyHis tags (1:2,500 dilution, Invitrogen) or anti GST (diluted 1:5,000, Santa Cruz Biotechnologies) and western analysis was detected using ECL detection methods. All in vitro binding assays were repeated three times.

Immunoprecipitation. Transfection of tsA-201 cells was performed in 35 mm dishes using JETPEI (Q-Biogen). The mixture of DNA was as follows; 1 μg of $Ca_V1.2$-HA, 0.5 μg of α_2-δ_1, 0.5 μg of AKAP79-myc and 100 ng of β3-flag plasmid; for the β_3-flag alone, 100 ng of β_3-flag plasmid was used with 1.9 μg of pcDNA3.1 vector. The cells were incubated for 48 h and harvested in 1% CHAPS in PBS + protease inhibitor mix (Boehringer Mannheim). The mixture was vortexed and then centrifuged for 15 min. Anti-FLAG beads (Sigma) were added and incubated overnight at 4°C. Beads were washed 3x (10 min incubations) with 1% CHAP in PBS. 40 μl of 200 mM Glycine (pH 2.5) was added and incubated at room temperature for 5 min. The supernatant was removed after a brief centrifugation and neutralized with 1 μl of 1 M Tris (pH 12). The samples were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Primary antibody incubation was performed overnight with a mouse anti-flag M2 antibody (1:1,000; Sigma) in 3% bovine serum albumin in PBS. Blots were washed 3 times and incubated subsequently using a sheep anti-mouse secondary HRP (Amersham NXA931; 1:10,000) for 45 min. This was followed by 3 washes with PBS. ECL was used to detect HRP activity (ECL western Blot detecting reagents-Amersham). Subsequently for detection of the myc epitope, a primary anti-myc antibody (clone 9E10) at a 1:1,000 dilution was used following the same incubation and washing procedure. Mouse secondary HRP was used at a concentration of 1:5,000. The HA epitope was detected by using a rat anti-HA primary antibody (1:1,000; Roche) with a subsequent incubation with a goat anti-rat-HRP secondary (1:5,000; Jackson ImmunoResearch).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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