Effective Association of Kv Channel-interacting Proteins with Kv4 Channel Is Mediated with Their Unique Core Peptide*

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Kv channel-interacting proteins (KChIPs) and neuronal calcium sensor-1 (NCS-1) have been shown to interact with Kv4 channel α-subunits to regulate the expression and/or gating of these channels. Here we examine the specificity and sites of these proteins for interaction with Kv channel proteins. Immunoprecipitation and green fluorescent protein imaging show that KChIPs (but not NCS-1) effectively bind to Kv4.3 protein and localize at the plasma membrane when channel proteins are coexpressed. Coexpression with chimeric proteins between KChIP2 and NCS-1 reveals that the three regions of KChIP2 (the linker between the first and second EF hands, the one between the third and fourth EF hands, and the C-terminal peptide after the fourth EF hand) are necessary and sufficient for its effective binding to Kv4.3 protein. The chimera with these three KChIP2 portions slowed inactivation and facilitated recovery from inactivation of Kv4.3 current. These results indicate that the sequence difference in these three regions between KChIPs and NCS-1 determines the specificity and affinity for interaction with Kv4 protein. Because the three identified regions surround the large hydrophobic crevice based on the NCS-1 crystal structure, this crevice may be the association site of KChIPs for the channel protein.

Voltage-gated K⁺ (Kv) channels comprise a diverse group of membrane proteins. Whereas the principal structure of these channels is composed of four pore-forming subunits, association with auxiliary subunits is often required for high levels of functional channel expression and proper channel gating. For example, a class of EF-hand-containing Ca²⁺-binding proteins (KChIPs) plays essential roles in controlling the expression, gating, and modulation of Kv4 family channels (1). In heterologous expression systems, KChIPs significantly increase channel current density and protein levels and alter the gating of their associated channels, with the most notable change being facilitation of recovery from inactivation (1–8). The importance of KChIPs in functional channel expression in vivo is further demonstrated by the finding that deletion of the KChIP2 gene leads to loss of cardiac transient outward current (9). Furthermore, KChIPs are required for the modulation of Kv4 channel current by arachidonic acids (10). These findings indicate that KChIPs are essential components of transient voltage-gated K⁺ channels in cardiac myocytes and neurons.

Whereas the physical interaction and physiological roles of Kv4-KChIP channel complexes have been well documented, recent studies indicated that neuronal calcium sensor-1 (NCS-1, also known as frequenin), a protein related to KChIPs, also influences Kv4 channels (11, 12). In Xenopus oocytes, coexpression of NCS-1 caused slower inactivation and faster recovery from inactivation of Kv4 current (12). Furthermore, the NCS-1-induced changes in channel gating required the N-terminal peptide of Kv4 protein, suggesting that NCS-1 and KChIPs interact with the same or similar site of the channel polypeptide. NCS-1 also significantly enhanced the densities of Kv4 currents. In HEK293 cells, NCS-1 did not affect the gating of coexpressed Kv4.2 or -4.3 currents but increased the densities of these currents (11). Finally, anti-NCS-1 antibody appeared to coimmunoprecipitate Kv4 channel proteins from mouse brain (12) and heart extracts (11). Therefore, NCS-1 may also play an important role in controlling cardiac and neuronal Kv4 channels.

Although KChIPs and NCS-1 exhibit similarities in channel association sites and enhancement of Kv4 current density and alterations in channel gating, they possess distinct primary structures; the amino acid identity in the core region between any KChIP and NCS-1 is ~40%, whereas those between any two KChIPs are more than 70%. Moreover, yeast two-hybrid screening with the N-terminal peptide of Kv4.2 detected specific interaction with KChIPs but not NCS-1 (1). These observations suggest that KChIP-Kv4 and NCS-1-Kv4 complex formation may involve distinct mechanisms. To elucidate these molecular interactions, we performed biochemical, imaging, and electrophysiological analyses. In this paper, we show that KChIPs exhibit significantly more effective binding to Kv4.3 protein than NCS-1. Our data further identify three regions of KChIP2 polypeptide that mediate this effective interaction with the channel protein.

MATERIALS AND METHODS

Isolation of Rat NCS-1 cDNA—Rat NCS-1 cDNA (GenBank® accession number L27421) was obtained by reverse transcriptase PCR with primers (5′-GGAAATCCAACAGCAAGTTGAAGC-3′ and 5′-gcccgtcgta-
gagggacagcgc-3′) using total RNA isolated from the whole brain of adult male Sprague-Dawley rats. The obtained PCR product was cloned into pGEM-T vector (Promega) and sequenced.

Constructions—Rat KChIP2 splicing variant (GenBank® accession numbers AF269263, 269284, and 269285) and NCS-1 cDNAs were subcloned into an Emerald-C1 expression vector that had been constructed by replacing the enhanced green fluorescent protein of EGFP-C1 (Clontech) with Emerald (Packard) by a PCR-based method.
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KChIP2 (but Not NCS-1) Efficiently Binds to Kv4.3 Proteins—Previous work has shown that N-terminally GFP-tagged KChIPs strongly bind to Kv4.3 proteins and localize to plasma membrane when channel proteins are coexpressed in HEK293 cells (8). Therefore, GFP-tagged KChIPs and NCS-1 were first used to investigate association of these molecules with Kv channel proteins (Fig. 1A). As expected, communoprecipita-

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Taken together, the two EF-hand linkers between the first and second EF hands, and between the third and fourth EF hands, and the C-terminal portion after the fourth EF hand (Regions II, IV, and V) of KChIP2 polypeptide are necessary for its effective association with Kv4.3 proteins.

To determine whether some or all of the identified regions of KChIP2 are sufficient for its association with Kv4.3 proteins, we generated NCS-1 chimeras containing these KChIP2 peptides instead of its own (Fig. 4D). Introduction of the two C-terminal regions (Regions IV and V) of KChIP2 into the corresponding portions of NCS-1 did not transfer the ability to interact effectively with Kv4.3 proteins (Chimera 13). However, a NCS-1 chimaera with all of the three identified KChIP2 peptides (Chimera 14) appeared to be as effective as the original KChIP2 and Myc-Kv4.3 protein but not any other combinations. The larger faint and smaller strong bands of Kv1.4 proteins represent mature glycosylated and non-glycosylated forms, respectively. Also, a doublet of smaller truncated Kv2.1 proteins was routinely seen in our transient transfection. Kv4.3 proteins were present as a doublet in addition to a minor truncated form and aggregates (seen in the bottom panel). B, Myc-Kv4.3 was coexpressed with NCS-1, empty vector (without NCS-1 (+)), or GFP-KChIP2. Lysates and precipitated materials with anti-Myc antibody were analyzed with immunoblotting with anti-Myc, anti-NCS-1, or anti-GFP antibody. *, anti-NCS-1 antibody exhibits strong reaction to endogenous proteins in HEK293 cells; **, secondary anti-rabbit IgG weakly cross-reacts with mouse IgG light chain.

**Fig. 1.** KChIP2 (but not NCS-1) specifically and effectively binds to Kv4.3 protein. A, GFP-tagged KChIP2, NCS-1, or GFP empty vector were coexpressed with Myc-tagged Kv1.4, Kv2.1, or Kv4.3 in HEK293 cells. The anti-GFP antibody was used for immunoprecipitation (IP). The top and middle panels show immunoblots (IB) of cell extracts (Lysate) with anti-Myc and anti-GFP antibody, respectively. The bottom panel represents immunoblot of the precipitated materials with anti-Myc antibody. Note that the reciprocal immunoprecipitation and immunodetection also detected specific and robust association between GFP-KChIP2 and Myc-Kv4.3 protein but not any other combinations. The larger faint and smaller strong bands of Kv1.4 proteins represent mature glycosylated and non-glycosylated forms, respectively. Also, a doublet of smaller truncated Kv2.1 proteins was routinely seen in our transient transfection. Kv4.3 proteins were present as a doublet in addition to a minor truncated form and aggregates (seen in the bottom panel). B, Myc-Kv4.3 was coexpressed with NCS-1, empty vector (without NCS-1 (+)), or GFP-KChIP2. Lysates and precipitated materials with anti-Myc antibody were analyzed with immunoblotting with anti-Myc, anti-NCS-1, or anti-GFP antibody. *, anti-NCS-1 antibody exhibits strong reaction to endogenous proteins in HEK293 cells; **, secondary anti-rabbit IgG weakly cross-reacts with mouse IgG light chain.

Kv4.3 Channels Alter Subcellular Localization of NCS-1 Chimera with the Three KChIP2 Peptides—We have shown previously that coexpression of Kv4.3 proteins dramatically alters subcellular localization of GFP-tagged KChIPs (8). Upon expression of Kv4.3 proteins, KChIPs with palmitoylation sites predominantly localize at plasma membrane, whereas those without the fatty acylation sites accumulate in intracellular compartments. To determine whether coexpression of Kv4.3 proteins similarly influences subcellular localization of NCS-1 chimera with the three KChIP2 peptides (Chimera 14), we examined localization of GFP-tagged proteins in the presence and absence of excess Kv4.3 proteins by confocal microscopy (Fig. 5). As has been observed in our previous work (8), GFP-KChIP2 was uniformly distributed throughout the cytosol in the absence of associating channels. In addition, a small fraction of the fusion protein was detected at the plasma membrane. In contrast, GFP-NCS-1 was distributed diffusely in the cytosol regardless of the presence or absence of Kv4.3 proteins. Similar to the original NCS-1, the NCS chimera with the three KChIP2 peptides (Chimera 14) by itself was seen mostly in the cytosol. Importantly, coexpression of Kv4.3 proteins localized this chimeric fusion protein to the plasma membrane and other intracellular compartments. Un-
like the original KChIP2 protein, more intracellular accumulation was evident, possibly because of the lack of palmitoylation of the N-terminal peptide. Thus, introduction of the three identified KChIP2 peptides causes NCS-1 to localize to the plasma membrane and other membrane-associated compartments upon coexpression of Kv4.3 proteins. These results also indicate that the association of this NCS-1 chimera with Kv4.3 proteins detected biochemically is not because of formation of nonspecific aggregates, nor is it because of a gross alteration in the subcellular localization of the protein.

The Chimera with the Three KChIP2 Regions Influences Kv4.3 Channel Gating—We also examined whether the chimera with the three identified KChIP2 peptides affects Kv4.3 channel gating in a similar manner to that of the original KChIP2. Our previous work indicated that KChIP2 produces two prominent gating effects (8), which are (i) slowing of inactivation and (ii) facilitation of recovery from inactivation. In contrast, a recent study showed that NCS-1 does not influence gating of Kv4.3 channels in a mammalian cell line (11). Therefore, we tested the effects of KChIP2, NCS-1, and Chimera 14 on these two parameters (Fig. 6). As expected, KChIP2 produced slower inactivation and faster recovery from inactivation of Kv4.3 current. Coexpression of NCS-1 produced no apparent changes in the two parameters. Importantly, we found that coexpression of Chimera 14 produced changes in the two parameters that are nearly identical to those seen with KChIP2. Thus, the chimera with the three KChIP2 peptides is capable of producing changes in Kv4.3 channel gating.

DISCUSSION

Structural and functional similarities between KChIPs and NCS-1 suggested that the two proteins interact with the N-terminal peptide of Kv4 channel proteins (11, 12). However, the present study revealed that the interaction of NCS-1 with Kv4.3 protein is significantly weaker and/or less efficient than that of KChIP2. Our analysis with chimeric proteins consisting of various regions of KChIP2 and NCS-1 identified three re-
regions of KChIP2 peptide that are necessary and sufficient for its effective association with Kv4.3 proteins. These three regions are the linker between the first and second EF hands, the one between the third and fourth EF hands, and the C-terminal peptide after the fourth EF hand. These results indicate that the sequence difference in these three regions between KChIPs and NCS-1 determines the efficiency of their binding to Kv4.3 proteins. On the basis of the crystal structure of human NCS-1 protein (14), these three peptides surround the large hydrophobic crevice (Fig. 7). Hence, this hydrophobic crevice of KChIP may be the site for its association with the N-terminal peptide of Kv4 channel proteins.

Previous studies have shown that NCS-1 is present in association with Kv4 proteins in brain and heart. However, we were unable to detect significant association of NCS-1 with Kv4.3 protein in this study. NCS-1 appeared to be incapable of forming stable complexes with Kv4.3 regardless of the presence or absence of a tag at its N terminus. This discrepancy might be because of the experimental conditions used by different groups, although there is no apparent difference in solution compositions. Another possibility for the complex formation previously observed between NCS-1 and Kv4 proteins is that NCS-1 binds indirectly to Kv4 proteins. For example, NCS-1 may interact with Kv4 proteins by forming complexes with other endogenous proteins that are present in brain and heart tissues. In any case, our results suggest that the direct inter-

Fig. 4. Three regions (II, IV, and V) of KChIP2 are necessary and sufficient for its binding to Kv4.3 protein. Myc-tagged Kv4.3 channel protein and GFP-tagged KChIP2, NCS-1, or KChIP2/ NCS-1 chimeras were coexpressed in HEK293 cells. Immunoprecipitation was carried out using anti-Myc antibody. The top and middle panels indicate immunoblots of total cell extracts (Lysate) with anti-Myc and anti-GFP antibody, respectively. The bottom panels show immunoblots of immunoprecipitated materials (IP) with anti-GFP antibody. A and B, different portions of the C-terminal (A) or N-terminal (B) peptide of KChIP2 were replaced with the corresponding regions of NCS-1. C, each region (II, III, IV, and V) of KChIP2 was substituted with the corresponding portion of NCS-1. D, one or more of three EF-hand linker regions of NCS-1 were replaced with the corresponding portions of KChIP2. Note that the NCS-1 chimera with the two EF-hand linkers (Regions II and IV) and C-terminal peptide (Region V) of KChIP2 associates with Kv4.3 proteins in a manner similar to the original KChIP2.

Fig. 5. Kv4.3 proteins localize the NCS-1 chimera with three regions of KChIP2 (Regions II, IV, and V) to plasma membrane. HEK293 cells were transfected with plasmids for GFP-tagged KChIP2, NCS-1, or Chimera 14 without (−) or with (+) Kv4.3 at the cDNA ratio of 1:10. Two days after transfection, cells were observed with a laser confocal microscope to examine the subcellular localization of GFP-tagged proteins.

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one between the third and fourth EF hands, and the C-terminal peptide after the fourth EF hand. These results indicate that the sequence difference in these three regions between KChIPs and NCS-1 determines the efficiency of their binding to Kv4.3 proteins. On the basis of the crystal structure of human NCS-1 protein (14), these three peptides surround the large hydrophobic crevice (Fig. 7). Hence, this hydrophobic crevice of KChIP may be the site for its association with the N-terminal peptide of Kv4 channel proteins.

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action of NCS-1 with Kv4 proteins is rather weak, and stable complex formation between these molecules requires additional supports.

In addition to the protein biochemical data, previous electrophysiological experiments showed variability in the effects of NCS-1 on Kv4 channel gating. Whereas NCS-1 slowed inactivation and facilitated recovery from inactivation of Kv4.2 current in *Xenopus* oocytes (12), it did not produce any appreciable effects on the gating of Kv4.2 or Kv4.3 channels in HEK293 cells (11). We also found that NCS-1 does not produce any effects on inactivation or recovery from inactivation. Furthermore, a recent study revealed that various channel auxiliary subunits, including MinK-related one-transmembrane proteins, Kvβ, and Na+ channel β-subunit, all increased the density and altered the gating of Kv4.3 channel in HEK293 cells (15). These findings further support the possibility that other proteins present in a certain expression system or in native cells significantly influence the physical interaction of NCS-1 with Kv4 channels and its effects on the gating of these channels.

Structures of several members of the KChIP-related Ca²⁺-binding protein family have been solved by x-ray crystallography and/or NMR (14, 16–21). These crystal and solution structures reveal that these proteins contain a hydrophobic crevice on one side and a chain of EF-hand motifs on the other. Left and right pictures represent views from the EF-hand and crevice sides, respectively. Note that the three regions of KChIP2 (Region II (green), IV (yellow), and V (red)) surround the large hydrophobic crevice.
Ca\textsuperscript{2+} binding. The oligomerization is mediated with specific portions of these proteins that face the EF-hand side (22). Thus, it is likely that this crevice is a major site for their association with effector proteins, whereas the EF-hand side may be involved in their oligomerization. In this study, we identified three regions of KChIP2 as determinants of the effective association of this protein with Kv4.3 protein. On the basis of NCS-1 crystal structure (14), these three regions surround the large hydrophobic crevice. Taken together, our findings further support the possibility that the hydrophobic crevice is the site of effector binding in the KChIP-related family of proteins.

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