The Identification and Characterization of a Noncontinuous Calmodulin-binding Site in Noninactivating Voltage-dependent KCNQ Potassium Channels*

Eva Yus-Nájera, Irene Santana-Castro, and Alvaro Villarroel‡

From the Instituto Cajal, Consejo Superior de Investigaciones, Avenida, Dr. Arce 37, 28002 Madrid, Spain

Received for publication, April 29, 2002, and in revised form, May 22, 2002. Published, JBC Papers in Press, May 24, 2002, DOI 10.1074/jbc.M204130200

We show here that in a yeast two-hybrid assay calmodulin (CaM) interacts with the intracellular C-terminal region of several members of the KCNQ family of potassium channels. CaM co-immunoprecipitates with KCNQ2, KCNQ3, or KCNQ5 subunits better in the absence than in the presence of Ca²⁺. Moreover, in two-hybrid assays where it is possible to detect interactions with apo-CaM but not with Ca²⁺-bound calmodulin, we localized the CaM-binding site to a region that is predicted to contain two α-helices (A and B). These two helices encompass ~85 amino acids, and in KCNQ2 they are separated by a dispensable stretch of ~130 amino acids. Within this CaM-binding domain, we found an IQ-like CaM-binding motif in helix A and two overlapping consensus 1–5-10 CaM-binding motifs in helix B. Point mutations in helix A or B were capable of abolishing CaM binding in the two-hybrid assay. Moreover, glutathione S-transferase fusion proteins containing helices A and B were capable of abolishing CaM binding in the two-hybrid assay. Also, mutations in four of the five known members of this family involved in the control of cellular excitability. Remarkably, mutations in four of the five known members of this family have been associated with different hereditary human disorders. While mutations in the KCNQ1 subunit (KvQT1) lead to arrhythmia in the human long QT syndrome, mutations in KCNQ2 or KCNQ3 are associated with a benign form of epilepsy. It has also been shown that KCNQ4 is mutated in a dominant form of progressive hearing loss (1).

With regards to the normal physiology of this protein family, the KCNQ2 and KCNQ3 subunits have been shown to form M-type potassium channels whose expression is restricted to neuronal tissue (2). Moreover, in some brain areas and neuronal tissues, KCNQ4 and KCNQ5 also contribute to the formation of M channels, suggesting that the different combinations of KCNQ subunits may be in part responsible for the diversity of M channel properties (1). The M current (I₉₅) is a subthreshold noninactivating voltage-dependent potassium current that is found in many neuronal cell types. The M current controls membrane excitability, and it has been shown to be modulated by a variety of intracellular signals that in turn dramatically affect the firing rate of neurons. Among those intracellular signals, Ca²⁺ has been shown to mediate the inhibition of I₉₅ by B₂ bradykinin receptors in sympathetic neurons (3). Indeed, intracellular Ca²⁺ can suppress the activity of M channels under conditions that do not support enzymatic activities such as phosphorylation (4). This phenomenon suggests that an intermediary might be involved in this Ca²⁺-dependent modulation.

In a search for candidates that might mediate the effects of Ca²⁺ in modulating I₉₅, we screened a human brain cDNA library using the yeast two-hybrid system. We found that calmodulin (CaM) binds to the C-terminal region of KCNQ channels. CaM is a small Ca²⁺-binding protein that acts as a ubiquitous intracellular Ca²⁺ sensor in the regulation of a growing diverse array of ion channels (5). Efforts to define common characteristics of CaM binding have indicated that it associates with short α-helical sequences within its targets (6–8). However, it appears that the interaction of CaM with KCNQ channels does not conform to this simple model. Rather, our data suggest that the CaM-binding site in KCNQ channels is formed by two α-helices that are separated by a stretch of ~130 amino acids. We hypothesize that those two helices come into close proximity in the tertiary structure, facilitating CaM binding.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—A cDNA generated by PCR encoding amino acids 310–844 of the human KCNQ2 subunit (9) was subcloned in frame with the GAL4 DNA-binding domain of the yeast vector pGBKKT7 (CLONTECH) to be used as bait in a yeast two-hybrid screen. The reporter yeast strain Y190 was sequentially transformed with this plasmid and with a human brain cDNA library subcloned in pACT2 (CLONTECH, catalog number HL 4004 AH, lot 5098, mRNA source: normal, whole brain from a 37-year-old Caucasian male, whose probable cause of death was trauma). We screened >2.5 × 10⁶ co-transformants that were selected on medium lacking histidine (in the presence of 25 mM 3-aminotriazole), leucine, and tryptophan and assayed for β-galactosidase activity.

* This work was supported by European Union Grant QLGT-1999-00827, Fondo de Investigaciones Sanitarias Grant FIS01/1136, Comunidad de Madrid Grant 08.5/001/2001.1, and Spanish Ministry of Education Grant SAF2000-0159. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 34-91-585-4718; Fax: 34-91-585-4754; E-mail: av@cajal.csic.es.

1 The abbreviations used are: CaM, calmodulin; apo-CaM, apo-calmodulin; Ca²⁺-free calmodulin; Ca²⁺-CaM, Ca²⁺-bound calmodulin; IQ, calmodulin binding motif; GST, glutathione S-transferase; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; aa, amino acids(s).
proteins was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside and assessed by the size and color of the colonies. E. coli (Invitrogen) was transformed into BL21 (Amersham Biosciences) and the synthesis of fusion proteins was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 30 °C. The cells were resuspended in chilled GST buffer that included protease inhibitors (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 8, plus 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin and leupeptine) and lysed by sonication at 4 °C. The lysate was recovered by immobilization on glutathione-Sepharose 4B beads (Amersham Biosciences). After extensive washing, the immobilized proteins were eluted in pull-down buffer (25 mM Hepes, 120 mM KCl, 5 mM NaCl, pH 7.5) with either 2 mM CaCl2 or 5 mM EGTA. Rat calmodulin (10 μg) was added to the beads and incubated for 45 min at room temperature. After three washes, the proteins were recovered, separated by 15% SDS-PAGE in the presence of 5% EGTA, and transferred to Problon nitrocellulose (Schleicher & Schuell) for Western blotting. The nitrocellulose was blocked with 5% nonfat dry milk in 0.05% Tween 20 in phosphate-buffered saline, incubated with the primary antibody (monoclonal anti-CaM from Upstate, diluted 1:2000 in blocking buffer) overnight at 4 °C, washed, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad) diluted 1:5000 in blocking buffer. Antibody binding was detected using enhanced chemiluminescence and ECL hyperfilm (Amersham Biosciences).

Antibody Production—Divergent sequences from the intracellular N- and C-terminal regions of the different KCNQ channels were used to generate GST fusion proteins that were then used to produce antisera in rabbits. The specificity of the antisera produced was tested in immunoblots of cell lysates of cells stably or transiently expressing different KCNQ subunits. A full description of the characterization of these antisera will be published elsewhere.2

Immunoprecipitation—Stable (kindly provided by B. S. Jensen, NeurOSearch) or transient HEK293 cells expressing human KCNQ subunits were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in 5% CO2. Transient expression was achieved with the KCNQ expression plasmid using LipofectAMINE 2000 (Invitrogen). For immunoprecipitation experiments, confluent 60-mm dishes were washed twice with ice-cold phosphate-buffered saline and solubilized for 1 h in 400 μl of immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, pH 7.5) including protease inhibitors as above, and phosphatase inhibitors (1 mM NaF, 1 mM β-glycerophosphate, and 5 mM pirophosphate). The cell lysates were centrifuged at 12,000 × g for 20 min to remove insoluble material, and the protein concentration was determined with the Bio-Rad protein assay. The lysate was diluted 10-fold in immunoprecipitation buffer, and bovine serum albumin was added to a final concentration of 1 mg/ml. Rabbit anti-KCNQ subunit-specific antibodies were added at 4 μg/ml and incubated for 3 h at 4 °C with agitation. Immunocomplexes were recovered with 40 μl of equilibrated protein A-agarose (Santa Cruz) and washed with immunoprecipitation buffer. The proteins were eluted in Laemmli’s buffer and resolved in 8% SDS-PAGE for the channels or 15% SDS-PAGE for CaM. The rabbit antiserum to KCNQ channels was used at 1:500 dilution, and the peroxidase-conjugated protein A/G (Pierce) was used at 1:5000 dilution.

Fluorescence Determination of CaM—CaM (100 μg/ml) was diluted 10-fold in 100 mM Tris-HCl, 20 mM CaCl2, pH 8.5. Dansyl chloride dissolved in acetone (2.17 mg/ml) was added (12.5 μl) to achieve a final concentration of 100 μM. The mixture was incubated at room temperature in the dark for 2 h, vortexing every 20 min. Unincorporated dansyl was eliminated using a 1-ml G-25 Sepharose column. The concentration of dansyl-calmodulin used in all experiments was 200 nM, unless noted otherwise.

Fluorescence spectra were recorded in a Perkin-Elmer fluorescence spectrophotometer in a final volume of 3 ml (light path, 1 cm). Dansyl-CaM was diluted in binding buffer (25 mM Tris-Cl, 120 mM KCl, 5 mM NaCl, with either 5 mM EGTA or 2 mM Ca2+, pH 7.4). The excitation wavelength was 340 nm, and the emissions were collected from 450 to 600 nm (0.5-nm steps).

RESULTS

CaM Interacts with the Intracellular C-terminal Region of KCNQ Channels—Potassium channels that are members of the KCNQ protein family have been implicated in pathological conditions affecting the nervous system and in the physiological regulation of excitable cells. Indeed, the activity of channels containing KCNQ subunits can affect aspects of the behavior of the cell as important as their firing rate. To identify proteins capable of modulating the activity of these channels, we have performed a yeast two-hybrid screen of a human brain cDNA library using the intracellular C-terminal region of human KCNQ2 as bait (534 amino acids).

We identified 32 positive clones that grew in the absence of histidine (25 μM 3-aminotriazole added), leucine, and tryptophan and that presented β-galactosidase activity. Of these, 19 clones were identified as the product of the gene CALM2, and 13 clones were identified as the product of the gene CALM3. Both genes encode variants of CaM and have an identical amino acid sequence. This interaction was confirmed in a second assay where CaM was used as bait and the C-terminal region of KCNQ2 or KCNQ3 was used as prey, demonstrating that the association of CaM with KCNQ subunits is independent of which protein is fused to the binding or activation domain.

Other regions of the KCNQ2 intracellular domain were also studied to determine whether they too were capable of interacting with CaM. We tested the N-terminal region (Met1–Arg100), the loop connecting the second and third transmembrane domains (Arg144–Ile173), and the loop connecting the fourth and fifth transmembrane domain (Leu208–Ala238). We also tested the pore region (Glu254–Leu283) that connects the fifth and sixth transmembrane domains because an interaction of the equivalent region of KCNQ1 with the C-terminal region of minK has been demonstrated in the two-hybrid assay (12). We were unable to observe any interaction between the constructs encoding these regions and CaM in the two-hybrid assay, despite the detection of these Myc-tagged hybrid proteins in immunoblots of lysates from transformed yeast (not shown).

The C-terminal domain of KCNQ2 channels commences with a region that is highly conserved between several members of this family and that is followed by a more divergent region. To study the domain to which CaM binds in more detail, we divided the C-terminal region of KCNQ2 and KCNQ3 into two overlapping parts and found that CaM interacted only with the initial, more highly conserved region (see Fig. 2). The overall similarity of this initial C-terminal region as defined by the Clustal method of DNA Star software ranges from 38 to 45% for KCNQ2–5 subunits, and the similarity between KCNQ1 and the other KCNQ channels ranges from 22 to 25%. Using this C-terminal region as bait (or the whole C-terminal region; not shown), we found that each member of the KCNQ channel family was capable of interacting with CaM. Furthermore, when the interaction was quantified with a liquid β-galactosidase assay, the binding of CaM appeared to be stronger for the KCNQ1 and KCNQ3 C-terminal regions than for those of KCNQ2, KCNQ4, or KCNQ5. The values relative to the quan-
Calmodulin Binds to KCNQ Channels

Several consensus sites for CaM binding have already been described (6, 7). A closer analysis of the region that contains helix A showed that it includes a sequence that resembles the IQ CaM binding motif and that this is conserved among several members of the KCNQ family (Fig. 3A). Mutations within the IQ motif have been shown to abolish the interaction between CaM and neurogranin in a two-hybrid assay (16) or to alter the Ca²⁺-dependent regulation of ion channels (17). We introduced several point mutations to determine whether amino acids within the IQ domain are necessary for the interaction of KCNQ2 with CaM. These mutations included Ile⁴³⁰ → Ala, Ile⁴³⁰ → Glu, Ser⁴³² → Asp, and Ala⁴³³ → Asp.

Amino acid alignment of the IQ motifs indicates that Ala⁴³³ of KCNQ2 corresponds to Ser⁴³⁶ of neurogranin (not shown). Mutating Ser⁴³⁶ → Ala of neurogranin makes the IQ motif of neurogranin resemble that of KCNQ2 and does not affect (or may even increase) CaM binding. However, mutating Ser⁴³⁶ → Asp, thereby introducing a negative charge that mimics the effect of protein kinase C, abolishes the interaction between neurogranin and CaM in the two-hybrid assay (16). Similarly, we found that the interaction between CaM and the KCNQ2 bait was lost in the equivalent Ala⁴³³ → Asp mutant. The interaction was also disturbed when the Ile⁴³⁰ → Ala and Ile⁴³⁰ → Glu mutations were introduced, and the Ser⁴³² → Asp mutation appeared to partially perturb CaM binding because the β-galactosidase assay took longer to develop and gave a weaker signal (Fig. 3E). Thus, it appears that the IQ binding motif is necessary to sustain CaM binding in the yeast two-hybrid assay.

Helix B contains two overlapping 1–5–10 CaM binding motifs (Fig. 3C and Ref. 7). We determined whether the introduction of negative charges in this region affected CaM binding in a manner similar to that used when they are introduced into key positions of the IQ binding motif. Within helix B of KCNQ2 there are three protein kinase C phosphorylation consensus sites (Ser⁵¹¹, Ser⁵²³, and Ser⁵³⁰), and thus we investigated the effect of introducing a negative charge (mutating Ser → Asp) at these positions to mimic the effect of phosphorylation. We also evaluated the effect of mutating serine 406, which is also a potential target for protein kinase C but that lies in a region not required for CaM binding. As expected, the introduction of an aspartate at position 406 did not alter CaM binding (not shown). In contrast, the interaction with CaM was lost when serine 511 was mutated to aspartate (Ser⁵¹¹ → Asp) but appeared to be only slightly affected (the signal was fainter than for the wild type) when the other serine residues were mutated (Fig. 3, D and E). These results suggest that protein kinase C might regulate the binding of CaM to KCNQ2 channels through the phosphorylation of Ser⁵¹¹.

To further characterize the interaction between CaM and the KCNQ channels, we performed GST pull-down experiments (Fig. 4). Different fragments of the C-terminal region of KCNQ2 were fused in frame to GST, and their binding to apo-CaM and Ca²⁺-CaM was compared with that of GST-neurogranin and GST fused to the C-terminal region of the NR1 subunit (18). The association of purified recombinant rat CaM to fusion proteins was analyzed both in the absence and in the presence of Ca²⁺. Western blots probed with a monoclonal anti-CaM antibody showed that the fragments including helix A (aa 310–451) or helix B (aa 445–548) bound CaM in the presence of Ca²⁺ (Fig. 4). In the absence of Ca²⁺, helix A did not bind CaM, whereas helix B appeared to show a weak interaction with CaM. In the two-hybrid assay, both of these regions appeared to be incapable of interacting with CaM; however, as discussed later, this can be explained by the failure to detect interactions with Ca²⁺-CaM with the two-hybrid assay.
A GST fusion protein containing the most C-terminal region of KCNQ2 (aa 549–844) was incapable of binding CaM in either the presence or the absence of Ca\(^{2+}\)/H11001. In contrast, CaM was pulled down independently of the presence of Ca\(^{2+}\)/H11001 (although more CaM was pulled down in absence of Ca\(^{2+}\)/H11001) when the GST fusion included a fragment that contained helices A and B (aa 310–548; Fig. 4), again indicating that CaM interacts directly with KCNQ channels.

The interaction of CaM with the C-terminal tail of KCNQ2 was studied by fluorimetry (Fig. 5). The fluorescence spectrum of CaM dansylated at Lys75 is shifted, and the intensity increases when the environment of the fluorophore becomes hydrophobic (19). Dansyl-CaM was incubated with GST, GST-helix A, GST-helix B, or GST-helices A/H11001 B fusion proteins, and the changes in fluorescence emission induced by the interaction were studied in the presence (2 mM Ca\(^{2+}\)/H11001) and absence of Ca\(^{2+}\)/H11001 (in the presence of 5 mM EGTA). As previously shown, GST alone had no effect on the emission spectrum of dansyl-CaM independent of the [Ca\(^{2+}\)] (20). In the presence of Ca\(^{2+}\) and equimolar concentrations of the GST fusion proteins that included helices A and B or helix B alone, modest changes in the fluorescent emission were detected. These changes were even more modest in the presence of the GST-helix A fusion protein. In contrast, a substantial enhancement in fluorescence was observed in the absence of Ca\(^{2+}\), with the GST-helices A+B fusion protein. Using a series of dansyl-CaM concentrations (50–400 nM), the EC\(_{50}\) estimated in the absence of Ca\(^{2+}\) for GST-AB binding to dansyl-CaM ranged from 186 to 320 nM. Under similar conditions, the change in fluorescence remained modest with the GST-helix B fusion and could not be detected with the GST-helix A fusion. In addition, no synergism was seen in the enhancement of fluorescent emission when both GST-helix B and GST-helix A were used together. Thus, the most significant changes in fluorescence were seen when both helices are part of the same polypeptide, suggesting that in the absence of Ca\(^{2+}\), this peptide folds in such a way that it provides a better CaM-binding site than helix B alone (Fig. 5).

The role of helices A and B was further examined by transiently expressing in HEK cells mutant KCNQ2 channels in which the helices were deleted and assessing their ability to associate with CaM by immunoprecipitation. CaM was not co-immunoprecipitated by antisera against KCNQ2 in cells expressing mutants devoid of helix A ([IQ Leu\(^{339}\)–Thr\(^{358}\), ΔIQ Trp\(^{359}\)–Met\(^{371}\), helix B ([Δφ Ser\(^{511}\)–Ser\(^{523}\)], or both ([ΔIQ+Δφ; Fig. 6). In contrast, the deletion of aa 372–493 did not appreciably inhibit CaM binding (Fig. 6A). In addition, we analyzed the point mutant Ser\(^{342}\)→Asp, which gave a weak signal in two-hybrid assay (Fig. 3E). With this mutant, CaM was only seen to co-immunoprecipitate with the channel in the absence of Ca\(^{2+}\). In contrast, the association of CaM was not observed with the Ser\(^{511}\)→Asp mutant in the presence or absence of Ca\(^{2+}\). Thus, the results of the co-immunoprecipitation experiments paralleled those obtained in the two-hybrid assay, reinforcing the proposal that both helices are necessary for interaction with CaM in the intact channel.
Determinants in CaM That Are Required for Binding to KCNQ—CaM is composed of four Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{-binding helix-loop-helix motifs, called EF hands. These are arranged in two pairs, each pair forming a distinct domain or lobe. The domains are arranged in a dumbbell-like conformation at the ends of a flexible central helix (aa 65–92). The N-terminal lobe (aa 1–77) contains EF hands 1 and 2, and the C-terminal lobe (aa 78–148) contains EF hands 3 and 4. It is thought that, in evolutionary terms, CaM arose by duplication of a gene that represented one lobe. As a consequence EF hands 1 and 3 are alike, and EF hand 2 is most similar to EF hand 4 (8). Ca\textsuperscript{2+}/H\textsubscript{11001} binds to the four EF hand motifs in a highly cooperative manner. First it associates with EF hands 4 and 3, and subsequently it associates with EF hands 2 and 1. The binding of Ca\textsuperscript{2+} to the EF hands results in the creation of a surface that serves as an interface for the association of CaM with the target protein. Each lobe can control distinct processes in the target protein (21, 22), and binding to a target can change the affinity for Ca\textsuperscript{2+}/H\textsubscript{11001} (23).

To investigate the role of the different EF hands, we studied the interaction of a mutated CaM where the first aspartate of EF hands 2, 3, and 4 has been replaced with alanine and another CaM mutated at EF hands 3 and 4. These Asp to Ala mutations in the EF hands greatly diminish or abolish their ability to bind Ca\textsuperscript{2+}/H\textsubscript{11001} (21, 24, 25). Both mutants were able to interact with the C-terminal region of KCNQ2 or KCNQ3, although less strongly than wild type CaM. In the liquid galactosidase assay the intensity of the signal obtained with the EF-2,3,4 mutant was 40% of wild type CaM (not shown, but see Fig. 7).

The determinants for this interaction were further studied using the low copy number pPC97 (bait) and pPC86 (prey-CaM) vectors. These plasmids produce more physiological levels of protein than the ones used to perform the previous experiments. Thus, this low copy number system allows for better discrimination of small changes in binding strength caused by the point mutations introduced into either the bait or the prey. It has been proposed that this two-hybrid assay only detects Ca\textsuperscript{2+}/H\textsubscript{11001}-independent interactions with CaM (21). While this still remains unclear (26), this may be an important technical consideration to bear in mind given the difficulties encountered in identifying the site of interaction of apo-CaM to some proteins (27). As mentioned earlier, we found that in vitro, helix A and helix B can associate with Ca\textsuperscript{2+}/H\textsubscript{11001}-CaM (Fig. 4), although we were
Calmodulin Binds to KCNQ Channels

We also studied the interaction of CaM with the full C-terminal region of SK2 potassium channel as well as neurogranin and the C-terminal region of P/Q calcium channel for reference (16, 21). As previously reported, we found that the C-terminal lobe of CaM (aa 78–148, EF-3,4) interacted with the C-terminal domain of SK2 (21). In contrast, full-length CaM (i.e. the four EF hands) was required to bind to the C-terminal AB region of KCNQ2 or KCNQ3 subunits.

CaM carrying different combinations of EF hands mutated at the first aspartate were tested for their ability to associate with the AB region of KCNQ2 (Fig. 7C). Although apo-CaM binds to both the SK (25) and KCNQ family of channels, there is no significant sequence homology in the C-terminal region of these K+ channels. It has been reported that CaM carrying mutations in any or all combinations of the EF hands associates with a fragment of the C-terminal region of SK2 (aa 390–487) (21). In the experiments reported here we used a longer bait (aa 390–707) that produced a weaker signal (i.e. the strength of the interaction of the hybrid proteins is closer to the threshold level of detection). With the full C-terminal region of SK2, the assay produced a signal that was difficult to detect when either EF hands 3 or 4 were mutated alone or in combination (M3, M4, and M34). Because it has been clearly demonstrated that EF hands 3 and 4 directly mediate the interaction of apo-CaM with SK2 channels (21, 23), these results indicate that the strength of binding is reduced when Asp to Ala mutations are introduced into these EF hands, sometimes to below the threshold of detection.

In contrast to SK, the interaction with the KCNQ2 or KCNQ3-AB region was lost or deficient when EF hands 1 or 3 were mutated (M1, M3, and M13), suggesting that these EF hands directly mediate the interaction of apo-CaM with KCNQ channels. In addition, the complementary M1 and M3 mutants (M234 and M124) retained the ability to interact with the KCNQ-AB region. Interestingly, whereas M1 did not bind to the KCNQ-AB region, the interaction was partially recovered when EF hands 1 and 2 were mutated simultaneously (M12 and M124), suggesting the existence of important cross-talk between EF hands 1 and 2 that influences the binding of CaM to KCNQ channels. Similarly, the interaction was recovered when EF hands 1 and 4 were mutated simultaneously (M14 and M124), indicating that both CaM lobes are functionally interconnected when interacting with the KCNQ-AB region.

FIG. 5. Changes in dansyl-CaM fluorescence induced by GST-Helix A, GST-Helix B, and GST-Helices A+B. Emission spectrum of dansyl-CaM (200 nM) alone and when mixed with GST fusion proteins in the absence (A) and presence of Ca2+ (B) is shown. The fluorescence spectrum of dansyl-CaM was determined upon excitation at 340 nm.

FIG. 6. Co-immunoprecipitation of KCNQ2 deletion mutants. A, membrane preparations from HEK293 cells transiently expressing KCNQ2 mutants were solubilized and immunoprecipitated with α-KCN2 antisera in the absence (−) or presence (+) of Ca2+ (with the addition of 5 mM EGTA or 2 mM Ca2+, respectively). The precipitated proteins were resolved in 8 or 15% polyacrylamide denaturing gels to detect the channels or CaM by Western blotting, respectively. The association between CaM and ΔIQ, AplIQ, and ΔΦ was detected neither in the absence nor the presence of Ca2+ condition. The deleted amino acids were: ΔIQ L339-T358, AplIQ W359-M371, and ΔΦ S511-S523. B, CaM was co-immunoprecipitated with the Ser245 → Asp in the absence of Ca2+, but an interaction was not detected with the Ser245 → Asp mutant in any Ca2+ condition. WT, wild type.

We tested fragments of CaM to determine which regions were required for binding to KCNQ2 and to KCNQ3 (Fig. 7B).

unable to detect this interaction in vivo with the two-hybrid assay. This is in keeping with Keen’s proposal (21). Moreover, using this assay, we were unable to detect an interaction of CaM with baits known to interact with Ca2+-CaM but not with apo-CaM (baits such as the C-terminal region of the NMDA receptor (18); the Eag potassium channel (28); and the CaM binding site of myosin light chain kinase (7)). Conversely, when neurogranin, which binds to apo-CaM but not to Ca2+-CaM, was used (7), a clear interaction could be seen (Fig. 7A).
DISCUSSION

We have demonstrated here that CaM binds to the intracellular C-terminal domain of neuronal KCNQ2, KCNQ3, and KCNQ5 transmembrane channels. Two-hybrid experiments suggest that CaM also binds to KCNQ1 and KCNQ4, but more experiments are necessary to unequivocally confirm this interaction. The voltage-dependent channels to which members of this family of proteins contribute have been implicated in a variety of physiological processes and pathologies. As a result, the modulation of the activity of these channels through intracellular signaling is important in maintaining the physiological homeostasis of nervous tissue (30). An example of this can be seen in the Ca\(^{2+}\)-dependent regulation of M channels (made up of KCNQ subunits) that influences the firing rate of the sympathetic cells in which they are expressed. Our results indicate that through its association with these proteins, CaM may mediate the Ca\(^{2+}\)-dependent modulation of channels that include KCNQ subunits.

In addition to demonstrating here that CaM associates with members of the KCNQ family, we have also defined its binding site. The binding site identified in this study is unusual in that it is composed of two discontinuous regions, helix A and helix B. Helix A contains an IQ-like binding motif, a motif (IQ\(_{X_{1}}XXR_{X_{2}}XXXXR_{X_{3}}\)) that mediates apo-CaM binding in a variety of proteins (31) and that contains positively charged residues at positions 6 and 11. When compared with other IQ motifs, in the

![Figure 7](image-url)
Calmodulin Binds to KCNQ Channels

As a result, this domain resembles the second “incomplete” IQ motif on myosin II, the region to which the regulatory myosin light chain (structurally similar to CaM) binds. A model of apo-CaM binding derived from this and other light chain structures bound to myosin IQ motifs reveals that the initial portion of this motif (IQXXXXR) is the most critical part. Moreover, it is this region that is specifically recognized by the loop between EF hands 3 and 4 that determines a semi-open lobe conformation (32). In accordance with this model, the interaction of KCNQ2 with CaM is destabilized when point mutations are introduced in the first part of the IQ motif.

On many occasions, the C lobe of CaM has been shown to be that which interacts with peptides, as also occurs with CaM-like proteins that associate with peptides. Moreover, the bound peptide essentially occupies the same position relative to the C-terminal EF hand domain (33). The finding that the C lobe is sufficient to bind to SK channels (21), P/Q Ca\(^{2+}\) channels, and neurogranin supports this finding. However, in contrast, it appears that to bind to KCNQ2 or KCNQ3 channels, both the C and N lobes are required.

Surprisingly, we found that when using the full-length C-terminal region of SK2 as bait, point mutations that abolish Ca\(^{2+}\) binding to EF hands 3 or 4 also abrogate the interaction with CaM. There is, however, ample biochemical, functional, and structural evidence to indicate that Ca\(^{2+}\) is not bound to EF hands 3 or 4 when CaM interacts with SK2. In addition, it has been shown that the Ca\(^{2+}\)-free C-terminal lobe is that which mediates the binding of CaM to the SK2 CaM-binding domain (21, 23). Our results indicate that mutating the first aspartate to alanine in the EF hands that directly mediate the interaction of apo-CaM with the target causes a reduction in the binding strength. By analogy with the SK2 CaM-binding domain, the observation that the interaction of CaM with KCNQ2 or KCNQ3 does not tolerate point mutations at EF hand 1 and (to a lesser degree) 3 suggests that binding to the KCNQ-BD is mainly mediated by the apo-EF hands 1 and 3. Interestingly, EF hand 1 is most similar to EF hand 3 (8), suggesting that both play a similar role in stabilizing the target complex.

The difficulties in identifying apo-CaM interactions have been highlighted by Erickson et al. (27). To overcome this problem, a very elegant technique has been developed, three-cube fluorescence resonance energy transfer, that demonstrates the preassociation of CaM with calcium channels in living cells (27). The yeast two-hybrid system is another viable alternative for approaching this problem. Our results provide further evidence that the two-hybrid assay is capable of detecting interactions between the Ca\(^{2+}\)-free form of CaM and target proteins such as the SK2 K\(^{+}\) channels (21). It should be born in mind that a limitation of the two-hybrid system is that the transmembrane segments of target proteins must be eliminated to allow targeting of the bait to the nucleus (34). However, the main advantage is that this is a relatively simple assay and that it does offer us the opportunity to study protein-protein interactions in a living cell (16).

The recent resolution of the structure of CaM associated with the SK-binding domain has shown that the binding domain is composed of two \(\alpha\)-helices connected by a short loop (23). The high probability that the two KCNQ regions contain \(\alpha\)-helices suggests that a similar conformation to the SK2 CaM-binding domain may also arise in KCNQ. However, some differences are evident. Although in SK channels the connecting loop is only 5 aa long, in KCNQ channels this varies from ~100 to ~150 aa. Secondly, the C lobe of CaM (EF hands 3 and 4) is sufficient for binding to SK channels (21), whereas the complete CaM molecule is necessary for binding to KCNQ2 or KCNQ3 channels. In essence, our data suggests that the C-terminal domain of KCNQ channels folds in such a way that helix A and helix B form a compact structure that can be engulfed between the N- and C-terminal lobes of apo-CaM (Fig. 8).

![Model of the interaction of CaM with KCNQ channels.](image)

The main features of the model are that helix A and helix B come into close proximity in the tertiary structure and are engulfed by CaM. The S6 transmembrane segment and pore region of only two subunits of a tetrameric potassium channel are shown for clarity. The relative orientation of helix A of the two subunits is a suggestion based on the proposed structure of cyclic nucleotide gated channels (36). The crystal structure of KcsA potassium channel (S6 and pore) (39) and the crystal structure of the SK potassium channel binding domain complexed with CaM (23) have been used as templates to draw this cartoon to scale.
CaM may also be important in other processes such as assembly or trafficking (37, 38). The functional analysis of mutants unable to bind CaM should help to unveil the role of CaM in KCNQ channel function.

Acknowledgments—We are very grateful to Drs. J. Maylie (Oregon Health Sciences University), T. Jentsch (Zentrum für Molekulare Neurobiologie Hamburg, Hamburg, Germany), L. M. Pardo and W. Stühmer (Max Planck Institute, Göttingen, Germany), A. Villalobo and J. Bernal (Instituto de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas, Madrid, Spain), W. A. Catterall (University of Washington, B. J. Jensen (NeuroSearch, Ballerup, Denmark), V. I. Teichmann (Weizmann Institute of Science, Rehovot, Israel), J. T. Stull (Instituto de Investigaciones Biologicas-Consejo Superior de Investigaciones Científicas, Madrid, Spain), W. A. Catterall (University of Washington), B. J. Jensen (NeuroSearch, Ballerup, Denmark), V. I. Teichmann (Weizmann Institute of Science, Rehovot, Israel), J. T. Stull (Instituto de Investigaciones Biologicas-Consejo Superior de Investigaciones Científicas, Madrid, Spain), W. A. Catterall (University of Washington), B. J. Jensen (NeuroSearch, Ballerup, Denmark), V. I. Teichmann (Weizmann Institute of Science, Rehovot, Israel), J. T. Stull

We acknowledge the help of Dr. Fernando Moro and Dr. M. Paz S. Pons (I Cajal, Madrid, Spain) for providing us with cDNAs and other materials used in this study. We thank Dr. Paula Bosch and Dr. Fernando Diaz for providing equipment and help in the fluorimetric assays. We acknowledge the help of Dr. Fernando Moro and Dr. M. Paz Regalado in some parts of the project. Dr. Mark Sefton for critical comments on the manuscript and editorial help, and Carmen Page and Uyen Le for technical assistance.

REFERENCES

1. Jentsch, T. J. (2000) Nat. Rev. Neurosci. 1, 21–30
2. Wang, H. S., Pan, Z., Shi, W., Brown, B. S., Wymore, R. S., Cohen, I. S., Dixon, J. E., and Hille, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7151–7156
3. Selyanko, A. A., and Brown, D. A. (1996) Neuron 16, 151–162
4. Saimi, Y., and Kung, C. (2002) Neuron 38, 289–311
5. O’Neil, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 59–64
6. Rhoads, A. R., and Friedberg, F. (1997) FASEB J. 11, 331–340
7. Jurado, L. A., Chockalingam, P. S., and Jarrett, H. W. (1999) Physiol. Rev. 79, 661–682
8. Biwer, C., Schroder, B. C., Kubisch, C., Berkovic, S. F., Propping, P., Jentsch, T. J., and Steinlein, O. K. (1998) Science 279, 403–406
9. Villarroel, A., and Regalado, M. P. (1997) Trends Genet. 13, 164
10. Hayashi, N., Matsubara, M., Takasaki, A., Titani, K., and Taniguchi, H. (1998) Protein Expression Purif. 12, 25–28
11. Rice, R., Attali, B., Chouabe, C., Abitbol, I., Guillemare, E., Barhanin, J., and Lazdunski, M. (1997) J. Biol. Chem. 272, 16713–16716
12. Schmitt, N., Schwarz, M., Perez, A., Abitbol, I., Attali, B., and Pongs, O. (2000) EMBO J. 19, 332–340
13. Smith, J. S., Iannotti, C. A., Dargis, P., Christian, E. P., and Aiyar, J. (2001) J. Neurosci. 21, 1096–1103
14. Schwake, M., Pusch, M., Kharkovev, T., and Jentsch, T. J. (2000) J. Biol. Chem. 275, 13343–13348
15. Prichard, L., Deloulme, J. C., and Storm, D. R. (1999) J. Biol. Chem. 274, 7689–7694
16. Zühlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Nature 399, 159–162
17. Joiner, W. J., Khanna, R., Schlichter, L. C., and Kaczmarek, L. K. (2001) Biochemistry 39, 1316–1323
18. El Far, O., Bofill-Cardona, E., Airas, J. M., O’Connor, V., Boehm, S., Freissmuth, M., Nanoff, C., and Betz, H. (2001) J. Biol. Chem. 276, 30662–30669
19. Keen, J. E., Khawaled, R., Farrens, D. L., Neelands, T., Rivard, A., Bond, C. T., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
20. Devillers, C., Usenri, H., Inoue, T., Michikawa, T., Kohda, K., Mikoshiba, K., and Yamamoto, T. (1999) J. Biol. Chem. 274, 20805–20810
21. Erickson, M. G., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) Neuron 31, 973–985
22. Geiser, J. R., van Tuinen, D., Breckenhoff, S. E., Neff, M. M., and Davis, T. N. (1991) Cell 65, 949–959
23. Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
24. Geiser, J. R., van Tuinen, D., Breckenhoff, S. E., Neff, M. M., and Davis, T. N. (1991) Cell 65, 949–959
25. Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
26. Hisatsune, C., Umemori, H., Inoue, T., Michikawa, T., Kohda, K., Mikoshiba, K., and Yamamoto, T. (1997) J. Biol. Chem. 272, 20805–20810
27. Erickson, M. G., Alseikhan, B. A., Peterson, B. Z., and Yue, D. T. (2001) Neuron 31, 973–985
28. Schonherr, R., Lober, K., and Heinemann, S. H. (2000) EMBO J. 19, 3263–3271
29. Lee, A., Wong, S. T., Gallagher, D., Li, B., Storm, D. R., Scheuer, T., and Catterall, W. A. (1999) Nature 395, 155–159
30. Marrion, N. V. (1997) Annu. Rev. Physiol. 59, 483–504
31. Houbiers, A., Silver, M., and Cohen, C. (1996) Structure 4, 1475–1490
32. Akins, R. A., Joseph, C., Kelly, G., Muskett, F. W., Frenkel, T. A., Nleitlsparsh, D., and Pastore, A. (2001) Nat. Struct. Biol. 8, 853–857
33. Niethammer, M., and Sheng, M. (1998) Methods Enzymol. 293, 104–122
34. Leviatan, I. B. (1999) Neuron 22, 645–648
35. Johnson, J. P. J., and Zagotta, W. N. (2001) Nature 412, 917–921
36. Gao, T., Bunemann, M., Gerhardtstein, B. L., Ma, H., and Hosey, M. M. (2000) J. Biol. Chem. 275, 25436–25444
37. Joiner, W. J., Khanna, R., Schlachter, L. C., and Kazmzarek, L. K. (2001) J. Biol. Chem. 31, 699–711
38. Doyle, D. A., Morais, C. J., Pietschn, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and Mackinnon, R. (1998) Science 280, 69–77