C-terminal Domain of Kv4.2 and Associated KChIP2 Interactions Regulate Functional Expression and Gating of Kv4.2*

Received for publication, May 19, 2006, and in revised form, June 29, 2006. Published, JBC Papers in Press, July 4, 2006, DOI 10.1074/jbc.M604843200

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The Kv4.2 transient voltage-dependent potassium current contributes to the morphology of the cardiac action potential as well as to neuronal excitability and firing frequency. Here we report profound effects of the Kv4.2 C terminus on the surface expression and activation gating properties of Kv4.2 that are modulated by the direct interaction between KChIP2, an auxiliary regulatory subunit, and the C terminus of Kv4.2. We show that increasing large truncations of the C terminus of rat Kv4.2 (wild type) cause a progressive decrease of Kv4.2 current along with a shift in voltage-dependent activation that is closely correlated with negative charge deletion. Co-expression of more limited Kv4.2 C-terminal truncation mutants (T588 and T528) with KChIP2 results in a doubling of Kv4.2 protein expression and up to an 8-fold increase in Kv4.2 current amplitude. Pulse-chase experiments show that co-expression with KChIP2 slows Kv4.2 wild type degradation 8-fold. Co-expression of KChIP2 with an intermediate-length C-terminal truncation mutant (T474) shifts Kv4.2 activation voltage dependence and enhances expression of Kv4.2 current. The largest truncation mutants (T417 and ΔC) show an intracellular localization with no measurable currents and no response to KChIP2 co-expression. Co-immunoprecipitation and competitive glutathione S-transferase-binding assays indicate a direct interaction between KChIP2 and the Kv4.2 C terminus with a relative binding affinity comparable to that of the N terminus. Overall, these results suggest that the C-terminal domain of Kv4.2 plays a critical role in voltage-dependent activation and functional expression that is mediated by direct interaction between the Kv4.2 C terminus and KChIP2.

Voltage-gated Kv4 α-subunits underlie the low threshold, rapidly inactivating transient outward K⁺ current termed Iₒ in cardiac myocytes and Iₐ in neurons. In the heart, Iₒ controls action potential configuration and duration (1–4), whereas in neurons Iₐ modulates synaptic processing (5), action potential propagation, and firing frequency (6). Recent work has demonstrated that Kv4 channel subunits form molecular complexes consisting of pore-forming α-subunits along with auxiliary subunits and associated scaffolding proteins (7). Included among the Kv4 β-subunits are the recently described EF-hand-containing Ca²⁺-binding Kv channel-interacting proteins (8). The Kv channel-interacting proteins bind to the N terminus of Kv4 α-subunits and produce isoform-dependent effects on Kv4 channel trafficking and gating properties (8, 9). In the heart, KChIP2 isoforms predominate and have a transmural expression profile in dogs and humans that parallels, and probably explains, a well recognized gradient in Iₒ (10–12). KChIP2 knock-out eliminates Iₒ, destabilizes repolarization, and creates a substrate for ventricular arrhythmias (4).

The N-terminal region of various K⁺ channel α-subunits has been identified as a binding domain for β-subunits (8), for Kv tetramerization (13), and for KChIP2-mediated regulation of gating and trafficking (9). The C terminus of Kv4 channel subunits has also been implicated in the localization and gating of the Kv4.2 channel. An interaction between a proline-rich C terminus domain and the actin-binding protein, filamin, contributes to the localization of Kv4.2 at synaptic densities (14). A 16-amino acid dileucine-containing motif has also been identified in the C terminus of Shal family K⁺ channels that is necessary for dendritic targeting of Kv4.2 channels (15). Two arginines (Arg⁴²⁶ and Arg⁴²⁹) in the C-terminal domain of Kv4.3 channel subunits are involved in voltage-dependent gating of Kv4.3 currents; replacement of these two arginines with alanines voltage-shifts both activation and inactivation gating (16). In addition, the Kv4 C-terminal domain interacts with Kv β-subunits (17) and scaffold proteins like PSD95 (18). Recent studies have shown that the ability of Kv4.2 to immunoprecipitate KChIP2 is heavily compromised when there are major deletions in the C terminus of Kv4.2 (19). Recent negative stain electron microscopy studies of Kv4.2 channels co-expressed with KChIP2 also suggest that KChIP2 may associate with the C terminus of Kv4.2 (32).

In the present study, we investigate in detail the role of the Kv4 C terminus by examining the consequences of a series of Kv4.2 C-terminal truncation mutants. We study the contribution of the C terminus to the expression and gating of Kv4.2. We also characterize the biochemical and functional properties of these mutant constructs with and without KChIP2 co-expression. The results indicate a direct binding between the C termi-
Preparation of cDNA Constructs—Full-length rat Kv4.2 WT was generated by PCR amplification using 5′ and 3′ primers with BglIII and NotI recognition sequences, respectively. The amplified product was digested with BglIII and NotI restriction enzymes and subcloned in-frame into HA-tagged phHA-CMV vector (Clontech). Full-length rat KChIP2 was PCR-amplified using 5′ and 3′ primers that introduced XhoI and NotI sites at the 5′ and 3′ ends and subcloned in-frame into phHA-CMV and p-c-myc-CMV (Clontech), respectively. Truncation mutant constructs were made by amplifying Kv4.2 WT plasmid with the same forward primer as for Kv4.2 WT and a series of reverse primers containing a NotI recognition site and in-frame stop codon with a partial Kv4.2 WT sequence that extends from the distal N terminus to each selected position in the post-transmembrane region on the C terminus (positions 406, 417, 474, 528, and 588), thereby truncating the more distal portion of the C terminus, as shown in Fig. 1 (top). These were correspondingly named ΔC (the T406 truncation of the entire Kv4.2 C terminus), T417, T474, T528, and T588. Deletion and negative charge neutralization mutants were constructed using QuikChange site-directed mutagenesis kits (Stratagene) and Kv4.2 WT as the PCR template. Kv4 N-terminal deletions were named according to the numbers of the residues deleted (e.g. Kv4.2ΔΔ2–31 means that the residues between 2 and 31 were removed). All PCR amplifications were conducted with a proofreading enzyme (Vent DNA Polymerase; New England Biolabs), and PCR products were purified with QIAquick gel extraction kits (Qiagen). Ligation reagents, restriction enzymes, and primers were purchased from New England Biolabs and Invitrogen, respectively. The fidelity of all constructs was confirmed by capillary electrophoresis-based sequencing (Sheldon Biotechnology Center, McGill University).

Cell Culture and Transfection—HEK293, COS7, and M2 cells were used and maintained as previously described (20). The interaction between Kv4.2 and KChIP2 was preserved in all cell lines. Cell cultures at 60–80% confluence were used for transfection. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Since the molecular mass of Kv4.2 is approximately double that of KChIP2, we transfected twice the amount of cDNA by weight of Kv4.2 over that of KChIP2 in an attempt to obtain a similar number of transcripts of Kv4.2 and KChIP2. Cells 24 h post-transfection were subjected to Western blot, pulse-chase analysis, immunoprecipitation, immunofluorescence, or electrophysiological analysis. No cells were used beyond 30 passages.

Immunoprecipitation and Western Blotting—Transfected HEK293 cells were washed twice with ice-cold PBS and lysed with buffer (50 mM Tris-HCl, 75 mM NaCl, 0.5% Nonidet P-40, protease inhibitors, pH 8.0). The lysate was centrifuged at 15,000 × g for 45 min to pellet insoluble material. The resulting supernatant was collected, and protein concentration was determined with a detergent-compatible assay (Bio-Rad). For immunoprecipitation, samples with equal amounts of protein and volume incubated overnight at 4 °C with monoclonal mouse anti-hemagglutinin (HA; Covance) or anti-c-myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies (1:100) were immobilized on Protein G-Sepharose beads for an additional 2 h. Immunoprecipitates were washed four times with ice-cold lysis buffer and eluted by incubation with 2× SDS sample buffer for 40 min at room temperature. For Western blot, samples were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% dry milk and incubated with monoclonal mouse anti-HA or anti-c-myc and/or anti-calcium/calmodulin-dependent serine protein kinase (CASK; Babco) antibodies, respectively. Immunoreactivity was detected by incubating membranes with goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) after extensive washes and visualized on Eastman Kodak Co. BMS film with ECL Plus (Amersham Biosciences). Immunoblots were quantified with an FC8000 densitometer, and the results are presented as arbitrary units normalized either to endogenous CASK or to relative controls.

GST Fusion Protein Pulldown Assays—For GST fusion proteins, the N (residues 1–183, ending at Met183) and C (residues 406–630) termini of Kv4.2 as well as four fragments of the C terminus (residues 406–474, 417–474, 474–630, and 417–630) were PCR-amplified and inserted in frame into bacterial PGEX-4T-3 expression vector (Amersham Biosciences). After sequence verification, plasmids were transformed into Escherichia coli BL21 cells. Clonal BL21 cells were grown to a density of ~0.8–1.0 A500 at 37 °C, and then protein expression was induced by adding 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (3-h incubation, 30 °C). After cell lysis, GST fusion proteins were purified following the manufacturer’s instructions.

Purified GST alone and GST fusion proteins were quantified by Coomassie Blue staining followed by analysis with an FC8000 imaging densitometer. Equal amounts of protein were added to 50 µl of 50% glutathione-Sepharose™ 4B bead slurry (Amersham Biosciences) in 500 µl of cell lysis buffer (1 mM EDTA, 0.5% Nonidet P-40, protease inhibitors in PBS, pH 8.0) and incubated on a rotator at 4 °C for 4 h. After three washes with lysis buffer, 100 µg of c-myc-KChIP2 lysate was added, rocked overnight at 4 °C, and washed four times. Competitive GST pulldown assays were performed by incubating various concentrations of purified N terminus (NT) and C terminus (CT) of Kv4.2 with fixed concentrations of GST-fused Kv4.2 N terminus (GST.Kv4.2NT) and C terminus (GST.Kv4.2CT), respectively, overnight at 4 °C with 100 µg of whole cell lysate from HEK293 cells transfected with c-myc-KChIP2. The purified N and C termini were obtained by cutting the GST.Kv4.2NT and GST.Kv4.2CT with thrombin and subsequently using glutathione-Sepharose™ 4B beads to remove the cleaved GST. Co-precipitates were eluted with 2× SDS...
sample buffer, resolved by SDS-PAGE, and analyzed by Coomassie Blue staining and Western blot.  

**Pulse-Chase Metabolic Labeling**—Transfected HEK293 cells were washed once with warmed PBS and starved for 1 h in serum-free Dulbecco’s modified Eagle’s medium lacking methionine and cysteine in the presence of 0.25% bovine serum albumin (Invitrogen). Cells were then metabolically labeled in the same medium containing 200 μCi/ml [35S]methionine/cysteine (PerkinElmer Life Sciences) for 1 h at 37 °C. The medium was then removed, and cells were rinsed with Dulbecco’s modified Eagle’s medium containing 2 mM unlabeled methionine and cysteine and chased in the same medium for different time intervals. Cells were lysed as described above, and equal amounts of protein were immunoprecipitated with anti-HA or anti-c-myc antibody, subjected to SDS-PAGE, and visualized by autoradiography followed by quantification with an FC8000 densitometer.  

**Immunolocalization**—COS7 cells transfected with Kv4.2 constructs singly or co-transfected with c-myc-KChIP2 were fixed on poly-L-lysine (Sigma)-coated glass coverslips with 2% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 30 min. Nonspecific antibody binding was suppressed in PBS containing 2% bovine serum albumin. Staining was performed with monoclonal rat anti-HA (Roche Applied Science) and/or mouse anti-c-myc antibodies (Santa Cruz Biotechnology) for 1 h, followed by Cy5 goat polyclonal anti-rat IgG (Abcam) and/or Alexa-Fluor 488 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) for 45 min, with extensive washing with PBS before and after the addition of the secondary antibody. Coverslips were mounted onto glass slides with Immuno-Fluore Mounting Media (ICN Biomedicals). Images were acquired with a Zeiss Axiovert 100-M microscope coupled to a Zeiss LSM-510 laser-scanning confocal system.  

**Electrophysiology**—M2 cells (a melanoma cell line) were used for all electrophysiological studies, because they are amenable to patch clamping. The plasmids (0.5 μg) indicated in Fig. 1 (top) and CD8 plasmid (0.1 μg) were co-transfected with KChIP2 (0.25 μg) into M2 cells. Immunomagnetic Dynabeads (Dynal) precoated with a monoclonal anti-CD8 antibody were used to select transfected cells for patch-clamp recording. To estimate selection efficiency, sister cells transfected with Kv4.2 WT were always recorded prior to mutant variants. Selection efficiency varied between 70 and 90%. The whole cell patch-clamp technique was performed on the stage of an inverted microscope (Zeiss IM35) with an Alembic VE-2 amplifier at room temperature as previously described (20). Micropipettes were filled with a solution containing 130 mM KCl, 1 mM MgCl2, 5 mM EGTA, 5 mM MgATP, and 10 mM Hepes (pH 7.2). The external solution contained 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.4). Voltage pulses (2 s) were imposed from a holding potential of −80 mV to voltages between −40 and +70 mV in increments of 10 mV. To assess voltage-dependent inactivation, test pulses to +50 mV were preceded by 1.5-s conditioning pulses to voltages between −140 and −10 mV. Current amplitude was measured from the peak current level to the steady-state end pulse level. Mean cell capacitance averaged 7.4 ± 0.3 pF ($n = 206$). Current densities are normalized to cell capacitance. Data were collected and analyzed with Clampex 8 and Clampfit 8 (Axon Instruments).
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FIGURE 2. C terminus truncations alter Kv4.2 channel gating and kinetics. A, voltage-dependent activation curves for WT and truncation mutants. Activation was assessed from recordings like those shown in Fig. 1A. Conductance (G) was estimated by dividing peak transient current by the estimated driving force. The activation curve was normalized by dividing G by $G_{\text{max}}$, the maximal value of G for each series. The curves are best Boltzmann fits to the data. B, correlation between half-activation potential and the number of positively (closed squares) and negatively charged (open triangles) residues removed from the C terminus for each truncation mutant. The shift in the voltage-dependent activation was closely fit by a linear relationship to the number of negative charges deleted ($y = -0.6313x - 9.6673; R^2 = 0.9717$) and to a lesser degree the number of positive charges deleted ($y = 0.7726x + 10.471; R^2 = 0.8567$). C, voltage-dependent inactivation of Kv4.2 WT and truncation mutants. From a −80-mV holding potential, a 1.5-s conditioning step was imposed to potentials between −140 and −10 mV, in increments of 10 mV, followed by a 2-s step to the +50 mV test potential (recordings shown in Fig. 4A). The curves are Boltzmann fits. D, time-dependent removal of inactivation (recovery) of Kv4.2 WT and truncation mutants, as determined with paired pulses from −80 to +30 mV ($P_1$ and $P_2$) delivered at a basic length of 10 s with variable $P_1-P_2$ intervals. Ratios of the current during $P_1$ ($I_1$) to the current during $P_2$ ($I_2$) were plotted as a function of $P_1-P_2$ intervals. The curves are best fit monoexponential functions.

Statistical Analysis—Data are presented as means ± S.E. Unpaired Student’s t test was used to compare means. A two-tailed $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Kv4.2 C Terminus Truncation Modifies Kv4.2 Current Density and Voltage-dependent Activation—Kv4.2 mutants were constructed by truncating from the distal end of the C terminus to residues 588 (T588), 528 (T528), 474 (T474), 417 (T417), and 406 (ΔC), respectively, as shown schematically in Fig. 1 (top). Fig. 1A shows representative families of outward currents in M2 cells expressing Kv4.2 WT and the various truncation mutants. With increasing C terminus truncation, the transient outward current amplitude decreased progressively, as summarized in Fig. 1B. Expression of truncation channels extending to T417 or beyond (ΔC) failed to produce any Kv4.2 transient outward current but instead showed currents similar to the endogenous (Endo.) currents obtained in control transfections with CD8 alone. C terminus truncations produced progressive depolarizations in transient current activation voltage dependence (Fig. 2A). The half-maximal activation voltage ($V_{1/2}$), estimated from the Boltzmann equation best fit to these activation curves, shifted by $\sim 30$ mV from $-17 \pm 4$ mV ($n = 7$) for Kv4.2 WT to $12 \pm 3$ mV ($n = 8, p < 0.001$) for T474, with no significant change in slope factor.

The voltage sensitivity of a channel is reflected by the equivalent charge movement associated with the activation curves (21). The numbers of equivalent charges ($z$) determined from the slope were not significantly different in Kv4.2 WT and the truncation mutants (data not shown). The change in the net charge of the C terminus truncation mutants is not correlated with the shift in voltage-dependent activation ($R^2 = 0.3213$). In contrast, there is a strong linear correlation between the depolarizing shift of voltage-dependent activation of the truncation mutants and the number of negatively charged residues deleted from the C terminus ($R^2 = 0.9717$), as shown in Fig. 2B; the correlation between positively charged residues and activation shift was slightly less robust ($R^2 = 0.8567$).

We evaluated the effect of neutralizing the six negative charge residues present in Kv4.2 WT that had been truncated in T588 (Glu$^{589}$/Glu$^{609}$/Asp$^{611}$/Asp$^{612}$/Glu$^{615}$/Glu$^{618}$). We found that neutralizing these six charges by site-directed mutagenesis to noncharged residues caused a positive shift in the activation $V_{1/2}$ from $-17 \pm 4$ mV ($n = 7$) for Kv4.2 WT to $-8 \pm 4$ mV ($n = 6, p = 0.02$ versus WT), not significantly different from the $V_{1/2}$ of $-4 \pm 4$ mV found with T588 ($n = 7, p < 0.01$ versus WT). This result suggests that these distal C-terminal negative charges influence voltage gating and probably account for the activation voltage shift in T588. We also created constructs with 10 and 18 negatively charged residues neutralized, corresponding to T528 and T474 truncations; however, these constructs failed to express measurable currents.

Fig. 2C shows normalized inactivation curves for the Kv4.2 truncation mutants. For less extensive truncations (T588 and T528), the $V_{1/2}$ and slope factor were not significantly different from Kv4.2 WT (see supplemental Fig. 1A). However, the largest truncation construct we studied that generated a measurable transient outward current, T474, had a $V_{1/2}$ of $-58 \pm 4$ mV ($n = 6$), significantly depolarized ($p < 0.05$) compared with the value ($-69 \pm 2$ mV, $n = 6$) for Kv4.2 WT. T474 was associated with a small but significant increase ($p < 0.05$) in the slope.
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factor of the Boltzmann function from $-11 \pm 1$ in Kv4.2 WT to $-14 \pm 1$ in T474 (see supplemental Fig. 1A). The inactivation kinetics of Kv4.2 WT and truncation mutant protein expression were well fit by the sum of two exponentials (see supplemental Fig. 1B). The fast inactivation time constants for T588 and T528, evaluated at $+50 \text{ mV}$, were unchanged from WT. On the other hand, both fast and slow time constants were significantly longer in T474 than Kv4.2 WT. The kinetics of the recovery from inactivation of Kv4.2 were unaffected by truncation of the Kv4.2 C terminus (Fig. 2D).

Kv4.2 C Terminus Truncation Does Not Alter Total Kv4.2 Protein Levels—The expression level of Kv4.2 protein was examined 24 h post-transfection using Western blot analysis. Kv4.2 WT protein appeared as a 65-kDa form (Fig. 3A). With increasing Kv4.2 C-terminal truncation, there was a progressive decrease in molecular mass and accordingly a downward shift of the mutant bands. The transfection efficiencies of Kv4.2 WT and mutants, as determined by Western blot analysis of co-transfected green fluorescent protein, were similar (data not shown). Loading was assessed by examining the expression of endogenous CASK by stripping each membrane and reblotting with anti-CASK antibody (Fig. 3A, bottom). We corrected for any differences in loading by normalizing target band intensities to those of CASK. Kv4.2 C terminus truncation did not significantly alter total channel protein expression relative to Kv4.2 WT, based on seven transfection and immunoblot experiments with each construct (Fig. 3B).

C Terminus Truncation Reduces the Modulation of Kv4.2 Function and Expression by KChIP2—We co-transfected KChIP2 with Kv4.2 WT and a series of C terminus truncation mutants into M2 cells at a 1:2 weight ratio (the molecular mass of Kv4.2 is approximately double that of KChIP2). A comparison of currents in the absence (gray recordings in Fig. 4A) and presence of KChIP2 indicate an approximately 3-fold increase in Kv4.2 WT current upon co-transfection with KChIP2 (black recordings). Similar current enhancement by KChIP2 was found for the smaller truncation mutants (T588 and T528; Fig. 4B), for which KChIP2 co-expression restored the current density to that of Kv4.2 WT. For the T474 mutant, current density increased ~3.3-fold but remained well below the level of Kv4.2 WT. No current enhancement resulted from KChIP2 co-expression for the larger truncation mutants, T417 and ΔC.

For comparison, we examined the effect of deleting the N-terminal fragment between residues 2 and 31 (Δ2–31), previously identified as an N terminus KChIP2 binding site (23). We found that Δ2–31 in the absence of KChIP2 had a current density (121 ± 20 pA/pF, $n = 7$) that was smaller than, but not significantly different ($p = 0.44$) from, Kv4.2 WT (172 ± 25 pA/pF, $n = 8$). When co-expressed with KChIP2, there was a significant increase in the density of Δ2–31 current (to 204 ± 41 pA/pF, $n = 5$) that was significantly less than the density of Kv4.2 WT when co-expressed with KChIP2 (461 ± 38 pA/pF, $n = 8$).

We then sought to determine the effects of KChIP2 on channel gating and kinetics of C terminus truncation mutants. Co-expression of Kv4.2 with KChIP2 shifted Kv4.2 WT activation voltage dependence in the negative direction (Fig. 4, C and D), positively shifted voltage-dependent inactivation (Fig. 4E and supplemental Fig. 1C), accelerated recovery from inactivation (Fig. 4F), and slowed inactivation (see supplemental Fig. 1D). Similar responses to KChIP2 co-expression were seen for the less severe truncation mutants T588 and T528. For the T474 mutant, the only significant effect noted was an acceleration in the recovery from inactivation, which nonetheless remained significantly slower than that of WT or the less extensive truncation mutants.

KChIP2 is known to increase the expression levels of Kv4 (9). Fig. 5 demonstrates that the increase in protein expression level induced by KChIP2 in Kv4.2 WT is severely compromised in the C terminus truncation mutants. As shown in Fig. 5A, KChIP2 significantly increased band intensities for Kv4.2 WT, T588, and T528. KChIP2 slightly shifted Kv4.2 WT and T588 band densities to increased $M$, values. The expression levels of Kv4.2 WT and the smaller truncation mutants T588 and T528 were approximately doubled by co-expression with KChIP2 (Fig. 5B), whereas the expression of larger truncation mutants was not significantly affected. Of note was the absence of any change in protein level for T474 (Fig. 5B), despite the fact that KChIP2 significantly enhanced functional expression (Fig. 4B). With truncation beyond T417, there was no rescue of transient outward current upon co-expression with KChIP2.
Evidence for a Direct Interaction between KChIP2 and the C Terminus of Kv4.2—The decrease in the modulation of Kv4.2 by KChIP2 resulting from C-terminal truncations suggests that KChIP2 could be physically associated with the C terminus of Kv4.2. The N terminus of Kv4.2 contains a region from amino acid residue 2 to 31 that is believed to be necessary for KChIP2 binding (9, 23). To test the association of KChIP2 with the different cytoplasmic domains of Kv4.2 in vivo, we carried out co-immunoprecipitation experiments with a deletion mutant of Kv4.2 lacking residues 2–31 (Kv4.2/H90042–31), with the T417 truncation mutant, with a mutant combining Kv4.2/H90042–31 and T417 (H90042–31/T417), and with a mutant consisting only of the Kv4.2 C terminus fragment (Kv4.2CT). A c-myc-tagged KChIP2 was transiently expressed alone or in combination with HA-tagged Kv4.2 WT, H90042–31, T417, H90042–31/T417, and Kv4.2CT in HEK293 cells. Cell lysates were incubated with anti-HA antibody, and the resultant immunoprecipitates were subjected to SDS-PAGE and Western blot. Fig. 6A shows a Western blot of the inputs corresponding to the respective immunoprecipitation experiments below. Probing the blot with anti-HA antibody revealed immunoreactive bands corresponding to Kv4.2 WT, Δ2–31, T417, Δ2–31/T417, and Kv4.2CT (Fig. 6B, top, arrows); no band was found from the lysates of cells expressing c-myc-tagged KChIP2 alone. Probing the same blot with anti-c-myc antibody demonstrated that KChIP2 was co-immunoprecipitated with all Kv4.2 constructs, including the C terminus fragment (Fig. 6B, bottom), with a
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A

WT + c-myc-KChIP2
Δ2-31
Δ2-31/T417
T417
Kv4.2CT

WT
Δ2-31
Δ2-31/T417
T417
Kv4.2CT

CASK

6% input
WB: anti-HA + anti-CASK

WT
Δ2-31
Δ2-31/T417
T417
Kv4.2CT

CASP

6% input
WB: anti-HA + anti-CASK

WT
Δ2-31
Δ2-31/T417
T417
Kv4.2CT

CASP

6% input
WB: anti-HA + anti-CASK

B

IgG
IP: anti-HA
WB: anti-HA

IP: anti-HA
WB: anti-c-myc

C

KChIP2 Optical Density

T417
Δ2-31/T417
Δ2-31
WT
Kv4.2CT
KChIP2

FIGURE 6. KChIP2 interacts with the Kv4.2 C terminus. A, a Western blot (WB) with a 6% input of HEK293 cell lysate (soluble fraction) expressing c-myc-KChIP2 alone (lane 6) or in combination with HA-tagged Kv4.2 constructs: WT (lane 4), Δ2-31 (lane 3), T417 (lane 1), Δ2-31/T417 (lane 2), and C terminus fragment (Kv4.2CT, lane 5) probed with anti-HA antibody. B, interaction between KChIP2 and the Kv4.2 C terminus demonstrated by in vivo co-immunoprecipitation (IP) with the same cell lysate as A. Western blot analyses were performed using both anti-HA (top) and anti c-myc antibodies (bottom). The arrows indicate the appropriate band for each construct. C, quantification of precipitated c-myc-KChIP2 based on optical densities for each construct. Data are pooled from five independent experiments for all constructs except for Kv4.2CT, which included three experiments.

particularly strong signal for Kv4.2 WT (lane 4). All mutations severely reduced the ability of Kv4.2 to immunoprecipitate KChIP2, including the N terminus deletion (Δ2–31, lane 3), the C terminus truncation (T417, lane 1), and the double mutant (Δ2–31/T417, lane 2). A barely visible KChIP2 band (lane 6) detected from the lysates of cells expressing c-myc-KChIP2 only could be due to nonspecific binding. A quantitative comparison (Fig. 6C) of mean band densities of co-immunoprecipitated samples for the various truncation mutants (based on multiple experiments of the type presented in Fig. 6B) shows that the binding levels of all mutants are significantly greater than the nonspecific binding level. Fig. 6C also shows that N-terminal (Δ2–31) and C-terminal (T417) truncation mutants had levels of binding that were not significantly different from each other but were both significantly greater than the double mutant Δ2–31/T417. This observation suggests that the ability of the 2–31 N-terminal domain to bind KChIP2 persists even in the truncation mutant T417 and that both N and C termini bind to KChIP2.

To determine the ability of different regions of the C terminus to bind KChIP2 directly, we performed in vitro protein-binding assays. GST fusion proteins constructed with four fragments of Kv4.2 C terminus (GST.406–474, 474–630, 417–474, and 417–630) as well as with the entire C terminus (GST.Kv4.2CT; 406–630) and N terminus (GST.Kv4.2NT; 1–183) were incubated with c-myc-KChIP2-expressing HEK293 whole cell lysate. Incubations of purified insert-free GST protein with c-myc-KChIP2-expressing cell lysates were used as negative controls. Purified complexes of GST fusion proteins and KChIP2 were resolved by SDS-PAGE. GST and GST fusion proteins were detected by Coomassie Blue staining (Fig. 7A, top), and KChIP2 signals were analyzed by Western blot with anti-c-myc antibody (Fig. 7A, bottom). KChIP2 not only bound to both the Kv4.2 N and C termini GST fusion constructs but also to the C terminus fragments, albeit to varying extents for different constructs (Fig. 7A, bottom, lanes 2, 3, 5, and 7). An index of the strength of binding was obtained by comparing the signal intensity of bound KChIP2 relative to the amount of GST fusion protein loaded per sample (Fig. 7B). Mean data indicate similar KChIP2 binding affinities for the N terminus (9.5 ± 0.5), the C terminus (9.7 ± 0.4), and a nearly full-length C terminus fragment (GST.417–630, 9.8 ± 2.4, n = 5, p = not significant). A smaller C terminus fragment (GST.474–630) showed a somewhat weaker but still substantial relative affinity for KChIP2 (6.4 ± 1.3, n = 5, p = 0.051 versus GST.417–630). The smallest C terminus fragments proximal to residue 474 (GST.406–474 and GST.417–474) showed much reduced binding affinities to KChIP2 compared with larger C terminus fragments but values significantly greater than GST alone.

We then conducted a homologous competitive protein-binding pull-down assay to assess the specificity and relative affinity of KChIP2 binding to the N and C termini. Fig. 7C shows that the amount of KChIP2 pulled down from the lysate of c-myc-KChIP2-expressing HEK293 cells by fixed concentrations of GST fusion N terminus (GST.Kv4.2NT) and C terminus (GST.Kv4.2CT) protein constructs was progressively reduced by increasing the concentration of purified N and C terminus protein, respectively. Fig. 7D provides an estimate of the relative binding affinities of N and C termini to KChIP2 obtained by plotting the level of KChIP2 pulled down (Fig. 7C, bottom), normalized to the level of GST fusion protein (Fig. 7C, top), as a function of the added concentration of competing N
or C terminus protein. The results suggest that KChIP2 binds to N and C termini with similar relative affinities.

Kv4.2 C Terminus Interaction with KChIP2 Stabilizes Kv4.2—The stability of Kv4.2 is enhanced when Kv4.2 is co-expressed with KChIP2 (9). Pulse-chase experiments were conducted in HEK293 cells metabolically labeled with [35S]methionine/cysteine and then chased at 0.5, 1, 2, 4, and 8 h. Samples were then incubated with anti-HA (Fig. 8A) or anti-c-myc antibody (Fig. 8B, top and bottom), fractionated by SDS-PAGE, and subjected to autoradiography. Fig. 8C shows the normalized percentage of signal remaining for each construct as a function of chase time. Monoeponential fits are shown as solid lines in Fig. 8C, with corresponding time constants in Fig. 8D. In the absence of KChIP2, Kv4.2 WT and the ΔC mutant had similar first order degradation time constants (τ values). Co-transfection with KChIP2 (Fig. 8B, top) shifted the Kv4.2 WT from a 65-kDa to a 70-kDa form after 2 h and showed only a modest decline in expression thereafter for up to 8 h. Co-expression with KChIP2 dramatically prolonged the stability of Kv4.2 WT, increasing the degradation τ from 1.3 ± 0.1 (n = 3) to 9.7 ± 0.6 h (p < 0.001, n = 5). However, KChIP2 had no effect on the degradation kinetics of ΔC. In the presence of KChIP2, the T528 truncation mutant displayed degradation kinetics similar to Kv4.2 WT, whereas the T474 mutant showed significantly more rapid degradation that was nonetheless ~2.4 times slower than that for ΔC (p < 0.01, n = 3).

Kv4.2 C Terminus Interaction with KChIP2 and Kv4.2 Cell Surface Expression—One explanation for the marked reduction in functional current in the C-terminal truncation mutants and N-terminal deletion mutant is a reduction in the cell surface expression of Kv4.2 protein. We evaluated this possibility by using confocal microscopy to determine the localization pattern of KChIP2 and various Kv4.2 HA-tagged constructs (Kv4.2 WT, T417, Δ2–31, and T417/Δ2–31) that had been co-transfected into COS7 cells. When HA–Kv4.2 WT is transfected alone, the staining is primarily intracellular with a small amount of cell surface staining (Fig. 9A). Expression of Δ2–31 alone displays a similar pattern to Kv4.2 WT with limited cell surface localization (Fig. 9A). In contrast, the C-terminal truncation mutant T417 expressed alone is largely restricted to the perinuclear area (Fig. 9A). c-myc–KChIP2 expressed alone shows a fairly diffuse intracellular distribution (Fig. 9A). Co-expression of HA–Kv4.2 WT and c-myc–KChIP2 together enhances the cell surface expression and enhances the co-localization of Kv4.2 WT and KChIP2 (Fig. 9B). In contrast, neither T417 nor the double mutant Δ2–31/T417 acquires the cell surface localization seen with Kv4.2 WT upon co-expression with KChIP2, retaining only limited intracellular perinuclear co-localization with KChIP2. A similar localization pattern was also found for ΔC (not shown). On the other hand, when Δ2–31 and KChIP2 are co-expressed, they show cell surface co-localization that is less extensive than the co-localization observed with KChIP2 and Kv4.2 WT (Fig. 9B).

**DISCUSSION**

In this study, we have shown that the Kv4.2 C terminus is a key determinant of a variety of important properties of the Kv4.2 channel, including voltage dependence, cell membrane expression, and KChIP2 interaction.

**Relation to Previous Findings Regarding Molecular Determinants of Kv4.2 Membrane Localization**—In the presence of KChIP2, Kv4.2 localizes to the cell surface, whereas in the absence of KChIP2 or with C-terminal truncations there is pronounced intracellular retention (Fig. 9, A and B). Shibata et al. (9) originally found similar effects resulting from the interaction between KChIP2 and the N terminus of Kv4.2. Our results indicate that these functions also require an intact C terminus. The intracellular retention phenotype of ΔC may be due to an altered conformation of
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Functional Consequences of C-terminal Mutations—A role for the C terminus in Kv4.2 gating is consistent with recent studies of potassium channel structure and function (28, 30–32). Hatano et al. (16) reported hyperpolarizing shifts in Kv4.2 activation (7–13 mV) for C-terminal point mutations at residues Arg426 and Arg429 but did not address voltage-dependence of Kv4.2 activation. In an earlier study on KAT1 channels, Marten and Hoshi (21) reported that increasing C terminus truncations decreased channel voltage sensitivity in a fashion strongly correlated with the number of positively and negatively charged residues deleted. They speculated that the distal C terminus resides in their C termini (26), and mutation or deletion (20) at C-terminal domains causes retention of ion channels within the ER. Neyroud et al. (27) previously showed that a fusion protein constructed by replacing the C terminus of surface antigen CD8 with Kv4.3 C terminus is highly expressed at the plasma membrane of infected ventricular myocytes.

We also demonstrated that that the C terminus could immunoprecipitate KChIP2, consistent with the findings of Callsen et al. (19), suggesting that the cytoplasmic C terminus harbors a KChIP2 binding site. This was confirmed with a GST-binding assay (Fig. 7, A and B), which demonstrates that KChIP2 can bind to the C terminus of Kv4.2 distal to residue 417. The co-immunoprecipitation data in Fig. 6C show that the combination of T417 and Δ2–31 co-immunoprecipitate significantly less KChIP2 than either T417 or Δ2–31 alone. This indicates that the N terminus retains the ability to co-immunoprecipitate some KChIP2 even in T417, arguing against the possibility that the effects of C-terminal truncation on KChIP2 binding are mediated by indirect effects on folding of the N terminus KChIP2 binding site. This is also reflected in the patch clamp data, which show that co-expression of Δ2–31 with KChIP2 caused a 1.7-fold increase in the Δ2–31 current density, consistent with a KChIP2 binding site on the Kv4.2 C terminus that mediates the increase in current density observed in Δ2–31. We found that the peak current density ofΔ2–31 in the absence of KChIP2 was not significantly different from Kv4.2 WT, consistent with a recent report (38). A recent study by Kim et al. (32) proposes that KChIP2 acts via four internal and four external columns that support the KChIP2 hanging gondola structure. They speculate that the C termini of Kv4.2 subunits may contribute to the formation of the distal portion of the four peripheral columns, whereas non-T1 N-terminal residues contribute to the proximal region. This may provide a region of interaction between the C terminus, N terminus, and KChIP2.

**FIGURE 8.** Kv4.2 C terminus interaction with KChIP2 stabilizes Kv4.2. HEK293 cells expressing HA-tagged Kv4.2 WT and ΔC mutant in the absence (A) and presence of c-myc-tagged KChIP2 co-transfection (B, top) as well as HA-tagged T474 and T528 in the presence of KChIP2 (B, bottom) were metabolically labeled for 1 h with [35S]methionine/cysteine and chased for the indicated time intervals. Lysates were subjected to anti-HA (A) or anti-c-myc (B) antibody immunoprecipitation (IP), followed by SDS-PAGE and autoradiography. C, average percentage of the Kv4.2 signal remaining as a function of chase time was fit with a monoeponential function (n = 3 for A; n = 5 and 3 for B, top and bottom, respectively). D, mean values for decay time constants for constructs with and without co-transfection with KChIP2. ***p < 0.001 for the time constants of Kv4.2 WT versus ΔC mutant, both in the presence of KChIP2 co-expression. **, p < 0.01 for the time constants of Kv4.2 ΔC474 versus ΔC mutant; *, p < 0.05 for the time constants of Kv4.2 T528 versus T474 in the presence of KChIP2 co-expression. NS, not significant; Ctrl, control.
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with the number of negatively charged residues removed. These results suggest that the C terminus may interact with the S4 voltage sensor, consistent with a close relation between the C-terminal polypeptide and inner helices, which must undergo large movements to gate the channel (33).

Based upon structural studies of Shaker, it appears that the C terminus lies along the side of the tetramerization domain (T1) (28). It has been reported that the T1 domain is coupled to voltage-dependent activation of Kv channels (34) and that the T1 domain may modulate channel activity by stabilizing the closed conformation (35). Truncation of the Kv4.2 C terminus may thus reduce the C terminus-T1 interaction, thereby influencing the conformational change at the T1 interface and stabilizing the channel in the closed state. It is also speculated that the C terminus may comprise a portion of the peripheral columns of Kv4.2 (32), which seem to be unique for KChIP2-bearing structures, and C terminus truncation may disrupt this structure, leading to a change in channel gating. This contrasts with the proposed structure of the mammalian Kv1.2-β2 complex, which lacks the peripheral columns and predicts a considerable distance between the C terminus and the β-subunit (33). By comparison with Kv1.2 (33), the Kv4.2-KChIP2 complex is squatter, and the KChIP2 domain appears to be in much closer proximity to the transmembrane domain and the C terminus than the β-subunits. Moreover, the C terminus of Kv4.2 is nearly triple the length of the Kv1.2 C terminus, which would facilitate the interaction between the C terminus and KChIP2.

We found that a hyperpolarizing shift in the voltage dependence of Kv4.2 activation was induced by KChIP2. A study of the three-dimensional structure of the C terminus of Shaker K⁺ channels demonstrated that upon Kvβ2 subunit binding to the T1 domain, the conformation of the C terminus changes, bringing a large part of the C terminus into close contact with Kvβ2 (28). Similarly, single particle reconstruction studies on Kv4.2 co-expressed with KChIP2 revealed that KChIP2 density is tightly integrated with the N and C termini of Kv4.2 (32). Both studies imply that Kvβ2 or KChIP may interact with the C termini of the Kv channels and perhaps thereby influence channel gating properties.

The shift we found in the activation voltage dependence contrasts with the rather minimal effect of C terminus truncation upon the voltage dependence of channel inactivation. Jerng and Covarrubias (36) showed that a positively charged domain (residues 420–550) in the C terminus of mouse Kv4.1 is involved in inactivation gating, since deletion of this region abolished the rapid phase of inactivation. Subsequently, Ohya et al. (37) demonstrated that deletion of 149 amino acids from the distal C-terminal domain of rat Kv4.3 significantly slowed channel recovery from inactivation but did not have any effect upon the kinetics of channel inactivation or the voltage dependence of channel activation or inactivation. Recently, Hatano et al. (16) showed that replacement of two arginines (Arg426 and Arg429) with alanes in rat Kv4.3 strongly retarded the recovery from inactivation but did not have any effect upon the kinetics of channel inactivation or the voltage dependence of channel activation or inactivation. Similarly, single particle reconstruction studies on Kv4.2 co-expressed with KChIP2 revealed that KChIP2 density is tightly integrated with the N and C termini of Kv4.2 (32). Both studies imply that Kvβ2 or KChIP may interact with the C termini of the Kv channels and perhaps thereby influence channel gating properties.

The shift we found in the activation voltage dependence contrasts with the rather minimal effect of C terminus truncation upon the voltage dependence of channel inactivation. Jerng and Covarrubias (36) showed that a positively charged
pressed with KChIP2, we found an accelerated recovery from inactivation for the smaller C terminus truncations. However, this effect was reduced for T474. These findings are similar to those of Callsen et al. (19), who also found an acceleration of the removal of inactivation induced by KChIP2, except that their effect was highly sensitive to truncation of the C terminus. The variability among the findings of these studies may be due to a number of factors, including 1) the different heterologous expression systems used, including Xenopus oocytes (36), mammalian cells (16, 37), and HEK293 cells (16), compared with M2 cells in the present study; 2) the different isoforms of Kv4.x channel family from different species that were studied (mouse Kv4.1 versus rat Kv4.3 and Kv4.2); and 3) variations in the extent of C terminus deletion and the localization of amino acid mutations.

**Novel Findings and Potential Importance**—This study demonstrates a role for the C terminus of Kv4.2 in determining Kv4.2 channel function and gating, highlighting the importance of the negative charge of the C terminus in channel voltage-dependent activation. Our results show for the first time a direct physical interaction between the Kv4.2 C terminus and KChIP2 that is required to confer the KChIP2 modulation of Kv4.2 cell surface expression, stability, and gating. It has been suggested that is required to confer the KChIP2 modulation of Kv4.2 cell surface expression, stability, and gating. It has been suggested that normal expression and gating of Kv4.2 C Terminus and KChIP2 Interactions Regulate Kv4.2

**Acknowledgments**—We thank Dr. Yuan Qing Zhao for technical support, Dr. Armin Akhavan for initial ideas, and Dr. Roxana Atanasiu for helpful suggestions.

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