

Calmodulin Regulates Assembly and Trafficking of SK4/IK1 Ca²⁺-activated K⁺ Channels*

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William J. Joiner^{‡§}, Rajesh Khanna^{§¶}, Lyanne C. Schlichter^{¶**}, and Leonard K. Kaczmarek[‡]

From the [‡]Departments of Pharmacology and Cellular and Molecular Physiology, Yale University School of Medicine New Haven, Connecticut 06520 and the [¶]Division of Cellular and Molecular Biology, Toronto Western Research Institute, University Health Network, and Department of Physiology, University of Toronto, Toronto, Ontario M5T 2S8, Canada

Calmodulin (CaM) regulates gating of several types of ion channels but has not been implicated in channel assembly or trafficking. For the SK4/IK1 K⁺ channel, CaM bound to the proximal C terminus (“Ct1” domain) acts as the Ca²⁺ sensor. We now show that CaM interacting with the C terminus of SK4 also controls channel assembly and surface expression. In transfected cells, removing free CaM by overexpressing the CaM-binding domain, Ct1, redistributed full-length SK4 protein from the plasma membrane to the cytoplasm and decreased whole-cell currents. Making more CaM protein available by overexpressing the CaM gene abrogated the dominant-negative effect of Ct1 and restored both surface expression of SK4 protein and whole-cell currents. The distal C-terminal domain (“Ct2”) also plays a role in assembly, but is not CaM-dependent. Co-immunoprecipitation experiments demonstrated that multimerization of SK4 subunits was enhanced by CaM and inhibited by removal of CaM, indicating that CaM regulates trafficking of SK4 by affecting the assembly of channels. Our results support a model in which CaM-dependent association of SK4 monomers at their Ct1 domains regulates channel assembly and surface expression. This appears to represent a novel mechanism for controlling ion channels, and consequently, the cellular functions that depend on them.

In neurons, small-conductance Ca²⁺-activated K⁺ (SK)¹ channels participate in the slow after-hyperpolarization that regulates action-potential frequency, spike frequency adaptation, and long term potentiation (1–4). In non-excitable tissues,

a related isoform, called IK (due to its intermediate conductance) contributes to volume regulation and the secondary immune response in lymphocytes (5–9), salt and water transport in colonic and airway epithelia (10, 11), activation of brain microglia (12), and pathophysiological conditions, such as Diamond-Blackfan anemia and sickle cell anemia in erythrocytes (13, 14).

The existence of a Ca²⁺-activated K⁺ (KCa) conductance was first demonstrated in erythrocytes by Gardos (15). The gene underlying this conductance was cloned by us and others. It encodes a KCa channel that has been variously called SK4, IK1, KCa4, and KCNN4 (14, 16–18). This channel, like other members of the SK family, is tethered tightly at its proximal C terminus (Ct1 domain) to calmodulin (CaM), which opens the channel in the presence of elevated intracellular Ca²⁺ (7, 19, 20). The demonstration that SK channels and CaM can be activated by divalent metal ions in the same order of preference (21) has been interpreted to mean that CaM is both necessary and sufficient to account for Ca²⁺-dependent gating of SK channels. Recently, an x-ray crystallographic study suggested a structural model for gating of SK channels that involved coordinated, Ca²⁺-dependent interactions between CaM and Ct1 domains (22).

Beyond the role of Ct1 in CaM binding and channel gating, very little is known about domains involved in SK function, such as channel assembly. SK channels lack the conserved N-terminal tetramerization domains of voltage-gated K⁺ channels (23–25) and the C-terminal multimerization domains of human ether-a-go-go-related gene (26) and big Ca²⁺-activated K⁺ channels (27). Here we provide the first evidence that multimerization of SK channel subunits is regulated by the same molecule responsible for channel gating: CaM. We find that CaM regulates the surface expression of SK4/IK1 protein by regulating the multimerization of channels through inter-subunit interactions between apposed Ct1 domains.

EXPERIMENTAL PROCEDURES

SK4 and Deletion Mutants Expressed in CHO Cells—For simplicity, we will call the channel SK4 in the remainder of this paper. Cloning and stable expression of human SK4 in CHO cells have been previously described (16). In the present paper, the full-length human channel was tagged at its C terminus with either a hemagglutinin (HA) or FLAG epitope and subcloned into pCDNA3 (Invitrogen, Carlsbad, CA). Construction of SK4.Flag and some epitope-tagged deletion constructs has been previously described (7). Briefly, the deletion mutants were called NM (encoding N terminus + six transmembrane domains), Ct1, Ct2 (distal C terminus), Ct1Ct2 (entire C terminus), NMct1 (N terminus + transmembrane domains + proximal C terminus) and NMct2 (N terminus + transmembrane domains + distal C terminus). They were constructed in the following manner: silent EcoRV and XhoI restriction sites were sequentially added just upstream of the leucine zipper domain of Ct2 in the SK4 cDNA using the Morph Mutagenesis kit (5'-3', Arapaho, CO). Then, after replacing the stop codon with an XhoI site by PCR, the resulting amplification product was ligated to a pair of com-

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§ Both authors contributed equally to this work.

¶ Supported by an Ontario Graduate Scholarship and a University of Toronto Margaret J. Santalo Scholarship. Current address: Dept. of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1751.

** To whom correspondence should be addressed: MC9-415, Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario M5T 2S8, Canada. Tel.: 416-603-5800 ext. 2052; Fax: 416-603-5745; E-mail: schlicht@uhnres.utoronto.ca.

¹ The abbreviations used are: SK channel, small-conductance KCa channel; KCa channel, Ca²⁺-activated K⁺ channel; Ct1, proximal C terminus; CaM, calmodulin; CHO, Chinese hamster ovary; HA, hemagglutinin; NM, N terminus + 6 transmembrane domains; Ct2, distal C terminus; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; Kv channel, voltage-gated K⁺ channel; GFP, green fluorescent protein.

plementary oligonucleotides encoding either the FLAG or HA epitope and flanked by *XhoI* and *NotI* sites. The region of these cDNAs encoding Ct1Ct2 was then amplified by PCR using primers containing a consensus translation initiation sequence. The full-length SK4 (NMct1Ct2) and the translatable Ct1Ct2 protoconstructs were then cut internally with either *EcoRV* or *XhoI* and re-ligated to excise specific SK4 regions; *i.e.* cutting with *EcoRV* generated *NMct2.Flag* and *Ct2.Flag*, whereas cutting with *XhoI* generated *NMct1.Flag* and *Ct1.Flag*. For electrophysiology experiments involving the co-expression of the wild-type channel construct with deletion mutants, the FLAG epitope was removed from the deletion mutants by PCR, and the products were subcloned into pTRACER (Invitrogen).

CHO cells were obtained from the American Type Culture Collection (Manassas, VA). For some experiments, they were stably transfected with pCDNA3 into which wild-type or HA-tagged SK4 cDNA had been subcloned. CHO cells were grown in Iscove's modified Dulbecco's medium with 10% fetal bovine serum, hypoxanthine + thymidine, and antibiotic/antimycotic. Stable cell lines were isolated and characterized (7, 16) and one clonal population expressing SK4 was chosen based on high channel expression (large whole-cell currents). Control cell lines expressing Kv3.1 (28) or a Kv1.3-GFP fusion protein² were generated by similar means. All tissue culture reagents were from Life Technologies, Inc.

Whole-cell Patch Clamp Recording of Wild-type SK4 and Co-expressed Deletion Mutants—Recordings were made at room temperature (22–25 °C) with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), and electrodes (3–5 megaohms) were fabricated from World Precision Instruments glass (Sarasota, FL). The bath solution consisted of (in mM): 140 NaCl, 1 CaCl₂, 5 KCl, 29 glucose, and 25 HEPES (pH 7.4). The pipette solution contained (in mM): 30 KCl, 100 K-gluconate, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2), supplemented with 4.27 mM CaCl₂ to obtain a free Ca²⁺ concentration of 1.0 μM (7) based on a stability constant of 16.2 for gluconate. All chemicals were the highest purity available from Sigma. Data were acquired at a sampling rate of 10 kHz and filtered at 2 kHz prior to analysis with pCLAMP6.0 software. On-line series-resistance compensation (80–90%) was used to reduce voltage errors produced by large currents.

Prior to recording from cells in the whole-cell configuration, CHO cells stably expressing human SK4 were transiently transfected with the pTRACER vector or with pTRACER harboring one of the deletion mutants: *NM*, *Ct1*, or *Ct1Ct2*. For transient transfection, 1 μg of cDNA in 100 μl of Optimem was mixed with 6 μl of LipofectAMINE in 100 μl of Optimem and incubated at room temperature for 30 min. Each transfection mixture was supplemented with 800 μl of Optimem and transferred to a 35-mm Petri dish containing CHO cells (~10% confluent) stably expressing SK4. After a 5-h incubation, the transfection medium was supplemented with 10% fetal bovine serum and incubated for another 12 h. Then, the medium was replaced with complete normal culture medium (as above). Cells were assayed for SK4 channel expression 3–15 h later (20–32 h after transfection) by measuring the current density (current/capacitance) of green fluorescent cells.

For CaM overexpression, cDNAs used for transfection were supplemented with 0.5 μg of either pCDNA3 alone or pCDNA3 containing the mouse CaM cDNA. The CaM cDNA was obtained by searching GenBank™ for expressed sequence tags (ESTs) containing full-length CaM open-reading frames and then purchasing EST 481929 from Research Genetics (Huntsville, AL). After sequencing to confirm its identity, it was subcloned between the *EcoRI* (5') and *NotI* (3') sites of pCDNA3. For some recordings 10 μM recombinant chicken CaM protein (Calbiochem, La Jolla, CA) was added to the pipette solution.

Immunofluorescence and Confocal Microscopy—Immunofluorescence was performed as previously described (12). CHO cells transiently transfected with various SK4 constructs and grown on glass coverslips were washed twice with PBS and fixed for 5 min in PBS containing 4% paraformaldehyde and 4% sucrose. The fixative was removed by washing (3×, 10 min each) in PBS. Then the cells were permeabilized with 0.25% Triton X-100 for 5 min followed by washing in PBS (2×, 5 min each). Nonspecific antibody binding was blocked by incubating the cells overnight in PBS containing 10% bovine serum albumin. After washing in PBS (3×, 10 min each) the cells were incubated (2 h, room temperature) with either mouse monoclonal anti-HA (Roche Molecular Biochemicals) or rat monoclonal anti-HA antibodies (Sigma) in 3% bovine serum albumin. After washing antibody-labeled cells in PBS (3×, 10 min each), they were incubated in secondary antibody (fluorescein isothiocyanate-conjugated anti-rat or anti-mouse, Vector Inc., Burl-

ingame, CA) for 30 min at room temperature. The cells were washed in PBS (3×, 10 min each), then rinsed briefly in distilled water, and mounted in SlowFade (Molecular Probes, Eugene, OR). Images were obtained with a 40× quartz objective on an inverted laser scanning confocal microscope (Bio-Rad MRC1000). All transfections and imaging were done in a "blind" fashion.

Co-immunoprecipitating FLAG-tagged and HA-tagged SK4 Subunits—Stably expressed HA-tagged SK4 was assayed for interaction with the FLAG-tagged wild-type or deletion mutants described earlier (7). Using standard protocols (above), HA-tagged SK4-expressing cells were transiently transfected with 1 μg of FLAG-tagged construct, plus either 1 μg of CaM/pCDNA3 or 1 μg of pCDNA3 vector alone. After 1–2 days, cells were lysed in cold ionic-detergent buffer (1% deoxycholate, 0.5% Nonidet P-40, 0.1% SDS), and FLAG-tagged protein complexes were precipitated with an anti-FLAG antibody (Sigma). Precipitates were electrophoresed under denaturing conditions, transferred to nylon, and Western blotted with an anti-HA antibody (Roche Molecular Biochemicals) followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Cedarlane, Hornby, ON) and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Biotinylation of Cell-surface Proteins—After some of the transient transfections described above, CHO cells were assayed for surface expression of full-length or deletion mutants of SK4 using a biotinylation assay (29). All procedures were conducted at 4 °C, except as indicated. At 24–36 h posttransfection, CHO cells were washed with ice-cold phosphate-buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-CM) and then incubated for 30 min at 4 °C with 500 μl of 0.5 mg/ml sulfo-succinimidyl 6-(biotinamido)-hexanoate (EZ-link-Sulfo-NHS-LC-biotin; Pierce Chemical Co.) Since the de-protonated form of the primary amine of lysine acts as a target for the sulfate group on biotin, this reagent selectively biotinylates lysine residues in proteins. It is cell-impermeant because of the negative charge on the sulfate group. The reaction was stopped by washing twice in PBS, then in 50 mM glycine to quench unbound biotin. The cells were then washed with PBS-CM and lysed in 1% SDS for 10 min at 65 °C. The lysate was diluted with four volumes of Triton X-100 lysis buffer and incubated for a further 60 min on ice. Biotinylated proteins were isolated by incubating solubilized CHO membranes (100 μg of protein) with Neutravidin-agarose (400 μl of a 50% slurry) (Pierce) pre-equilibrated in lysis buffer. After rocking the reaction mixtures gently for 12–16 h at 4 °C, biotin-neutravidin-agarose complexes were harvested by centrifugation and washed three times with a 20-fold excess of lysis buffer and once with 10 mM Tris-HCl (pH 8.0) and 300 mM NaCl. The beads were resuspended in 100 μl of SDS sample buffer and boiled for 5 min prior to SDS-polyacrylamide gel electrophoresis. This was followed by immunoblotting with an anti-HA antibody to detect total HA-tagged proteins at the cell surface or an anti-β-tubulin antibody (Oncogene Research) to confirm that intracellular proteins were not biotinylated.

Statistics—Where appropriate, data are expressed as mean ± S.E. for the number of cells or experiments indicated. To test for significant differences (*p* < 0.05) between two conditions, a Student's *t* test was used. For current amplitudes, statistical analyses were performed using analysis of variance, and differences were considered significant for *p* < 0.05 in a Student-Newman Keul posttest.

RESULTS

A CaM-dependent Dominant-Negative Effect of Ct1 Overexpression—We first tested whether overexpressing each of the domains, *NM*, *Ct1Ct2*, or *Ct1*, affects the formation of functional full-length channels. Each of these constructs or the control GFP-expressing vector (pTRACER) was transfected into CHO cells that stably expressed SK4, and whole-cell currents were recorded (Fig. 1A). The current at +60 mV was divided by cell capacitance to normalize for cell size and expressed as average current density (Fig. 1B). Either *Ct1Ct2* or *Ct1* greatly reduced the SK4 current density compared with vector-transfected cells; whereas, the *NM* region had no effect. This dominant-negative effect appeared to be specific to SK4, since *Ct1* co-transfection did not reduce the voltage-activated Kv3.1 current in stably transfected CHO cells (Fig. 1B).

Because the *Ct1* domain binds CaM (7), this raised the possibility that *Ct1* overexpression reduced the current by acting as a CaM "buffer", thereby reducing the amount of CaM available for channel gating. If so, gating should be restored by including excess CaM protein in the patch pipette during

² R. Khanna, unpublished data.

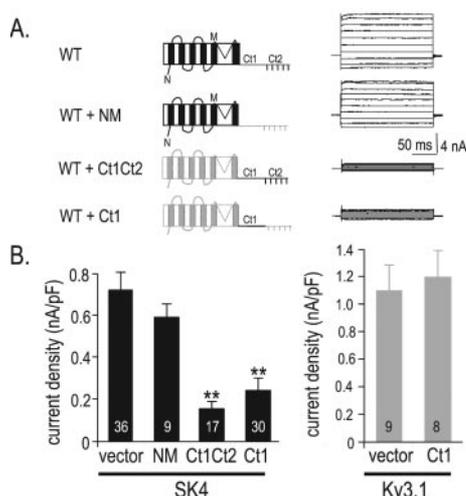


FIG. 1. The C terminus of SK4 acts as a dominant negative when co-expressed with a full-length channel construct. *A*, CHO cells that stably expressed full-length (WT, wild-type) human SK4 were transiently transfected with a recombinant deletion construct or empty pTRACER vector, and then whole-cell currents were recorded. *Left*, the transiently transfected constructs encoded the N terminus plus transmembrane domains (NM), the complete C terminus (Ct1Ct2) or the Ct1 domain (Ct1). *Right*, representative whole-cell currents recorded from green fluorescent cells during 200-ms long steps between -120 and $+60$ mV from a holding potential of -70 mV. *B*, summary of experiments as in part *A*. The amplitude at $+60$ mV was divided by the capacitance of each transfected cell to calculate the current density (mean \pm S.E.; number of cells is indicated on each bar). SK4 currents that were significantly smaller than the vector-transfected controls are indicated (**, $p < 0.001$, Student-Newman-Keuls posttest). *Right*, as a control for specificity, CHO cells stably expressing Kv3.1 were transiently transfected with vector alone or with the Ct1 construct of SK4. There was no effect on Kv3.1 current density calculated at $+40$ mV ($p > 0.05$, one-way analysis of variance).

whole-cell recording. This strategy was ineffective (Fig. 2); the $\sim 56\%$ reduction in current caused by Ct1 overexpression was not relieved by infusing CaM protein into transfected cells ($p < 0.01$). In contrast, transfecting cells with CaM cDNA prevented the inhibition by co-transfected Ct1; the current was not significantly different from controls ($p > 0.05$). These data suggest that Ct1 overexpression inhibits SK4 channels by limiting the amount of cellular CaM available at an early stage of channel assembly and/or trafficking, rather than by stripping channels at the plasma membrane of their Ca^{2+} -binding gating subunits.

To address the role of Ct1 in trafficking, CHO cells were co-transfected with SK4.HA, with or without Ct1 and CaM (as above) and assayed for localization by immunofluorescence microscopy. Expression of full-length SK4 was mainly at the cell surface, appearing as a ring around the cell periphery (Fig. 3A), which in *z*-plane scans (not shown) appeared as a halo around the cell. The SK4 channel protein was localized to the cell surface whether overall channel protein expression was moderate (Fig. 3A, left) or high (right). Simultaneous overexpression of either Ct1 (Fig. 3B) or Ct2 (Fig. 3D) resulted in cytoplasmic retention of full-length SK4 subunits; *z*-plane scans (not shown) confirmed staining throughout the cytoplasmic and perinuclear regions. Consistent with the ability of Ct1 to titrate CaM availability, co-expression of CaM with Ct1 restored the surface expression of full-length SK4 subunits and prevented their cytoplasmic retention (Fig. 3C). As expected from the lack of CaM binding to the Ct2 domain (7), overexpression of CaM did not reverse the effect of overexpression of Ct2; the SK4 protein staining remained diffuse and intracellular (Fig. 3E).

Because confocal microscopy cannot prove that channels are inserted into the cell membrane, a cell-surface biotinylation

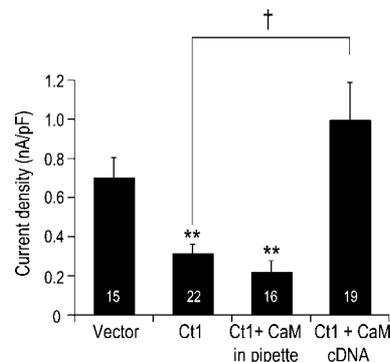


FIG. 2. CaM overexpression reverses the Ct1 dominant-negative action. Transient transfection of vector or Ct1 into CHO cells stably expressing SK4 currents. All transiently transfected cells were identified by co-transfection with green fluorescent protein. Whole-cell current densities (\pm S.E.) were measured as described in Fig. 1. Ct1 transfection reduced the SK4 current (**, $p < 0.01$; Student-Newman-Keuls posttest). The current was reduced to a similar extent when $10 \mu\text{M}$ CaM protein was present in the pipette solution during whole-cell recording (**, $p < 0.01$). In cells co-transfected with CaM cDNA and Ct1, the SK4 current was much larger than with Ct1 alone (†, $p < 0.01$).

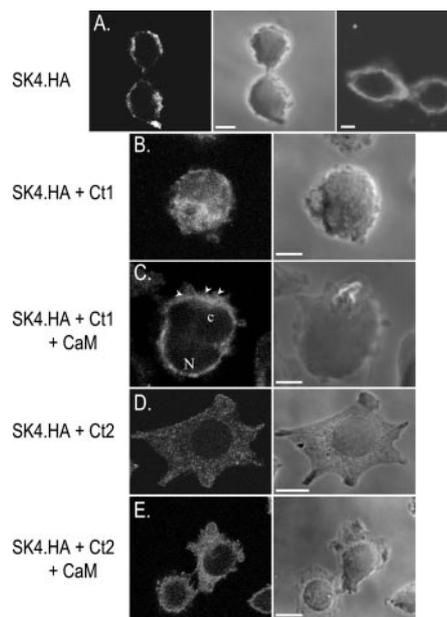


FIG. 3. Surface expression of SK4 protein is conferred by CaM binding to the proximal C terminus (Ct1) but not to the distal C terminus (Ct2). CHO cells were co-transfected with cDNA encoding full-length SK4.HA and untagged Ct1 or Ct2 in the presence or absence of exogenous CaM cDNA. Following paraformaldehyde fixation, the cells were permeabilized and labeled with an anti-HA primary antibody and finally a fluorescein isothiocyanate-conjugated anti-rat Ig secondary antibody. Representative confocal fluorescence (left panels) and corresponding phase images (right panels) are shown for the indicated transfections. *A* has an additional confocal image at the right. Arrows in *C* indicate some regions of high surface expression of SK4 (N, nucleus; c, cytoplasm). Scale bars: 5 μm (A-C); 10 μm (D and E).

assay was performed. CHO cells were first transfected with full-length SK4. After 24–36 h, non-permeabilized cells were treated with cell-impermeant sulfo-NHS-LC-biotin. Labeled proteins were harvested by overnight incubation with Neutravidin beads, and the precipitates were analyzed by Western analysis with an anti-HA antibody. A specific SK4-sized band (~ 48 kDa) was detected (Fig. 4A, lane 3), indicating robust cell-surface expression. Bands were absent from control cells (vector-transfected, lane 1) or when cells were not exposed to biotin (lane 2). Biotinylation itself did not affect SK4 expression, as judged by Western analysis of whole-cell lysates (lower

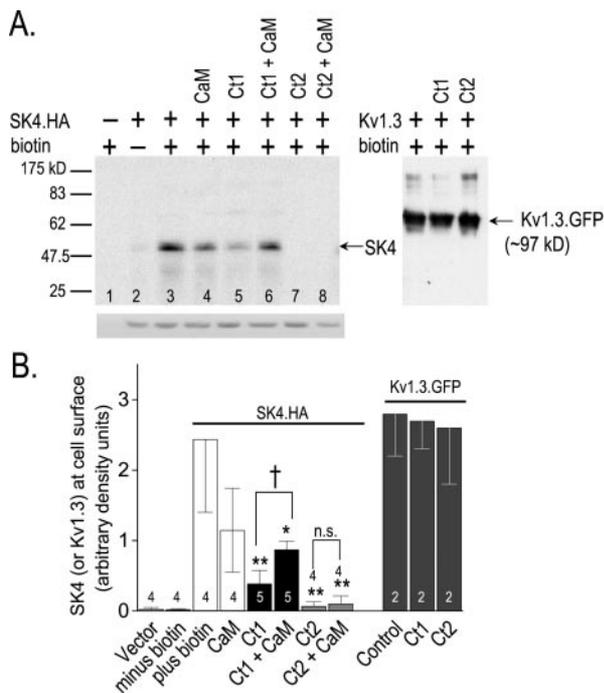


FIG. 4. Overexpression of *Ct1* or *Ct2* reduces SK4 surface expression; CaM partially reverses the effect of *Ct1*. Cell-surface SK4 protein expression was monitored using a biotinylation assay (see “Experimental Procedures” for details). CHO cells were co-transfected with full-length SK4.HA (or Kv1.3.GFP) and untagged *Ct1* or *Ct2* in the presence or absence of CaM overexpression. Following biotinylation, the cell-surface proteins were harvested from cell lysates and the precipitates were analyzed with an anti-HA antibody or an anti-GFP antibody. **A, left panel**, representative Western blot showing biotinylated SK4 protein (~48 kDa) for each of the transfection conditions used. Bands were absent from control cells (vector-transfected, lane 1) or when cells were not exposed to biotin (lane 2). The blot below shows SK4 expression in cell lysates from the same CHO cells (5 μ g of protein loaded per well). **Right panel**, as a specificity control, *Ct1* or *Ct2* was transiently transfected into CHO cells that stably expressed a Kv1.3-GFP fusion protein. A representative Western blot illustrates Kv1.3.GFP expression monitored with an anti-GFP antibody. **B**, summary of the quantity of biotinylated SK4 or Kv1.3.GFP protein (mean \pm S.E.), calculated by densitometry from at least three independent experiments, each performed in duplicate. Values that differ from channel + vector controls are indicated (*, $p < 0.05$; **, $p < 0.01$). A significant difference with or without CaM co-transfection is indicated (\dagger , $p < 0.05$).

bands). As an internal control, blots were stripped and probed with a β -tubulin antibody to determine whether intracellular proteins were biotinylated: no β -tubulin bands were observed (not shown).

Only *Ct1* binds CaM, but since we previously observed that the *Ct2* deletion mutant also failed to express current (7), we next asked whether both regions might be involved in SK4 trafficking. CHO cells were co-transfected with full-length SK4.HA and either *Ct1* or *Ct2*. Co-expression of *Ct1* decreased the amount of biotinylated SK4 (lanes 3 versus 5) at the cell surface by about 84% (Fig. 4A, $p < 0.01$, Student's *t* test). *Ct2* co-expression (lane 7) decreased biotinylated SK4 at the cell surface by ~97% ($p < 0.01$). These effects were specific for SK4 since surface biotinylation of another channel protein, Kv1.3, was unaffected by co-expression with *Ct1* or *Ct2* (Fig. 4A, right panel, data summarized in Fig. 4B). The biotinylation results are in excellent agreement with the confocal microscopy images above, since Western analysis of CHO cell lysates (blot beneath Fig. 4A, left) showed that none of the conditions significantly altered the overall expression of SK4 channel protein. Thus, *Ct1* and *Ct2* exert their dominant-negative effect by reducing cell-surface SK4 expression, not total channel protein. The dominant-negative effect of *Ct1* on SK4 cell-surface expression

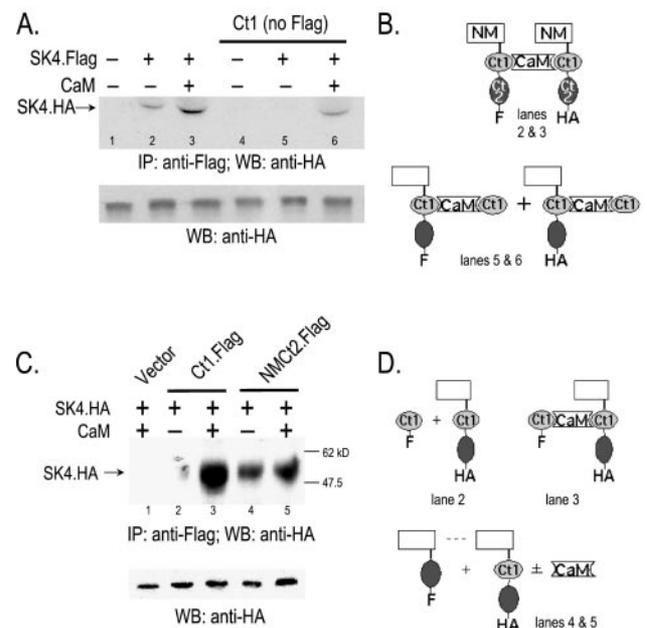


FIG. 5. CaM enhances SK4 subunit interactions. **A, upper**, cells stably expressing HA-tagged SK4 were co-transfected with combinations of FLAG-tagged SK4, with or without CaM or *Ct1*. Proteins were immunoprecipitated with an anti-FLAG antibody, followed by Western analysis with an anti-HA antibody (see “Experimental Procedures” for details). **Lower**, immunoblots labeled with anti-HA from equal aliquots of cell lysates from each transfection as a control for gel loading (40 μ g of protein loaded in each well). This example is representative of at least three experiments with similar results. **B**, proposed model of *Ct1*-CaM interactions corresponding to the experiments indicated by each lane in A. **C**, evidence that SK4 subunit interactions result from cross-linking of *Ct1* domains by CaM. CHO cells were co-transfected with full-length SK4.HA and *Ct1*.Flag, *NM**Ct2*.Flag or vector alone. **Upper**, immunoprecipitation with anti-FLAG antibody and Western analysis with an anti-HA antibody (as in A). **Lower**, control for gel loading: immunoblots labeled with anti-HA from equal aliquots of cell lysates for each transfection condition. **D**, proposed model for *Ct1* cross-linking by CaM.

was relieved by co-transfecting with CaM (Fig. 4A, lanes 5 versus 6); the level of biotinylated SK4 at the cell surface was ~2.3-fold higher than without CaM (Fig. 4B; $p < 0.05$). In contrast, SK4 surface expression was not restored by co-transfecting *Ct2* with CaM (lanes 7 versus 8): biotinylated SK4 protein remained at ~4% of the control value.

Domain-Domain Interactions in SK4—To assay more directly for interactions between channel subunits, CHO cells that stably expressed HA-tagged SK4 were first transiently transfected with a cDNA-encoding FLAG-tagged SK4. At 36–48 h posttransfection, cells were lysed and proteins were immunoprecipitated with an anti-FLAG antibody. The precipitates were separated by SDS-polyacrylamide gel electrophoresis and an anti-HA antibody was used for Western blotting. There was a clear band of the predicted size of an SK4 monomer with an HA epitope (~48 kDa) (Fig. 5A, lane 2), indicating a stable association between HA- and FLAG-tagged SK4 subunits. There was no band if the immunoprecipitating antibody was omitted (not shown) or if SK4.Flag was not included in the transfection mixture (lane 1). Notably, co-transfecting CaM cDNA significantly increased the amount of co-immunoprecipitated protein (lanes 3 versus 2), implying that CaM facilitates channel assembly.

Because CaM binds to the *Ct1* domain, assembly may involve linking of *Ct1* domains in adjacent SK4 subunits (7, 19). If so, multimerization should be inhibited by overexpressing *Ct1* to titrate the available CaM. When untagged *Ct1* was overexpressed, the anti-FLAG antibody no longer immunoprecipitated SK4.HA (lane 5). However, simultaneously over-

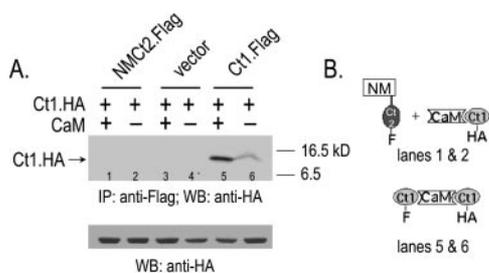


FIG. 6. CaM-dependent interactions between *Ct1* domains of SK4. *A*, CHO cells were co-transfected with *Ct1.HA* and vector, *NMct2.Flag*, or *Ct1.Flag*. Following immunoprecipitation with an anti-FLAG antibody, the precipitates were immunoblotted with an anti-HA antibody. *Top panel*, *Ct1* domains co-assemble in the presence of CaM (lanes 5 and 6). A construct lacking *Ct1* did not co-assemble with *Ct1* (lanes 1 and 2). *Bottom panel*, equal aliquots of cell lysate from each transfection (40 μ g of protein per well) resulted in expression of the same amount of HA-tagged *Ct1* protein. *B*, model proposing that *Ct1* alone is cross-linked by CaM and that without CaM, no interaction can occur between the channel monomers.

expressing CaM restored interaction between HA-tagged and FLAG-tagged SK4 (lane 6). Again, none of the transfection combinations significantly changed the overall SK4 protein expression levels (lower panel). Fig. 5B shows a general model of monomer interactions based on these results. We envision that the endogenous CaM in CHO cells allows some channels to assemble, but that more assembly takes place when more CaM is available (lanes 2 versus 3). *Ct1* overexpression appears to reduce channel assembly by buffering CaM (lane 5), a process that can be overcome by overexpressing CaM (lane 6). Our results support a model wherein CaM enhances SK4 self-association.

To determine which channel domains interact, CHO cells that stably expressed full-length SK4.HA were transfected with vector, *Ct1.Flag* or *NMct2.Flag* (Fig. 5C). Without CaM co-transfection, a small amount of SK4.HA co-immunoprecipitated with *Ct1.Flag* protein (lane 2). However, CaM co-expression dramatically increased the amount of co-immunoprecipitated product (lane 3). Interestingly, *NMct2.Flag*, which lacks the *Ct1* domain, also co-immunoprecipitated with full-length SK4 (lane 4), but this interaction was not increased by CaM (lane 5). Similar overall amounts of SK4 protein were expressed under all conditions (lower panel). As indicated in the model in Fig. 5D, our results imply that more than one region is involved in channel assembly, but that *Ct1* confers the CaM-dependence on channel assembly.

We further examined interactions between *Ct1* and channel protein domains by transiently transfecting *Ct1.HA* with either *NMct2.Flag* or *Ct1.Flag* (Fig. 6) and performing co-immunoprecipitations as before. In some batches, CaM was also co-transfected. *Ct1* did not co-precipitate with the *Ct1*-lacking polypeptide, *NMct2*, whether or not CaM was over-expressed (lanes 1 and 2), implying weak or non-existent interactions between the CaM-binding domain (*Ct1*) and other domains. In contrast, *Ct1.Flag* co-precipitated with *Ct1.HA* (lane 6), an interaction that was greatly enhanced by CaM overexpression (lane 5). Western analysis of cell lysates showed no changes in total *Ct1* expression (lower panel). Together, our data support a model (Fig. 6B) whereby CaM facilitates assembly of SK4 channels through interactions between the *Ct1* domains of the monomers.

DISCUSSION

Role of the C Terminus and CaM in Gating of SK Channels—Models of voltage-gated K⁺ (Kv) channels that place the gate near the interface between S6 and the C terminus (30, 31) have been advanced by recent x-ray structural analysis of the inner

mouth of the pore and the adjacent C-terminal region of bacterial K⁺ channels (27, 32). The C-terminal domains of many K⁺ channels have a region analogous to the recently crystallized “regulator of K⁺ conductance” (RKC) domain (27). In at least one K⁺ channel the gating mechanism involves a dimer structure; the proximal C termini of adjacent subunits of BK channels form a hydrophobic dimer cleft (27).

Interactions with CaM modulate the gating behavior of several channels (for review, see Ref. 33), but SK channels are highly unusual in having CaM as an integral component of the gating mechanism (19, 20, 34, 35). For SK4, the CaM-binding site (19, 20) is in a proximal region of the C terminus called *Ct1* (7), immediately following S6. The gate of SK channels has been proposed to consist of four apposed *Ct1* regions that constrict the central pore when Ca²⁺ is not bound (20) then open the channel when Ca²⁺ binds to and causes a conformational change in the four bound CaM molecules. Recent x-ray structural data on SK2 showed the direct interaction of CaM with a CaM-binding domain: two α helices from each proximal C terminus of adjacent SK2 monomers appear to be linked by CaM to form a channel dimer when Ca²⁺ is bound to the N-lobe of CaM (22).

Role of the C Terminus in Assembly and Trafficking of SK4—Unlike gating, little is known about domains involved in the assembly or trafficking of SK channels. SK channels lack domains homologous with the N-terminal tetramerization domains of many voltage-gated K⁺ channels (23–25, 36). However, an N-terminal SK tetramerization domain (of undetermined sequence) was inferred from suppression of functional channel activity when N-terminal fragments of SK2 or SK3 were co-expressed with wild-type channel proteins (9). Channel C termini can also contain regions critical for cell-surface expression, such as the unique endoplasmic reticulum-to-Golgi forward trafficking signals of some voltage-gated (37) and inwardly rectifying (38) K⁺ channels. Significantly, C-terminal deletions can prevent formation of functional HERG channels (26).

For SK4 channels to be functional, four subunits must bind to each other. However, it is not known if this occurs before the channels are assembled and trafficked to the cell surface. We previously found that SK4 constructs lacking either the CaM-binding *Ct1* domain or the conserved leucine zipper domain (*Ct2*) failed to produce functional channels (7). We proposed that failed gating could account for the lack of active channels in the *Ct1*-lacking mutant but that the *Ct2* domain was necessary for channel formation. This led us to ask whether the C terminus harbors a domain critical for assembly and/or trafficking. We have now found that both *Ct1* and *Ct2* act as dominant negatives when co-expressed with full-length SK4 protein. For *Ct1*, the dominant-negative effect is not simply due to buffering of CaM gating subunits since the current was not restored by adding excess CaM protein to the recording pipette. Although *Ct1* and *Ct2* both reduced surface expression of SK4, only for *Ct1* was this effect relieved by co-expressing CaM, consistent with the lack of CaM binding to *Ct2*. Our co-immunoprecipitation results show that *Ct1* domains can interact with each other and with full-length SK4 monomers and that these interactions are greatly enhanced by CaM. Furthermore, channel constructs that lack *Ct1* still associate with full-length monomers, but not with *Ct1* monomers, and neither interaction is affected by overexpressing CaM. The simplest interpretation of these data is that without binding of CaM to the *Ct1* domain, SK4 channels do not assemble properly. Our interpretation of the *Ct1* dominant-negative effect is that *Ct1* buffers CaM, making it unavailable for mediating subunit interactions. CaM overexpression restores the CaM availability and allows the *Ct1* regions of

full-length SK4 proteins to assemble into functional channels that are then properly targeted to the cell surface.

The *Ct2* domain also appears to contain a motif essential for assembly and cell-surface expression of SK4. Although deleting 25 amino acids from the distal region of the C terminus of SK4 produced non-functional channels (39) no information was presented on whether this reflected a defect in channel assembly, trafficking, or gating. A leucine zipper domain is conserved across all mammalian SK channels, and is contained in the *Ct2* domain of SK4 (16). Since leucine zipper domains can mediate intersubunit interactions in other proteins (40), we previously hypothesized that this domain is involved in binding SK subunits together (7, 16). We have now found that *Ct2* is required for cell-surface channel protein expression and *Ct2* overexpression prevents the membrane localization of full-length SK4 subunits. Not surprisingly, since we previously observed no CaM binding to *Ct2* (7), we find that the dominant-negative effect is not reduced by CaM overexpression. Consistent with the view that *Ct2* is important for proper folding and trafficking, a preliminary report showed that leucine-to-alanine substitutions in the leucine zipper motif prevent surface expression of SK4, possibly due to retention in the endoplasmic reticulum (41).

Role of CaM in SK4 Channel Assembly and Trafficking—In principle, two very different processes could account for the dominant-negative effect of *Ct1* overexpression: i) a decrease in the CaM binding necessary for gating and ii) a decrease in CaM-*Ct1* interactions involved in channel assembly. Our results support the second process. In the first scenario, buffering CaM with excess *Ct1* is expected to reduce the number of CaM-occupied channels. Channels that lack CaM should never open because binding of four CaM molecules is required for channel gating (19, 20, 35). If so, adding purified CaM protein to the patch pipette to restore CaM availability should reverse the *Ct1*-mediated dominant-negative suppression of current; it did not. Instead, it appears that CaM must be present during channel assembly and trafficking. *Ct1* overexpression greatly reduced SK4 currents and localization on the plasma membrane, and this was overcome by simultaneously overexpressing CaM for 20–32 h. Not only did *Ct1* domains co-immunoprecipitate with each other, an interaction that was greatly increased by CaM, but *Ct1* did not co-immunoprecipitate with other regions of the channel whether or not CaM was present. Thus, titrating CaM availability with excess *Ct1* interfered with assembly of functional SK4 tetramers.

Combined with x-ray crystallographic studies of *Ct1*-CaM interactions (22), our data are consistent with a model in which assembly is mediated by each CaM molecule bridging two *Ct1* domains. This suggests a novel CaM-dependent mechanism for regulating ion channel activity and cell function. CaM-dependent assembly of SK channels could be an important feedback mechanism for regulating the number of functional channels on an intermediate time scale. The consequent hyperpolarization of cells in the presence of elevated intracellular Ca^{2+} might, in turn, regulate certain cellular functions. For example, hyperpolarization could protect against neurotoxic hyperexcitability or promote T-cell proliferation by sustaining the Ca^{2+} influx required to generate the secondary immune response.

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REFERENCES

- Goh, J. W., and Pennefather, P. S. (1987) *J. Physiol.* **394**, 315–330
- Pedarzani, P., Mosbacher, J., Rivard, A., Cingolani, L. A., Oliver, D., Stocker, M., Adelman, J. P., and Fakler, B. (2001) *J. Biol. Chem.* **276**, 9762–9769
- Sah, P. (1996) *Trends Neurosci.* **19**, 150–154
- Sah, P., and Davies, P. (2000) *Clin. Exp. Pharmacol. Physiol.* **27**, 657–663
- Schlichter, L. C., and Sakellaropoulos, G. (1994) *Exp. Cell Res.* **215**, 211–222
- Vandorpe, D. H., Shmukler, B. E., Jiang, L., Lim, B., Maylie, J., Adelman, J. P., de Franceschi, L., Cappellini, M. D., Brugnara, C., and Alper, S. L. (1998) *J. Biol. Chem.* **273**, 21542–21553
- Khanna, R., Chang, M. C., Joiner, W. J., Kaczmarek, L. K., and Schlichter, L. C. (1999) *J. Biol. Chem.* **274**, 14838–14849
- Ghanshani, S., Wulff, H., Miller, M. J., Rohm, H., Neben, A., Gutman, G. A., Cahalan, M. D., and Chandy, K. G. (2000) *J. Biol. Chem.* **275**, 37137–37149
- Fanger, C. M., Rauer, H., Neben, A. L., Miller, M. J., Wulff, H., Rosa, J. C., Ganellin, C. R., Chandy, K. G., and Cahalan, M. D. (2001) *J. Biol. Chem.* **276**, 12249–12256
- Devor, D. C., Singh, A. K., Lambert, L. C., DeLuca, A., Frizzell, R. A., and Bridges, R. J. (1999) *J. Gen. Physiol.* **113**, 743–760
- McCann, J. D., Matsuda, J., Garcia, M., Kaczorowski, G., and Welsh, M. J. (1990) *Am. J. Physiol.* **258**, L334–L342
- Khanna, R., Roy, L., Zhu, X., and Schlichter, L. C. (2001) *Am. J. Physiol. Cell Physiol.* **280**, C796–C806
- Brugnara, C., De Franceschi, L., Armsby, C. C., Saadane, N., Trudel, M., Beuzard, Y., Rittenhouse, A., Rifai, N., Platt, O., and Alper, S. L. (1995) *Ann. N. Y. Acad. Sci.* **763**, 262–271
- Ghanshani, S., Coleman, M., Gustavsson, P., Wu, A. C., Gargus, J. J., Gutman, G. A., Dahl, N., Mohrenweiser, H., and Chandy, K. G. (1998) *Genomics* **51**, 160–161
- Gardos, G. (1958) *Biochim. Biophys. Acta* **30**, 653–654
- Joiner, W. J., Wang, L. Y., Tang, M. D., and Kaczmarek, L. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11013–11018
- Ishii, T. M., Silvia, C., Hirschberg, B., Bond, C. T., Adelman, J. P., and Maylie, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11651–11656
- Logsdon, N. J., Kang, J., Togo, J. A., Christian, E. P., and Aiyar, J. (1997) *J. Biol. Chem.* **272**, 32723–32726
- Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsencko, S., Maylie, J., and Adelman, J. P. (1998) *Nature* **395**, 503–507
- Fanger, C. M., Ghanshani, S., Logsdon, N. J., Rauer, H., Kalman, K., Zhou, J., Beckingham, K., Chandy, K. G., Cahalan, M. D., and Aiyar, J. (1999) *J. Biol. Chem.* **274**, 5746–5754
- Cao, Y. J., and Houamed, K. M. (1999) *FEBS Lett.* **446**, 137–141
- Schumacher, M. A., Rivard, A. F., Bachinger, H. P., and Adelman, J. P. (2001) *Nature* **410**, 1120–1124
- Shen, N. V., and Pfaffinger, P. J. (1995) *Neuron* **14**, 625–633
- Xu, J., Yu, W., Jan, Y. N., Jan, L. Y., and Li, M. (1995) *J. Biol. Chem.* **270**, 24761–24768
- Tu, L., Santarelli, V., Sheng, Z., Skach, W., Pain, D., and Deutsch, C. (1996) *J. Biol. Chem.* **271**, 18904–18911
- Kupersmidt, S., Snyder, D. J., Raes, A., and Roden, D. M. (1998) *J. Biol. Chem.* **273**, 27231–27235
- Jiang, Y., Pico, A., Cadene, M., Chait, B. T., and MacKinnon, R. (2001) *Neuron* **29**, 593–601
- Wang, L.-Y., Gan, L., Perney, T. M., Schwartz, I., and Kaczmarek, L. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 1882–1887
- Roquemore, E. P., and Banting, G. (1998) *Mol. Biol. Cell* **9**, 2125–2144
- Liu, Y., Holmgren, M., Jurman, M. E., and Yellen, G. (1997) *Neuron* **19**, 175–184
- Holmgren, M., Shin, K. S., and Yellen, G. (1998) *Neuron* **21**, 617–621
- Doyle, D. A., Morais-Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* **280**, 69–77
- Levitan, T. B. (1999) *Neuron* **22**, 645–648
- Hirschberg, B., Maylie, J., Adelman, J. P., and Marrion, N. V. (1998) *J. Gen. Physiol.* **111**, 565–581
- Keen, J. E., Khawaled, R., Farrens, D. L., Neelands, T., Rivard, A., Bond, C. T., Janowsky, A., Fakler, B., Adelman, J. P., and Maylie, J. (1999) *J. Neurosci.* **19**, 8830–8838
- Shen, N. V., Chen, X., Boyer, M. M., and Pfaffinger, P. J. (1993) *Neuron* **11**, 67–76
- Li, D., Takimoto, K., and Levitan, E. S. (2000) *J. Biol. Chem.* **275**, 11597–11602
- Ma, D., Zerangue, N., Lin, Y. F., Collins, A., Yu, M., Jan, Y. N., and Jan, L. Y. (2001) *Science* **291**, 316–319
- Gerlach, A. C., Syme, C. A., Giltinan, L., Adelman, J. P., and Devor, D. C. (2001) *J. Biol. Chem.* **276**, 10963–10970
- Iozzo, R. V. (1997) *Crit. Rev. Biochem. Mol. Biol.* **32**, 141–174
- Syme, C. A., Gerlach, A. C., Giltinan, L., Watkins, S. C., and Devor, D. C. (2001) *Biophys. J.* **80**, 138 (abstr.)
- Khanna, R., Chang, M. C., Roy, L., and Schlichter, L. C. (1999) *Physiologist* **42**, (Abstr. 21)
- Joiner, W. J., Khanna, R., Schlichter, L. C., and Kaczmarek, L. K. (2000) *Soc. Neurosci. Abstr.* **25**, 984