

Small Conductance Ca^{2+} -activated K^+ Channels and Calmodulin

CELL SURFACE EXPRESSION AND GATING*

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Small conductance Ca^{2+} -activated K^+ channels (SK channels) are heteromeric complexes of pore-forming α subunits and constitutively bound calmodulin (CaM). The binding of CaM is mediated in part by the electrostatic interaction between residues Arg-464 and Lys-467 of SK2 and Glu-84 and Glu-87 of CaM. Heterologous expression of the double charge reversal in SK2, SK2 R464E/K467E (SK2:64/67), did not yield detectable surface expression or channel activity in whole cell or inside-out patch recordings. Coexpression of SK2:64/67 with wild type CaM or CaM1,2,3,4, a mutant lacking the ability to bind Ca^{2+} , rescued surface expression. In patches from cells coexpressing SK2:64/67 and wild type CaM, currents were recorded immediately following excision into Ca^{2+} -containing solution but disappeared within minutes after excision or immediately upon exposure to Ca^{2+} -free solution and were not reactivated upon reapplication of Ca^{2+} -containing solution. Channel activity was restored by application of purified recombinant Ca^{2+} -CaM or exposure to Ca^{2+} -free CaM followed by application of Ca^{2+} -containing solution. Coexpression of the double charge reversal E84R/E87K in CaM (CaM:84/87) with SK2:64/67 reconstituted stable Ca^{2+} -dependent channel activity that was not lost with exposure to Ca^{2+} -free solution. Therefore, Ca^{2+} -independent interactions with CaM are required for surface expression of SK channels, whereas the constitutive association between the two channel subunits is not an essential requirement for gating.

Small conductance Ca^{2+} -activated K^+ channels (SK channels)¹ are fundamental components of cell excitability. SK channels are voltage-independent and activated by elevated intracellular Ca^{2+} levels that occur during an action potential. In many neurons, SK channels remain open after the action potential and contribute to a post-hyperpolarization, thereby

influencing interspike interval and burst duration (1–4). SK channels are also important in peripheral tissues, regulating hormone release from gland cells (5, 6) and smooth muscle tone (7–9).

Four genes comprise the SK channel gene family. SK1, 2, and 3, are expressed in the central nervous system and peripheral tissues, whereas expression of the structurally and functionally similar intermediate conductance channel, IK1, is limited to peripheral tissues (10–12). SK and IK channel α subunits share the serpentine architecture of voltage-gated K^+ channels, each bearing six transmembrane domains, with the N and C termini residing within the cell. The SK subunits are highly homologous but vary in their extreme N- and C-terminal domains. The only striking primary sequence homology between SK channel subunits and other K^+ channels is in the pore region located between the fifth and sixth transmembrane domains.

Functional SK channels are heteromeric complexes of four pore-forming α subunits and calmodulin (CaM) that mediates Ca^{2+} gating. In inside-out patches containing cloned SK channels, Ca^{2+} -dependent gating persists in the absence of applied CaM, suggesting that CaM is constitutively bound to the native complex (13). Structure-function studies are consistent with this model and have shown that the interaction occurs at the CaM binding domain (CaMBD), a highly conserved stretch of 92 amino acids residing in the proximal region of the intracellular C terminus of the α subunits (14, 15). The structure of the complex between the CaMBD and Ca^{2+} -CaM partitions CaM into distinct functional domains. Through interactions with the CaMBD, CaM adopts an extended conformation with the globular N- and C-lobes that harbor the E-F hand motifs separated by an elongated linker region. Ca^{2+} binding to the N-lobe E-F hands 1 and 2 of CaM is necessary and sufficient for Ca^{2+} gating (14, 16). Residues in the linker domain and the C-lobe maintain Ca^{2+} -independent interactions, including salt bridges between Arg-464 and Lys-467 on the CaMBD and Glu-84 and Glu-87 on CaM. Indeed, the spatial orientation of the residues in E-F hands 3 and 4, usually the higher affinity Ca^{2+} binding sites, is disrupted by extensive interactions with CaMBD residues and cannot adopt a chelating configuration. Therefore, it is likely that these interactions account for the constitutive association between the proteins (16).

The constitutive association between the channel subunits and CaM permits rapid gating in response to Ca^{2+} (13, 17). To determine whether the constitutive association between the CaMBD and CaM is required for SK channel gating, mutations that disrupt the constitutive interaction were introduced into SK2. Expression studies show that SK channels can undergo Ca^{2+} -CaM-dependent gating in the absence of a constitutive association with CaM. Surprisingly, Ca^{2+} -independent inter-

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¹ The abbreviations used are: SK channel, small conductance Ca^{2+} -activated potassium channel; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein; CaM, calmodulin; IK channel, intermediate conductance Ca^{2+} -activated potassium channel; CaMBD, calmodulin binding domain; SK2:64/67, SK2 R464E/K467E; CaM:84/87, CaM E84R/E87K; CaM1,2, CaM3,4, and CaM1,2,3,4, calmodulins with mutations in the first position of the indicated E-F hands, effectively abolishing Ca^{2+} binding.

actions with CaM are required for cell surface expression of SK channels.

MATERIALS AND METHODS

Molecular Biology—Proteins expressed in transfected cells were cloned in the cytomegalovirus-based vector, pJPA. Site-directed mutagenesis was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The tandem triple myc epitope (EQKLISEEDL) was inserted at the S3-S4 loop of rSK2 using complementary oligonucleotides cloned into a *Bam*HI site that had been introduced by site-directed mutagenesis at position 246 of rSK2. All sequences were verified by DNA sequence analysis.

Electrophysiology—COSm6 or HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (all from Invitrogen, Carlsbad, CA). Cells were transfected with pJPA expression plasmids encoding CD4, the indicated SK2 channel, and CaM (ratios of DNAs were 1:8:8, respectively) using calcium phosphate for HEK293 cells or lipofection (Qiagen, Valencia, CA) for COSm6 cells. Recordings were performed at room temperature 1–3 days after transfection. Transfected cells were identified by CD4 antibody-coated microspheres (Dynabeads, M-450 CD4, Dynal, Oslo, Norway). When filled, pipettes prepared from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL) had resistances of 1.8–3 M Ω . Voltage-clamp recordings were performed with an Axopatch-1B patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 5 kHz (–3 db). For whole-cell recordings pipettes were filled with (in mM) 140 KCl, 10 HEPES, 1 MgCl₂, 10 EGTA, with pH adjusted to 7.2 with KOH, after adding CaCl₂ to 100 μ M. The bath solution was 30 KCl, 110 NaCl, 10 HEPES, 1 MgCl₂, 1 CaCl₂ (with pH adjusted to 7.2 with NaOH). For excised patch recordings, the pipette solution was (in mM) 30 KOH, 120 NaOH, 10 HEPES, and 1 MgCl₂, with pH adjusted to 7.2 with methanesulfonic acid. Excised patches were superfused with an intracellular solution containing (in mM): 150 KOH, 10 HEPES, and 1 EGTA, supplemented with CaCl₂, with pH adjusted to 7.2 with methanesulfonic acid; the amount of CaCl₂ required to yield the indicated concentrations was calculated according to Fabiato and Fabiato (1979). Current amplitudes were measured at –80 mV unless otherwise indicated.

Rat CaM and the indicated mutants were cloned into pET23b, expressed in BL21 (DE3), and purified on a low substitution phenyl-Sepharose column (Amersham Biosciences, Piscataway, NJ). CaMs were added to the bath solution at 10 μ M immediately prior to use. The SK-MLCK M13 peptide (KRRWKKNFIAVSAANRFKISSSGAL) was synthesized by Genemed Synthesis (South San Francisco, CA).

Immunocytochemistry—COSm6 cells were grown to ~15% confluency in a 60-mm dish on microscope cover glasses and incubated for 5 h with the transfection mixture of 2.75 μ g of DNA (ratio of GFP:SK2:CaM, 1:5:5) in 1 ml of DMEM and 8 μ l of DMRIE-C reagent (Invitrogen) in another 1 ml of DMEM. The mixture was incubated at room temperature for 20 min prior to cell treatment. After transfection, cells were washed and fed with complete medium and incubated at 37 °C in a 5% CO₂. Immunocytochemistry was performed 1–2 days post-transfection.

Non-permeabilized immunostaining was performed by incubating the cells at 37 °C with 1:250 dilution of anti-myc monoclonal antibody (Invitrogen) in complete medium for 1 h. After three washes in complete medium and two washes in PBS⁺ (1 \times phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂), cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After quenching with two washes with 50 mM NH₄Cl in PBS⁺, cells were washed once with PBS⁺. Nonspecific binding was then blocked by incubating the cells with 10% bovine serum albumin (BSA) in PBS⁺ at room temperature for 30 min. The excess BSA was removed, and the secondary antibody (1:500 dilution of Texas Red-conjugated horse anti-mouse IgG (H+L), Vector, Burlingame, CA) was applied at 4 °C overnight. Cells were then washed three times with PBS⁺ and mounted (ProLong Antifade Kit, Molecular Probes, Eugene, OR) for imaging.

For permeabilized labeling, cells were washed with PBS⁺ and fixed with 4% paraformaldehyde at 4 °C for 30 min. After washing three times with ice-cold PBS⁺, cells were permeabilized with 0.2% Triton X-100 in PBS⁺ at room temperature for 15 min. To remove excess Triton X-100, cells were washed five times with PBS⁺ at room temperature. Nonspecific binding was then blocked by incubating the cells with 10% BSA in PBS⁺ at room temperature for 30 min, and primary antibody was then added and incubated at 4 °C overnight. The next day, the cells were washed and incubated with the secondary antibody at room temperature for 1 h. Cells were washed again, and mounting was performed as described for non-permeabilized cells. Images were

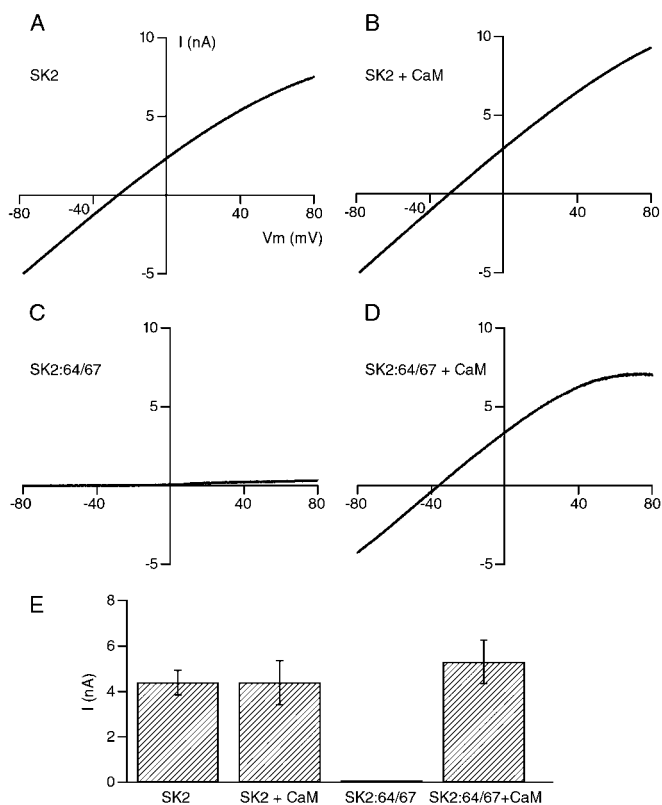


FIG. 1. SK2:64/67 requires coexpressed CaM for function. A–D, representative whole-cell voltage clamp recordings from HEK293 cells transfected with the indicated expression plasmids, 5 min after whole-cell patch formation. To activate SK channels, Ca²⁺ (100 μ M) was dialyzed into the cell through the patch pipette. The traces show responses to 2-s voltage ramp commands from –80 to 80 mV. E, whole-cell current amplitudes \pm S.E. measured at –80 mV ($n = 5$).

acquired with epifluorescence using an optical microscope (Axioplan2, Zeiss, Thornwood, NY) and the program OpenLab (Improvision, Lexington, MA).

RESULTS

SK2:64/67 Channels Require Cotransfected CaM for Function—The crystal structure of the CaMBD-Ca²⁺-CaM complex from SK2 revealed strong electrostatic contacts between the SK channel CaMBD residues Arg-464 and Lys-467 and CaM Glu-84 and Glu-87 in the CaM linker region close to the C-lobe implicated in constitutive CaM binding (16). Consistent with this observation, the CaMBD peptide harboring the double charge reversal R464E/K467E did not retain CaM in Ca²⁺-free pull-down assays, whereas the Ca²⁺-dependent interaction was still detected (14). To further investigate the role of the constitutive interaction between SK channels and CaM, whole-cell recordings were performed from cells transiently transfected with SK2 wild type or SK2:64/67 mutant channels, with or without cotransfected CaM (Fig. 1). Five minutes after whole-cell patch formation with Ca²⁺ (100 μ M) in the patch pipette, cells transfected with wild type SK2 and recorded in asymmetrical K⁺ showed large currents (-4.4 ± 0.5 nA, $n = 5$, Fig. 1A) that were reversed in response to voltage ramp commands close to the predicted K⁺ reversal potential (–40 mV). Cotransfection with CaM did not obviously affect current responses (-4.4 ± 1.0 nA, $n = 5$, Fig. 1B). Point mutation charge reversals at either of Arg-464 or Lys-467 resulted in functional channels that were not obviously different from wild type (not shown), whereas only small currents that were not different from mock transfected cells were recorded from cells transfected with SK2:64/67 (-30.0 ± 10.0 pA, $n = 5$, Fig. 1C). However, when SK2:64/67 was cotransfected with CaM, robust

currents were recorded within 1 min of whole-cell patch formation and increased to a steady state by 5 min (-5.3 ± 1.0 nA, $n = 5$, Fig. 1D), indicating that SK2:64/67 channels retain an ability to interact with Ca^{2+} -CaM.

Exogenous CaM Rescues SK2:64/67 Activity in Excised Patches—To more closely examine the interaction between CaM and SK2:64/67, inside-out patches were excised from transiently transfected COS cells into Ca^{2+} -containing solution ($10 \mu\text{M}$, $E_{\text{K}} = -40$ mV). In the absence of cotransfected CaM, currents measured at -80 mV (-31.3 ± 5.7 pA, $n = 13$) were not different from mock transfected cells (-23.3 ± 6.8 pA, $n = 4$, $p = 0.5$, unpaired t test). In contrast, patches from cells cotransfected with SK2:64/67 and CaM yielded channel activity immediately after excision (-638.3 ± 109.3 pA, $n = 19$), but channel activity diminished even while maintaining the patches in Ca^{2+} solution such that within 3 min the current measured at -80 mV decayed to $40.2 \pm 5.7\%$ of the initial current amplitudes (-294.9 ± 81.8 pA, $n = 19$). If patches were excised into Ca^{2+} solution (-887.5 ± 214.7 pA, $n = 10$) and then exposed to Ca^{2+} -free solution (0 Ca^{2+}) for 1 min, SK currents disappeared (-38.4 ± 12.3 pA, $n = 10$), and, different from wild type, a return to Ca^{2+} solution did not re-evolve SK currents (-42.0 ± 9.9 pA, $n = 10$).

One possible reason for the rapid decay of SK2:64/67 channel activity in patches and the inability to repeatedly activate the channels by exposure to Ca^{2+} is that the channels are weakly associated with CaM prior to and immediately after excision into Ca^{2+} solution, but channel activity is lost as CaM dissociates from the channels. To test this possibility, patches from cotransfected cells were excised into Ca^{2+} solution, and currents were evoked as described above. Ca^{2+} -dependent channel activity was abolished by exposure to Ca^{2+} -free solution. However, when exposed to Ca^{2+} solution additionally containing $10 \mu\text{M}$ CaM (Ca^{2+} -CaM solution), $56.7 \pm 12.8\%$ of the initial channel activity was reconstituted ($n = 10$, Fig. 2, A and B). Upon re-exposure to 0 Ca^{2+} solution and return to Ca^{2+} solution, currents could not be evoked, nor were currents evoked by exposure to CaM solution in the absence of Ca^{2+} .

The time course of Ca^{2+} -CaM binding and unbinding was examined by three sequential applications of Ca^{2+} -CaM solution separated by exposure to Ca^{2+} solution lacking CaM (Fig. 2C). The amplitude of the currents sampled at -80 mV increased upon addition of Ca^{2+} -CaM and decreased upon switching to Ca^{2+} solution without CaM. Fitting the data with single exponentials yielded time constants of 1.2 ± 0.2 , 1.7 ± 0.3 , and 1.8 ± 0.4 min for Ca^{2+} -CaM association and 0.7 ± 0.1 , 1.4 ± 0.4 , and 2.1 ± 0.8 min for Ca^{2+} -CaM dissociation ($n = 7$). The maximum current amplitudes decreased over the course of the experiment, consistent with channel rundown (18).

To determine whether CaM could associate with SK2:64/67 channels in the absence of Ca^{2+} , patches were excised into Ca^{2+} solution, verifying functional channels, and then exposed to 0 Ca^{2+} solution during which time CaM dissociated as confirmed by subsequent exposure to Ca^{2+} solution. Patches were then exposed to Ca^{2+} -free CaM ($10 \mu\text{M}$) for 5 min. Upon switching to Ca^{2+} solution lacking CaM, currents were rapidly evoked, indicating that CaM had assembled with the channels during the Ca^{2+} -free incubation (Fig. 3A). After CaM dissociation and decay of the currents, reapplication of Ca^{2+} -CaM for 5 min again rescued the currents (Fig. 3B). The percentage of the current evoked upon exposure to Ca^{2+} following Ca^{2+} -free CaM compared with the current subsequently evoked by Ca^{2+} CaM was $29.7 \pm 3.8\%$ ($n = 4$). This result shows that Ca^{2+} -free CaM can associate with SK2:64/67 channels and suggests that the CaM affinity is increased by the presence of Ca^{2+} .

CaM antagonists such as the M13 peptide (19) or calmidazolium (20) do not interfere with the Ca^{2+} -dependent gating process of wild type SK2 (13). It is likely that the constitutive interaction of CaM with SK2 does not present an exposed hydrophobic domain on CaM for antagonist binding (16). However, the interaction between SK2:64/67 and CaM is less stable in the absence of Ca^{2+} . Therefore, patches containing wild type SK2 or SK2:64/67 channels were exposed to Ca^{2+} -CaM solution (1.2 ± 0.3 nA for wild type, $n = 9$; -486.0 ± 187.2 pA for SK2:64/67, $n = 10$) and then exposed to the same solution

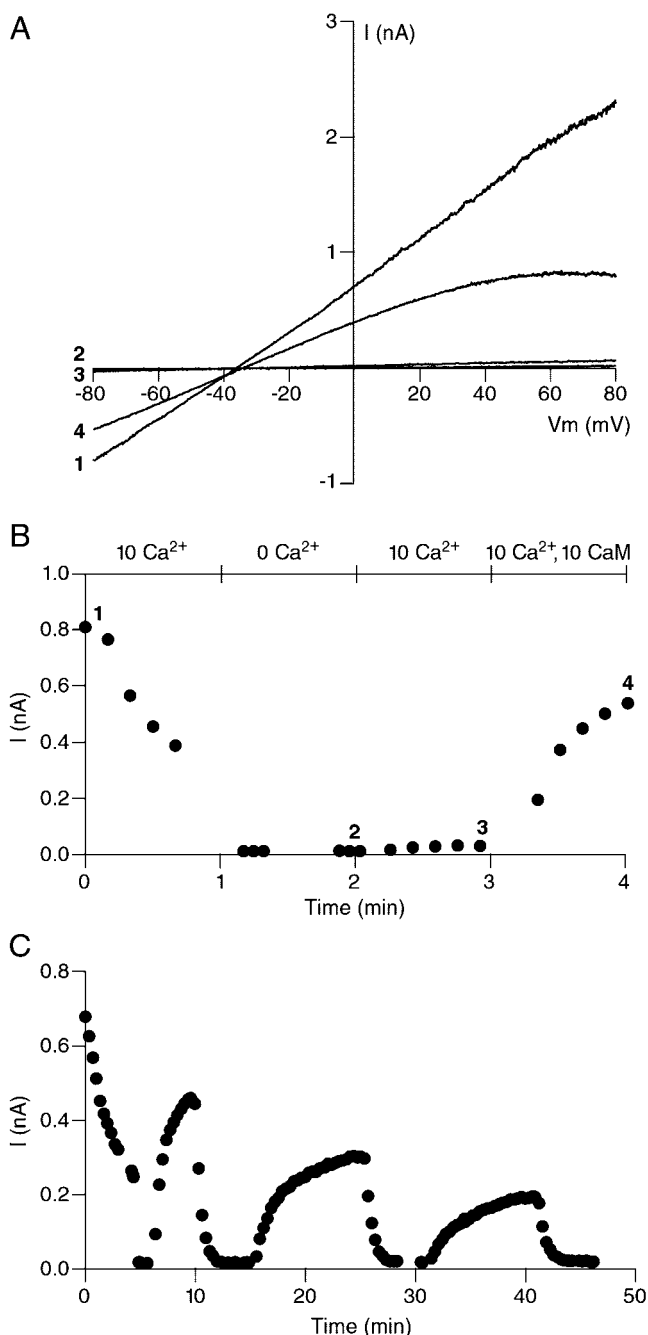


FIG. 2. Ca^{2+} -CaM rescues SK2:64/67 in excised patches. A, currents recorded from a representative inside-out patch excised from a COS cell cotransfected with SK2:64/67 and CaM. The patch was excised into Ca^{2+} solution ($10 \mu\text{M}$, trace 1), then exposed to 0 Ca^{2+} solution (trace 2), and returned to Ca^{2+} solution (trace 3), before Ca^{2+} -CaM ($10 \mu\text{M}$) was applied (trace 4), rescuing channel activity. B, diary plot of the current measured at -80 mV from the patch shown in A. The numbers correspond to the current responses shown on the left. C, diary plot of the currents measured at -80 mV from a separate patch exposed to three sequential applications of Ca^{2+} -CaM ($10 \mu\text{M}$) separated by exposure to Ca^{2+} solution ($10 \mu\text{M}$) lacking CaM.

zolium (20) do not interfere with the Ca^{2+} -dependent gating process of wild type SK2 (13). It is likely that the constitutive interaction of CaM with SK2 does not present an exposed hydrophobic domain on CaM for antagonist binding (16). However, the interaction between SK2:64/67 and CaM is less stable in the absence of Ca^{2+} . Therefore, patches containing wild type SK2 or SK2:64/67 channels were exposed to Ca^{2+} -CaM solution (1.2 ± 0.3 nA for wild type, $n = 9$; -486.0 ± 187.2 pA for SK2:64/67, $n = 10$) and then exposed to the same solution

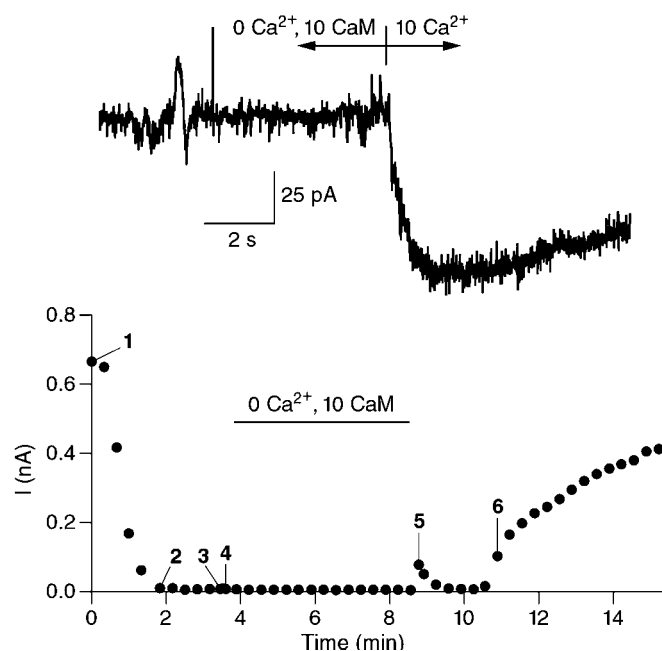


FIG. 3. Ca^{2+} -free CaM associates with SK2:64/67. *Top*, continuous recording at -80 mV from an inside-out patch containing SK2:64/67 channels. After a 5-min application of CaM in the absence of Ca^{2+} , currents were evoked upon exposure to a solution lacking CaM but containing $10 \mu\text{M}$ Ca^{2+} (corresponds to point 5, below). *Bottom*, diary plot of the patch shown above. The patch was excised into Ca^{2+} solution ($10 \mu\text{M}$, 1), then exposed to 0Ca^{2+} solution (2), and returned to Ca^{2+} solution (3), before Ca^{2+} -free CaM ($10 \mu\text{M}$) was applied (4). Exposure to Ca^{2+} without CaM (5) evoked a current that decayed within 1.5 min. Channel activity was rescued (6) by a second application of Ca^{2+} -CaM.

additionally containing the M13 peptide ($100 \mu\text{M}$). In contrast to wild type SK2 channels, the M13 peptide blocked the ability of Ca^{2+} -CaM to activate SK2:64/67 channels (-1.1 ± 0.3 nA for wild type, $n = 9$; -32.4 ± 7.9 pA for SK2:64/67; $n = 10$). Interestingly, the rate of current inhibition (0.28 ± 0.04 min, $n = 8$) was greater than removal of CaM from the bath solution (see Fig. 2C) suggesting that the excess M13 peptide enhanced the dissociation of SK2:64/67-bound CaM.

Compensatory Mutations in CaM Restore Association with SK2:64/67 Channels—To test whether the double charge reversal E84R/E87K in CaM (CaM:84/87) might compensate for the R464E/K467E double charge reversal in SK2, cells were cotransfected with SK2:64/67 and CaM:84/87. Inside-out patches exposed to Ca^{2+} , displayed SK currents (-1.7 ± 0.6 nA, $n = 8$) that disappeared upon subsequent exposure to 0Ca^{2+} solution (-43.3 ± 12.2 pA, $n = 8$). Distinctly different from cotransfection of SK2:64/67 with wild type CaM, returning the patch to Ca^{2+} solution reactivated the channels, rescuing $62.2 \pm 12.0\%$ of the initial current (Fig. 4).

Cotransfection of wild type SK2 with CaM:84/87 resulted in channels that activated upon patch excision into Ca^{2+} solution (-594.2 ± 225.4 pA, $n = 8$) and rapidly decreased without CaM in the bath solution. Unlike coexpression of SK2:64/67 with wild type CaM, currents did not completely disappear, plateauing at $53.2 \pm 6.4\%$ of the initial current. This current may reflect channels that have assembled with wild type CaM. Following exposure to 0Ca^{2+} solution, re-exposure to Ca^{2+} solution evoked $62.2 \pm 5.3\%$ of the initial current. Subsequent exposure to Ca^{2+} -CaM solution rescued the currents (-740.7 ± 317.6 , $n = 8$) (Fig. 4B). These results suggest that the E84R/E87K charge reversals in CaM destabilize the interaction between SK2 and CaM, and that in patches, CaM:84/87 is lost and may be replaced by wild type CaM.

Ca^{2+} Dependence—Coexpression studies with wild type SK2

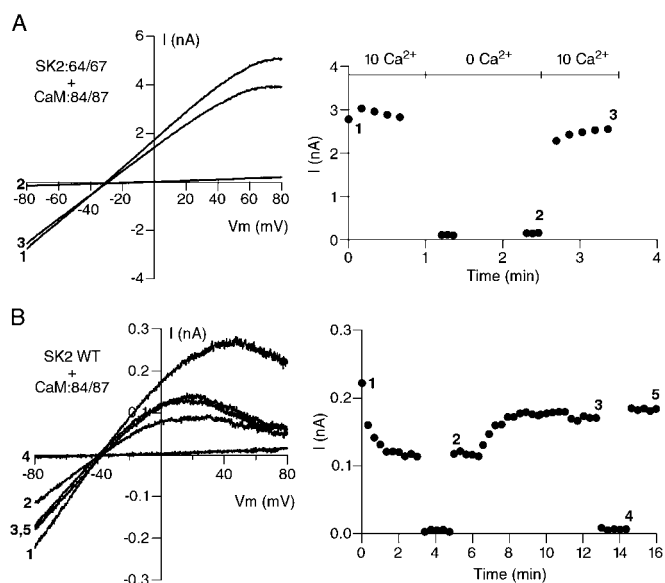


FIG. 4. CaM:84/87 compensates for SK2:64/67. *In A*: *Left*, currents recorded from a representative inside-out patch excised from a COS cell cotransfected with SK2:64/67 and CaM:84/87. The patch was excised into Ca^{2+} solution ($10 \mu\text{M}$, trace 1), then exposed to 0Ca^{2+} solution (trace 2), and returned to Ca^{2+} solution (trace 3). Ca^{2+} alone is sufficient to reactivate the channels; application of Ca^{2+} -CaM is not required. *Right*, diary plot of the current measured at -80 mV from the patch shown on the left. The numbers correspond to the current responses shown on the left. *In B*: *left*, currents recorded from a representative inside-out patch excised from a COS cell cotransfected with wild type SK2 and CaM:84/87. The patch was excised into Ca^{2+} solution ($10 \mu\text{M}$, trace 1), then exposed to 0Ca^{2+} solution, returned to Ca^{2+} solution (trace 2), and exposed to Ca^{2+} -CaM (trace 3) before returning to 0Ca^{2+} solution and exposure to Ca^{2+} (trace 5). *Right*, diary plot of the current measured at -80 mV from the patch shown on the left. The numbers correspond to the current responses shown on the left.

and CaMs harboring point mutations in the E-F hand domains showed that E-F hands 1 and 2 are necessary and sufficient for Ca^{2+} gating. Mutations in E-F hands 3 and 4 (CaM3,4) that abolish Ca^{2+} binding do not alter Ca^{2+} sensitivity, whereas mutations in either E-F hands 1 or 2 (CaM1 or CaM2) shift the sensitivity from ~ 0.5 to $\sim 1 \mu\text{M}$, and the double mutant, CaM1,2, eliminated Ca^{2+} gating. Functional studies were confirmed by the crystal structure of the CaMBD- Ca^{2+} -CaM complex that showed E-F hands 1 and 2 occupied by Ca^{2+} ions, whereas E-F hands 3 and 4 were uncalcified (14, 16). To examine the Ca^{2+} dependence of SK2:64/67 channels, and whether exogenous application of Ca^{2+} -CaM reconstituted SK channel gating similar to wild type, cells were cotransfected with either WT CaM, CaM1,2, CaM3,4, CaM1,2,3,4, or CaM:84/87. 1–3 days later, patches were excised into an internal solution containing the same purified recombinant CaM ($10 \mu\text{M}$), and Ca^{2+} dose responses were performed by changing between internal solutions containing CaM with varying concentrations of Ca^{2+} (Fig. 5). For either wild type or SK2:64/67, neither CaM1,2 nor CaM1,2,3,4 supported Ca^{2+} gating (not shown). The Ca^{2+} sensitivity of SK2:64/67 with wild type CaM was right-shifted ($K_d = 0.82 \pm 0.07 \mu\text{M}$, $n = 5$) compared with wild type channels ($K_d = 0.51 \pm 0.02 \mu\text{M}$, $n = 7$, $p = 0.001$, unpaired t test). Application of CaM:84/87 to wild type SK2 channels right-shifted the Ca^{2+} dose response ($K_d = 0.82 \pm 0.06 \mu\text{M}$, $n = 9$) to the same extent as SK2:64/67 was right-shifted in the presence of wild type CaM ($p = 0.97$, unpaired t test). Coexpression of SK2:64/67 with CaM:84/87 rescued stable currents patches (see above) but with reduced Ca^{2+} affinity ($K_d = 1.38 \pm 0.06 \mu\text{M}$, $n = 8$). Application of CaM3,4 to either wild type or SK2:64/67 slightly left-shifted the apparent affinity compared with wild type CaM (not shown).

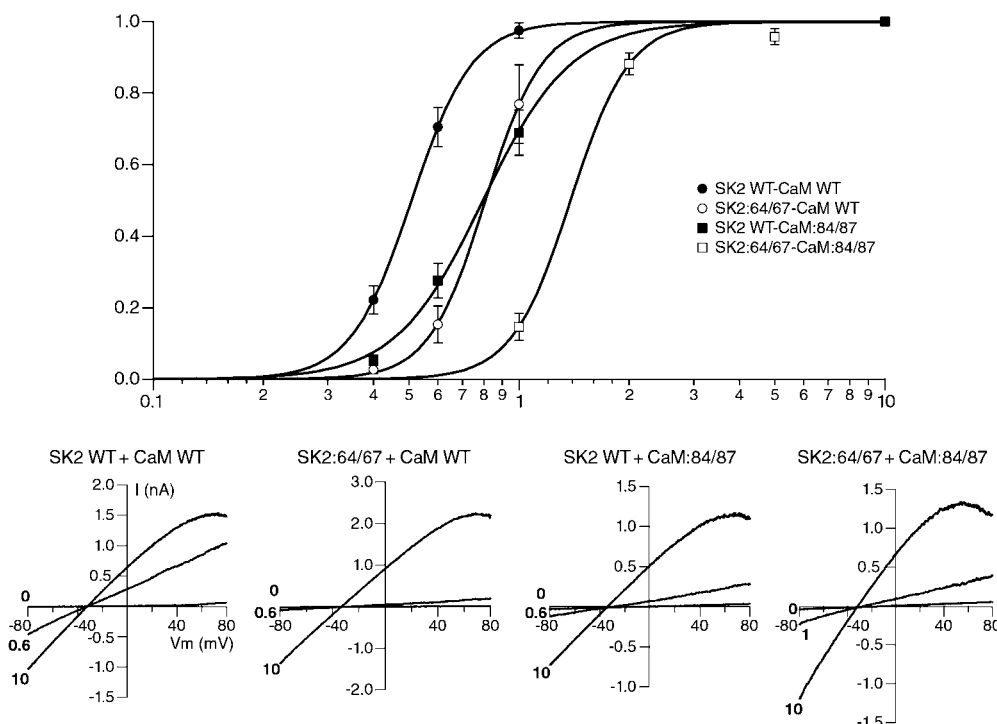


FIG. 5. **Ca²⁺ dose-responses.** *Top*, normalized Ca²⁺ dose-response relationships. The indicated SK2 channel and CaM were coexpressed, and the same purified recombinant CaM was applied with different Ca²⁺ solutions to the *inside face* of patches. Relative current amplitudes measured at -80 mV from $n \geq 5$ patches for each combination of channel and CaM were averaged and plotted *versus* the intracellular Ca²⁺ concentration. The averaged data were fitted with a Hill equation (*continuous lines*) yielding an EC₅₀ (μ M) and (Hill coefficient) of 0.51 ± 0.02 (5.6) for wild type SK2 with wild type CaM, 0.82 ± 0.07 (7.1) for SK2:64/67 with wild type CaM, 0.82 ± 0.06 (4.2) for wild type SK2 with CaM:84/87, and 1.38 ± 0.07 (6.4) for SK2:64/67 with CaM:84/87. *Bottom*, currents measured in response to voltage ramps in representative patches from cells coexpressing the indicated SK2 channel and CaM for 0, 0.6, 1, and 10 μ M Ca²⁺.

CaM Is Required for SK Channel Cell Surface Localization—Application of Ca²⁺ or Ca²⁺-CaM to patches from cells transfected with SK2:64/67 alone did not yield currents, whereas patches from cells cotransfected with CaM yielded robust channel activity. These results suggest that, in the absence of cotransfected CaM, SK2:64/67 channels may not be present on the cell surface. To test this possibility, three tandem copies of the myc epitope were inserted into the extracellular loop between transmembrane domains 3 and 4 in SK2 and SK2:64/67. Cells were transfected with the channel alone or in combination with CaM and then examined by immunocytochemistry with a monoclonal anti-myc antibody, either with or without permeabilizing the cells; cotransfected GFP was used to identify transfected cells. For SK2 with or without cotransfected CaM, channel protein was detected on the cell surface when cells were not permeabilized as well as in intracellular organelles after permeabilization (not shown). In contrast, SK2:64/67 protein was detected on the cell surface only when cotransfected with CaM, even though the protein was highly expressed as evidenced by the strong intracellular organelle staining in permeabilized cells (Fig. 6, *top, middle panels*).

To determine whether requires Ca²⁺-CaM, cells were cotransfected with wild type SK2 or SK2:64/67 and CaM1,2,3,4, a Ca²⁺-independent form of CaM (13). When cotransfected with CaM1,2,3,4, SK2:64/67 subunits were detected in the plasma membrane, demonstrating that Ca²⁺ binding to CaM is not essential for cell surface expression (Fig. 6, *lower panels*). Patches from these cells were excised into Ca²⁺ solution but did not yield SK currents (-31.4 ± 4.5 pA, $n = 9$). However, application of exogenous wild type Ca²⁺-CaM resulted in channel activation (-488.8 ± 85.1 pA, $n = 9$) suggesting that the channels were in the plasma membrane and initially associated with non-functional CaM1,2,3,4, which was replaced by the exogenous functional Ca²⁺-CaM. Consistent with this idea,

patches from cells cotransfected with wild type SK2 and CaM1,2,3,4 were detected in the plasma membrane, and excision into Ca²⁺ solution did not result in channel activation (-13.3 ± 4.4 pA, $n = 4$), nor could channel activity be restored by exogenous Ca²⁺-CaM (-11.1 ± 3.0 pA, $n = 4$). Therefore, wild type SK2 channels retain constitutively bound CaM1,2,3,4, which cannot bind Ca²⁺ and cannot be replaced by wild type CaM but are properly trafficked to the cell surface.

DISCUSSION

The results presented here show that Ca²⁺-independent interactions between the CaMBD and CaM are essential for cell surface expression and that the constitutive binding between the pore-forming α subunits of SK channels and CaM is not required for channel gating. The crystal structure of the complex between the CaMBD and Ca²⁺-CaM showed strong interactions between Arg-464 and Lys-467 on the channel and Glu-84 and Glu-87 on CaM, in the region implicated in constitutive association (16). This was supported by the lack of channel function when the double mutant was expressed. In addition, the purified CaMBD R464E/K467E peptide failed to retain purified CaM in pull-down assays in the absence of Ca²⁺ (14). These results show that the CaMBD R464E/K467E is different from wild type in its ability to retain CaM, but they do not distinguish between a complete lack of binding or a weakened affinity.

The crystal structure of the CaMBD-Ca²⁺-CaM and functional studies with mutant CaMs (14, 16) suggested a separation of function between the two lobes of CaM with the C-lobe mediating many of the Ca²⁺-independent interactions with CaMBD. Indeed, the shape of E-F hands 3 and 4 are altered through the multiple interactions with CaMBD residues and no longer coordinate Ca²⁺ ions. The N-lobe E-F hands 1 and 2 bound Ca²⁺ and were comparable to other Ca²⁺-CaM substrate

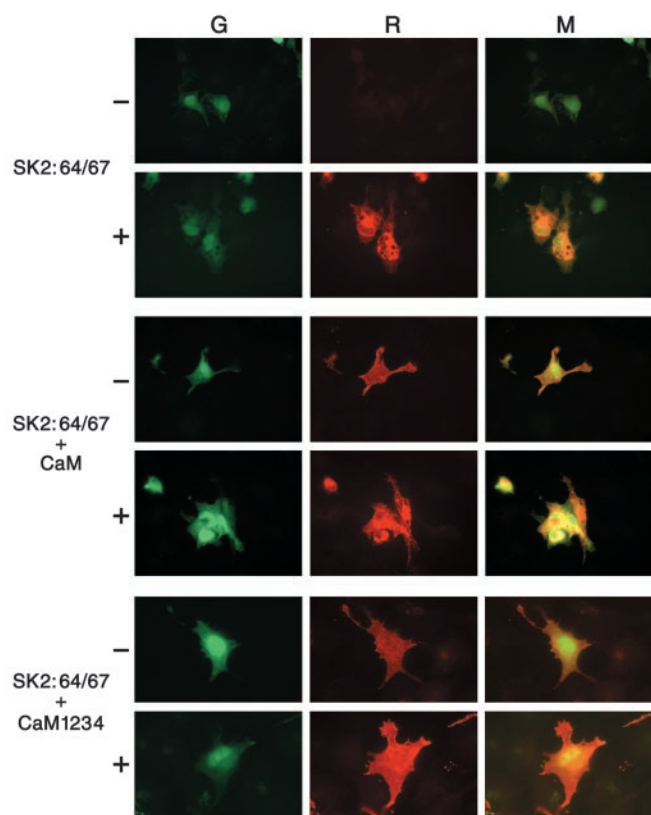


FIG. 6. **CaM is required for surface expression.** Immunocytochemistry of COS cells transfected with the indicated combinations of SK2:64/67, CaM, and a GFP expression plasmid. SK2:64/67 harbors three tandem copies of the myc epitope in the external loop between transmembrane domains 3 and 4. For each panel, transfected cells were visualized by expression of GFP (*G*; left), channel protein was detected with an anti-myc mouse monoclonal antibody and visualized by Texas Red-conjugated horse anti-mouse secondary antibody (*R*; middle), and the signals were merged (*M*; right). In each case, cells were examined either without (–) or with (+) the membrane permeabilization. *Top*, SK2:64/67 was not detected on the cell surface (–), but channel protein was detected inside permeabilized cells (+). SK2:64/67 was detected on the cell surface (–) as well as inside the cell (+) when transfected with wild type CaM (*middle*) or the Ca²⁺-independent CaM mutant, CaM1,2,3,4 (*bottom*).

structures (21–23). Therefore, if the double mutation R464E/K467E eliminated all Ca²⁺-independent interactions, Ca²⁺ gating in this channel might be mediated solely by interactions with either Ca²⁺-loaded N- or C-lobes. However, application of mutant CaMs with different combinations of intact E-F hands to SK2:64/67 channels showed that, just as for wild type channels, E-F hands 1 and 2 are required, whereas E-F hands 3 and 4 are dispensable. This implies that the double mutation R464E/K467E weakens the Ca²⁺-independent interactions between the channel subunit and CaM and that the reconstituted channels interact with exogenously applied CaM similar to that of wild type but with a reduced Ca²⁺ sensitivity. Therefore, it is likely that overexpressed CaM rescues whole cell SK2:64/67 channels and surface expression by overcoming the weakened affinity.

The compensatory mutations in CaM reconstituted tighter binding between the two double mutants, SK2:64/67 and CaM: 84/87, presumably by reinstating salt bridges between these positions. However, the reconstituted channels have reduced Ca²⁺ sensitivity, with apparent K_d values even more right-shifted than SK2:64/67 with WT CaM suggesting that, although the complex is stabilized, the conformational changes that open the channel gate subsequent to Ca²⁺ binding are compromised.

A role for CaM in SK channel trafficking was also found. Immunocytochemistry clearly demonstrated surface expression of SK2:64/67 only with cotransfected CaM, and functional studies showed that the channels carried associated CaM. Joiner *et al.* (24) had observed that overexpressing a part of the C-terminal domain of IK1 that included the CaMBD redistributed the channels to the intracellular compartments and overexpressing CaM redeposited them in the plasma membrane. The present results extend the implications for trafficking by showing that the Ca²⁺-independent interactions between the channel subunits and CaM are sufficient for cell surface expression. Patches from cells cotransfected with SK2:64/67 channels and CaM1,2,3,4 did not show channel activity when excised into Ca²⁺ solution, but channel activity was reconstituted upon subsequent application of Ca²⁺-CaM. Immunocytochemistry verified surface expression of SK2:64/67 channels when cotransfected with CaM1,2,3,4. This result is consistent with the ability of Ca²⁺-free CaM to associate with the channels in excised patches.

Because the CaM-dependent gating mechanism was described for SK channels, a variety of other channels have been shown to undergo Ca²⁺-free and Ca²⁺-dependent CaM interactions that alter their functions. For example, Ca²⁺-free CaM binds to an I-Q motif in the intracellular C-terminal domains of L-, N-, and P/Q-type voltage-gated Ca²⁺ channels (25–28), and similar to SK channels the different lobes of CaM mediate different functions (29). CaM binds to and modulates specific isoforms of voltage-gated Na⁺ channels (30, 31), cyclic nucleotide-gated channels (32, 33), and CaM is an auxiliary subunit of human gene transient receptor potential channels (34, 35). CaM also binds to and regulates the function of several ionotropic receptors, Ca²⁺ release channels, and TRP channels (see 36–38). CaM is not the only E-F hand Ca²⁺-binding protein that directly regulates ion channel function. Members of the Kv4 family of voltage-gated K⁺ channels interact with K-channel-interacting proteins that endow important biophysical properties as well as regulating trafficking to the plasma membrane (39). For SK channels, it remains to be determined just how the binding of CaM influences trafficking. However, the SK channels contain a conserved RKR motif in the intracellular N terminus, immediately preceding the first transmembrane domain. An analogous situation exists for functional K_{ATP} channels in which the channel forming Kir 6.2 subunits require association with SUR1 for surface expression. In this case, trafficking is regulated by the RKR endoplasmic reticulum retention signals present in each of the partner subunits that is exposed prior to co-assembly and buried once the two subunits form the macromolecular complex (40). However, mutagenesis of the RKR motif to AAR in SK2:64/67 did not result in surface expression in the absence of cotransfected CaM, although channel subunits were detected inside the cell (not shown).

CaM is highly expressed in almost all cell types, yet the concentrations and subcellular localization of free CaM may vary dramatically depending upon the state of phosphorylation, anchoring to the plasma membrane, or association with CaM storage proteins such as GAP-43 (41–43). Moreover, distinct CaM pools may be differentially mobilized, transiting long cellular distances, from dendrites to the nucleus (44). In addition, local CaM concentrations may also be translationally regulated as CaM mRNA is differentially distributed, a process that likely reflects the conservation of three non-allelic mammalian CaM genes encoding identical proteins but distinct 5' and 3' non-coding sequences. For example, one pool of CaM mRNA, derived from a specific CaM gene (*CALM1*) is abundant in the apical dendrites of cerebellar pyramidal cells and may give rise to local reservoirs of CaM; however, mRNAs derived

from *CALM1* and *CALM2* genes are found in neurite outgrowths in nerve growth factor-stimulated PC12 cells, and *CALM3*-derived transcripts reside within the cell body (45–47). Base upon these factors, and the number and concentrations of CaM-binding proteins, it is possible that CaM availability is rate-limiting for SK channel surface expression (43). In this case, metabolic processes that alter the concentrations of free CaM may dynamically regulate SK current density and cell excitability.

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