Calmodulin Mediates Calcium-dependent Activation of the Intermediate Conductance KCa Channel, IKCa1

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Small and intermediate conductance Ca2+-activated K+ channels play a crucial role in hyperpolarizing the membrane potential of excitable and nonexcitable cells. These channels are exquisitely sensitive to cytoplasmic Ca2+, yet their protein-coding regions do not contain consensus Ca2+-binding motifs. We investigated the involvement of an accessory protein in the Ca2+-dependent gating of hIKCa1, a human intermediate conductance channel expressed in peripheral tissues. Calmodulin was found to interact strongly with the cytoplasmic carboxyl (C)-tail of hIKCa1 in a yeast two-hybrid system. Deletion analyses defined a requirement for the first 62 amino acids of the C-tail, and the binding of calmodulin to this region did not require Ca2+. The C-tail of hSKCa3, a human neuronal small conductance channel, also bound calmodulin, whereas that of a voltage-gated K+ channel, mKCl.3, did not. Calmodulin co-precipitated with the channel in cell lines transfected with hIKCa1, but not with mKCl.3-transfected lines. A mutant calmodulin, defective in Ca2+ sensing but retaining binding to the channel, dramatically reduced current amplitudes when co-expressed with hIKCa1 in mammalian cells. Co-expression with varying amounts of wild-type and mutant calmodulin resulted in a dominant-negative suppression of current, consistent with four calmodulin molecules being associated with the channel. Taken together, our results suggest that Ca2+-calmodulin-induced conformational changes in all four subunits are necessary for the channel to open.

Ca2+-mediated signaling events are central to the physiological activity of diverse cell types. Opening in response to changes in intracellular Ca2+ ([Ca2+]i), Ca2+-activated K+ (KCa) channels play an important role in modulating the Ca2+

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The abbreviations used are: KCa, Ca2+-activated K+; BKCa, large conductance KCa; SKCa, small conductance KCa; IKCa, intermediate conductance KCa; C-tail, carboxy-terminal tail; CAM, calmodulin; ChTX, charybdotoxin; RBL, rat basophilic leukemia; TFP, trifluoperazine; WT, wild-type; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
proteins, and only about 40% identity with the SKCa1–3 gene products. Thus, hIKCa1 constitutes a distinct subfamily within the extended K⁺ channel supergene family.

The Ca²⁺ sensor for BKCa channels resides in a negatively charged Ca²⁺-bowl domain in the C-terminal of the α-subunit (28, 29). The β-subunit also contributes to the gating of these proteins (7). In marked contrast, the protein-coding regions of SKCa1–3 and hIKCa1 do not contain any EF-hand or Ca²⁺-bound motifs in their primary amino acid sequence, despite their exquisite Ca²⁺ sensitivity. This observation led us to speculate that the Ca²⁺ sensor for these channels either resides in a novel motif intrinsic to the channel or is provided by an accessory subunit that is tightly linked to channel activity. We investigated the latter possibility in a yeast two-hybrid system using hIKCa1 as our prototype. The Ca²⁺-binding protein calmodulin (CAM) was identified as a strong interacting partner of the C-tail of hIKCa1. Recently, CAM was shown to confer Ca²⁺ sensitivity to SKCa1 channel subfamily members (30). Here, we report that CAM binds to and is required for Ca²⁺-dependent activation of hIKCa1. Biochemical studies demonstrate that both hIKCa1 and hSKCa3 are prebound tightly to CAM in a Ca²⁺-independent fashion. Finally, we show by expression and patch-clamp recording that four CAMs are required to mediate the Ca²⁺-dependent channel activity of the hIKCa1 tetramer.

EXPERIMENTAL PROCEDURES

Clones, Mutants, and Vectors—We have previously reported the cloning of hIKCa1 (22, 23), hSKCa3 (15), and mKv1.3 (31). Drosophila wild-type (WT) and mutant (B1234Q) CAMs with differing Ca²⁺ sensitivities have been reported previously (32, 33). The B1234Q mutant has all four EF-hands mutated; glutamates 31, 67, 140 and 140 are replaced by glutamates (33). PAGA2 vector was a kind gift of Lutz Birnbaum (University of California, Los Angeles, CA). This vector is a pGEM5-based version of the pAGA vector, both of which contain the 5′-untranslated region of alfalfa virus RNA 4 and a 92-base pair poly(A) tail to increase stability of message and for efficient in vitro translation.

The segments of DNA encoding the C-terminal tails of hIKCa1 (nucleotides 1252–1678; GenBank™ accession AF227297), hSKCa3 (nucleotides 1632–2193; GenBank™ accession number AF031815) and mKv1.3 (nucleotides 1736–2112; GenBank™ accession number M30441) were subcloned into the PAGA2 vector using the polymerase chain reaction with engineered restriction sites. Both CAM clones were sequenced using vector-specific primers. The insert was confirmed by sequencing.

mKv1.3 cDNA was isolated from T-Lymphocytes that were grown nonconfluently on glass 1 day prior to use for cRNA injection and electrophysiological experiments. T-Lymphocytes were isolated from human peripheral blood and activated with phytohemagglutinin (DIFCO, Detroit, MI) as described previously (20). Prior to experimentation, cells were plated for 15 min on glass coverslips coated with poly-l-lysine (Sigma) and 10% heat-inactivated fetal calf serum (Summit Biotechnology, Fort Collins, CO) and grown in a humidified, 5% CO₂ incubator at 37 °C. Cells were plated to grow nonconfluently on glass 1 day prior to use for cRNA injection and electrophysiological experiments. T-Lymphocytes were isolated from human peripheral blood and activated with phytohemagglutinin (DIFCO, Detroit, MI) as described previously (20). Prior to experimentation, cells were plated for 15 min on glass coverslips coated with poly-l-lysine (Sigma). For other experiments, we stably transfected a yeast two-hybrid system using hIKCa1 as our prototype. The Ca²⁺-binding protein calmodulin (CAM) was identified as a strong interacting partner of the C-tail of hIKCa1. Recently, CAM was shown to confer Ca²⁺ sensitivity to SKCa1 channel subfamily members (30). Here, we report that CAM binds to and is required for Ca²⁺-dependent activation of hIKCa1. Biochemical studies demonstrate that both hIKCa1 and hSKCa3 are prebound tightly to CAM in a Ca²⁺-independent fashion. Finally, we show by expression and patch-clamp recording that four CAMs are required to mediate the Ca²⁺-dependent channel activity of the hIKCa1 tetramer.
an accuracy of 25%, based on intensity of bands in agarose gel electrophoresis. The cRNA was diluted with fluorescein isothiocyanate-dextran (Sigma) (average M, 10,000; 0.1% in 100 mM KCl). RBL cells were injected with an Eppendorf (Hamburg, Germany) microinjection system (Micro-manipulator 5171 and Transjector 5246) using injection capillaries (Femtotips®, Eppendorf) filled with the cRNA/fluorescein isothiocyanate solution, as described previously (35). Cells were visualized by fluorescence, and hIKCa1-specific currents were measured 4–8 h after injection. Cells measured in the whole cell configuration were normally bathed in normal Ringer solution containing 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose; adjusted to pH 7.4 with NaOH, with an osmolarity of 290–310 mOsM. In K+ Ringer solution, Na+ was replaced by K+. A simple syringe-driven perfusion system was used to exchange the bath solutions in the recording chamber. The internal pipette solution with 1 µM free Ca2+ contained 145 mM K+-aspartate, 8.5 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 10 mM K2 EGTA; adjusted to pH 7.2 with KOH, with an osmolarity of 290–310 mOsM. EGTA was omitted in the high Ca2+-internal solution containing 1 mM CaCl2. Pipettes were pulled from glass capillaries, coated with Sylgard (Dow-Corning, Midland, MI), and fire-polished to resistances measured in the bath of 2–5 MΩ. Membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA elektronik, Lambrecht, Germany) interfaced to a computer running acquisition and analysis software (Pulse and PulseFit; HEKA elektronik). Data were filtered at 1.5 kHz, and all voltages were corrected for a liquid junction potential of 80 mV. For characterization of the hIKCa1 current, voltage ramp stimuli were used to assess channel activation by elevated [Ca2+]i. RBL cells express an endogenous inwardly rectifying K+ channel that did not interfere with K+ currents seen at depolarized potentials. Experiments were performed at room temperature (21–25 °C). The CAM antagonists W7, trifluoperazine (TFP), and calmidazolium were purchased from Calbiochem (La Jolla, CA). ChTX was added to IK Ca and SK Ca channels, because their Ca2+ sensitivities and gating behavior are remarkably similar (10, 13, 20, 22, 24). An amino acid alignment of hIKCa1 with hSKCa1, rSKCa2, and hSKCa3 revealed that except for the pore and the transmembrane regions, the proximal half of the cytoplasmic C-tail was the most highly conserved (Fig. 1); the C-tail of hIKCa1 was therefore employed as the bait. We chose an activated human leukocyte cDNA library to screen for interaction partners because hIKCa1 has been previously shown to be highly up-regulated in activated lymphocytes (20, 22). A primary screen using the triple nutrient selection (Trp, Leu, His) resulted in the identification of several thousand positive clones. A subsequent subclass of 500 colonies yielded nine clones that were positive for β-galactosidase activity. Seven of these clones encoded CAM.

We next examined the ability of 35S-labeled in vitro translated hIKCa1-C-tail to bind CAM-Sepharose as assessed by running the product on an SDS-PAGE gel followed by autoradiography. As shown in Fig. 2A, a radiolabeled band of ~28 kDa is visible (hIKCa1) consistent with the size of the hIKCa1 C-tail (Fig. 2B), indicating that hIKCa1 and CAM interact. CAM also binds to the C-tail of the SKCa channel, hSKCa3 (Fig. 2A, ~22-kDa band (hSKCa3)), but not the C-tail of the voltage-gated K+ channel, mKv1.3 (Fig. 2A, mKv1.3). As an additional specificity control, we performed GST pull-down experiments; CAM did not bind GST alone but interacted with the GST-hIKCa1 C-tail (Fig. 3A). Thus, CAM interacts specifically with members of the IKCa and SKCa family.

Surprisingly, the C-tail fragments of hIKCa1 and hSKCa3 bound CAM efficiently in buffers containing 2 mM EDTA and no added Ca2+ (Fig. 2A, hIKCa1 and mKv1.3, right panel). Deletion analysis revealed that the shorter 1–98 and 1–62 fragments of the hIKCa1 C-tail also bound CAM efficiently in Ca2+-free conditions (Fig. 3A, bottom panel; Fig. 3B, right panel). Four additional deletion fragments (45–98, 37–77, 1–28, 1–9, and 1–4) were also detected when the fragment was incubated with GST-Sepharose beads alone (data not shown). B, an autoradiogram of 35S-labeled products, synthesized by coupled transcription translation (TnT) and loaded prior to incubation with CAM produced equivalent band intensities in all three lanes.
1–72, and 1–82) bound CAM, but only in the presence of 1 mM Ca$^{2+}$ (Fig. 3A, top panel; Fig. 3B, left panel), whereas two others (1–50 and 93–142) did not bind CAM at all (Fig. 3).

Thus, CAM interacts with the C-tails of hIKCa1 and hSKCa3 in the absence of Ca$^{2+}$, and this property resides in a domain within the first 62 residues of the hIKCa1 C-tail. The segment between residues 62 and 82 appears to mask the Ca$^{2+}$-independent interaction of CAM with hIKCa1, because the 1–72 and 1–82 fragments bind CAM only in the presence of Ca$^{2+}$, whereas residues 82–98 appear to reverse the negative effect of 62–82. Removal of as yet unidentified motifs between residues 1 and 37 appears to unmask a Ca$^{2+}$-dependent interaction with CAM. Interestingly, the 1–98 segment of the hIKCa1-C-tail, which contains the Ca$^{2+}$-independent and Ca$^{2+}$-dependent modulatory domains, shares a high degree of sequence similarity with the three members of the SKCa family (Fig. 1).

**CAM Co-precipitates with Full-length hIKCa1 in Transfected Cells** —The binding data described above suggest that CAM is pre-associated with the channel in cells with resting low [Ca$^{2+}$], If this were the case, it should be possible to co-precipitate CAM from cells expressing hIKCa1. To test this hypothesis, we expressed an N-terminal His-tagged fusion protein of hIKCa1 in COS-7 cells, prepared a crude membrane lysate in a Ca$^{2+}$-free solution, and passed the lysate through a nickel chelate column to allow the channel to bind to the column via a His-nickel interaction. The column was washed extensively, and the unbound fraction was collected in the flow-through. The His-tagged hIKCa1 channel (along with any prebound accessory proteins) was then eluted with 400 mM imidazole. We found that the His-tagged hIKCa1 channel, along with a rapidly activating IKCa current (Fig. 5), was co-precipitated with CAM from the column. To ensure that this was a specific CAM interaction, we tested whether CAM antagonists might disrupt IKCa currents activated by dialysis of human T cells with a pipette solution containing 1 mM Ca$^{2+}$. Whole cell recordings revealed two components of IKCa current, an immediately active voltage-gated K$^{+}$ current encoded by hKv1.3, along with a rapidly activating IKCa current (Fig. 5A, traces 1 and 2), as reported previously (20). The time course of the slope conductance ratio of this IKCa current is shown in Fig. 5B. Treatment with the CAM antagonist W7 (10 μM) had no effect on the IKCa current at physiological potentials. Although it blocked both currents at depolarized potentials (Fig. 5A), this suppression is voltage-dependent and is thought to be mediated by a direct effect on the channel, rather than via CAM modulation (36). Another CAM antagonist, TFP (10 μM), had no effect on IKCa currents when applied acutely; the slope conductance ratio, pre-TPF/post-TPF was 1.3 ± 0.2 in six cells. Intact cells were also pretreated with TFP, W7, or 2 μM calmidazolium for 15–30 min prior to recording, with no effect (not shown). Inclusion of 10 μM W7 inside the patch pipette in combination with such pretreatment also had no effect; the slope conductance ratio in seven drug-treated cells relative to untreated cells was 1.3 ± 0.3. Similar results were observed in COS-7 cells stably transfected with hIKCa1. We conclude that...
CAM antagonists do not interfere with the activation of hIKCa channels.

IKCa Channel Function Requires WT CAM—The association of hIKCa C-tail with CAM in very low Ca2+ (Figs. 2 and 4), as well as the inability of CAM antagonists to alter current through these channels (Fig. 5), supports the idea of a stable, nonconventional association between CAM and hIKCa1. Therefore, to study the interaction of these proteins, simultaneous new synthesis of each might be required. We injected combinations of cRNA encoding channel proteins plus cRNA encoding WT or mutant CAM into RBL cells, enabling us to investigate the effects and interactions of the resulting gene products using electrophysiological techniques. First, we characterized the physiological and pharmacological properties of hIKCa1 expressed after injection of the encoding cRNA alone. Robust currents exhibiting all the hallmarks of IKCa channels were seen 4–7 h postinjection (Fig. 6). The currents reversed near −80 mV in normal Ringer solution, and switching the bath solution to K+-Ringer (164.5 mM K+) shifted the reversal potential to −0 mV, as expected from the Nernst equation for a K+-selective channel (not shown). ChTX or clotrimazole reduced the current in a dose-dependent manner with the expected potency for IKCa channels in native tissue (20, 22, 24).

To determine whether CAM was responsible for the Ca2+-mediated gating of these channels, we exploited the availability of a Drosophila mutant (B1234Q) CAM. Drosophila and human CAM are identical at the amino acid level except at five positions. In B1234Q, glutamates (Glu31, Glu67, Glu107, and Glu140) of a calcium coordination positions in each of the four Ca2+ binding sites have been replaced by glutamine, resulting in a dramatically lower affinity for Ca2+ (33). We reasoned that co-expressing hIKCa1 along with B1234Q would result in a significant reduction of current amplitudes. In order for this hypothesis to be tested by co-expression, it was first important to show that B1234Q bound WT-CAM, or WT-CAM and a mutant CAM, B1234Q, normally. The apo form of B1234Q would therefore be expected to bind the hIKCa1 C-tail in a Ca2+-independent fashion. Consistent with this prediction, 35S-labeled Drosophila WT- and B1234Q-CAM bound to GST-hIKCa1 C-tail both in the presence (Fig. 7, lanes 4 and 5) and absence (lanes 9 and 10) of Ca2+. As did 35S-labeled hCAM (lanes 3 and 8). These CAMs did not bind GST alone in the presence or absence of Ca2+ (Fig. 7, lanes 1, 2, 6, and 7).

Co-injection into RBL cells of WT-CAM cRNA together with
cRNA encoding the hIKCa1 channel produced robust hIKCa1 currents in the whole cell mode with 1 μM free Ca\(^{2+}\) in the pipette. In marked contrast, co-injection of B1234Q cRNA with hIKCa1 cRNA resulted in an average 17-fold reduction in current amplitude. A, ramp currents in RBL cells injected with hIKCa1 in combination with WT-CAM (WT) or hIKCa1 in combination with B1234Q (MUT). B, comparison of the slope conductance of uninjected cells and those co-injected with hIKCa1 or KV1.3 and either WT-CAM (WT) or B1234Q (MUT). Each circle represents the measurement of slope conductance of currents in a single cell approximately 2 min after establishing the whole cell mode. The bold lines illustrate the mean slope conductance for all cells in each column. The difference between the mean slope conductance of cells microinjected with B1234Q RNA and those microinjected with WT-CAM RNA was statistically significant, as illustrated by the one-tailed Student's t test (p < 0.02). Cells co-injected with KV1.3 and MUT or WT-CAM showed no significant difference in current at +20 mV.

Fig. 8. Co-injection of hIKCa1 with mutant CAM inhibits IK\(_{Ca}\) currents. hIKCa1 cRNA was co-injected into RBL cells with either WT-CAM or B1234Q cRNA. Slope conductance was determined at potentials between −20 and −40 mV to avoid contamination with currents through the endogenous inward-rectifier channel present at potentials below −70 mV. A, ramp currents in RBL cells injected with hIKCa1 in combination with WT-CAM (WT) or hIKCa1 in combination with B1234Q (MUT). B, comparison of the slope conductance of uninjected cells and those co-injected with hIKCa1 or KV1.3 and either WT-CAM (WT) or B1234Q (MUT). Each circle represents the measurement of slope conductance of currents in a single cell approximately 2 min after establishing the whole cell mode. The bold lines illustrate the mean slope conductance for all cells in each column. The difference between the mean slope conductance of cells microinjected with B1234Q RNA and those microinjected with WT-CAM RNA was statistically significant, as illustrated by the one-tailed Student's t test (p < 0.02). Cells co-injected with KV1.3 and MUT or WT-CAM showed no significant difference in current at +20 mV.

As depicted by Fig. 9, curve 1. Similarly expanded equations can be written for cases in which two or three mutant CAM are allowed. The data are well fitted only by the equation in which a single mutant subunit is sufficient to disrupt hIKCa1 func-
RBL cells were measured in whole cell patch-clamp experiments 4–7 h after microinjection with hIKCa1 RNA + CAM RNA. The CAM RNA consisted of wild-type (WT) or B1234Q mutant (MUT) RNA injected separately or together in varying proportions, as indicated. All experiments were performed as a paired comparison between WT and MUT CAM RNA, or WT and the indicated ratio of WT:MUT CAM RNA, using a constant amount of hIKCa1 RNA for each pair. Nine pairs of experiments are shown in the table. Slope conductance values were determined as described in the Fig. 8 legend and are reported in nS as mean ± S.E. (number of cells), with data taken approximately 2 min after establishing the whole cell configuration. Similar ratios of slope conductance were obtained for all experiments employing a given proportion of WT:MUT CAM RNA (see Fig. 9). The day-to-day variations in slope conductance resulted from injections of different amounts of hIKCa1 RNA.

| Exp. | WT All MUT | 1 WT:1 MUT | 3 WT:1 MUT |
|------|------------|------------|------------|
|      | nS         | nS         | nS         |
| 1    | 1.98 ± 0.74 (8) | 0.12 ± 0.04 (6) |             |
| 2    | 15.1 ± 5.0 (10) | 1.03 ± 0.24 (6) |             |
| 3    | 22.52 ± 18.5 (6) | 1.12 ± 0.23 (4) |             |
| 4    | 38.2 ± 11.6 (5) |             | 2.34 ± 1.19 (6) |
| 5    | 5.37 ± 1.25 (6) |             | 0.25 ± 0.07 (11) |
| 6    | 22.5 ± 18.5 (6) |             | 1.70 ± 0.68 (5) |
| 7    | 22.8 ± 8.4 (10) |             | 13.8 ± 5.3 (10) |
| 8    | 0.68 ± 0.18 (5) |             | 0.31 ± 0.08 (7) |
| 9    | 19.4 ± 8.6 (4)  |             | 4.4 ± 1.6 (6)  |

**Fig. 9. Dominant-negative suppression of hIKCa1 currents by B1234Q.** RBL cells were co-injected with hIKCa1 cRNA and either WT-CAM alone, B1234Q-CAM alone, or a mixture of B1234Q- and WT-CAM cRNA. For each experiment, the base-line conductance (the mean slope conductance of un.injected cells) was subtracted and the resulting number was divided by the mean slope conductance of cells from the same experiment microinjected with WT-CAM. Each circle represents the ratio = S.E. of three independent experiments. The ratio of mutant CAM RNA to total CAM RNA is shown on the x-axis. Each experiment consisted of 4–10 cells measured 4–7 h postinjection. Error bars on the x-axis indicate the maximal anticipated error in RNA concentration. The solid line (line 0) represents a fit to the binomial distribution for the scenario in which no mutant subunits are allowed in a functional channel. Dotted lines (lines 1–3) show the fits to the same equation if one, two, or three mutant CAMs, respectively, binding a single hIKCa1 could form a functional channel.

**Fig. 10. Model depicting the site of interaction of CAM with the C-tail.** The inner helices of each channel subunit, shown as black cylinders, are arranged in a bundle, as suggested by the KcsA crystal structure (46). These helical rods cross at the bottom cytoplasmic surface (bundle crossing) and diverge at the extracellular surface to accommodate the P-regions. The upper end of the helices correspond to the S6 (M2 in KcsA) segments (black), and the region below the bundle crossing represents residues 1–10 of the hIKCa1 C-tail (white). Residues 11–62 of the critical C-tail CAM-binding region of each subunit are shown (in scale) as a separate single helix based on both Chou/Fasman and Robson algorithms for secondary structure predictions. Two CAM molecules (actual structure in the absence of Ca$^{2+}$) are shown in apposition to the C-tail; the other two subunits also associate with CAM. The spatial disposition of the CAMs does not imply interaction with the C-tail in any particular orientation.

**DISCUSSION**

In the present study, we have demonstrated that CAM is prebound to the cytoplasmic C-tail of the intermediate conductance KCa channel, hIKCa1, and mediates Ca$^{2+}$-dependent gating of these channels. The first 98 amino acids in the C-tail of hIKCa1 contain subdomains that are critical for both Ca$^{2+}$-dependent and Ca$^{2+}$-independent CAM binding. Although this region contains several positively charged and hydrophobic residues reminiscent of CAM-binding sites (37, 38), its lack of dependence on Ca$^{2+}$ for binding is noteworthy. We also show that known CAM antagonists W7 and TFP have no effect on hIKCa1 current, indicating a novel binding surface for the CAM-hIKCa1 interaction. Such Ca$^{2+}$-independent binding of CAM to its target protein, although uncommon, has been reported for some molecules, including nitric oxide synthase, neurogranin, neuromodulin, phosphorylase kinase, and unconventional myosins (39).

Our results with an IKCa channel parallel and complement those recently reported by Xia et al. (30) for SKCa channels. In that study, CAM was shown to associate tightly with the C tails of SKCa channels in the absence of Ca$^{2+}$. Co-expression in Xenopus oocytes of rSKCa2 and CAM mutants with lower Ca$^{2+}$ binding affinities resulted in a significant decrease in the Ca$^{2+}$ sensitivity of the expressed channel, thus providing the first evidence for a mechanism of Ca$^{2+}$-gating by SKCa channels.
Our finding of an identical mechanism for hIKCa1 expressed in mammalian cells confirms a common mechanism of Ca\(^{2+}\) gating for both SK<sub>Ca</sub> and IK<sub>Ca</sub> channels, despite their ~40% overall sequence identity. Not surprisingly, the region in the C-tail of hIKCa1 that we identified as being critical for CAM binding (1–98) shows a high degree of sequence similarity with the corresponding regions in the three members of the SK<sub>Ca</sub> subfamily of K\(^{+}\) channels.

In addition to demonstrating that CAM can bind to and mediate the function of hIKCa1, we have demonstrated a strong suppression of IK<sub>Ca</sub> conductance when the channel is co-expressed with a mutant CAM with four defective EF-hand motifs (B1234Q CAM). The fact that the mutant CAM is so effective at competing with the endogenous protein for binding to the channel subunits suggests some mechanism for coassembly of newly synthesized CAM protein with the cytoplasmic tails of new channel molecules as they are folded on the endoplasmic reticulum membranes. Alternatively, it is possible that there is, in essence, no pool of “free” CAM to compete with the plasmic reticulum membranes. Alternatively, it is possible that the tails of new channel molecules as they are folded on the endoplasmic reticulum membranes. Alternatively, it is possible that there is, in essence, no pool of “free” CAM to compete with the newly synthesized protein. At elevated [Ca\(^{2+}\)], this condition appears to apply, with free (non-target bound) CAM representing about 0.1% of total CAM protein (40). The situation at resting [Ca\(^{2+}\)], is less well understood. However, studies in muscle cells suggest that the levels of CAM and its target proteins are carefully co-regulated even at resting levels of [Ca\(^{2+}\)](41).

If each subunit of hIKCa1 channel tetramer binds one molecule of Ca\(^{2+}\)-free CAM, and if the concerted action of all four molecules is necessary for gating, then perturbing one interaction would impose a dominant-negative phenotype on the channel currents. Consistent with this hypothesis, the currents observed in cells microinjected with a 1:1 ratio of WT and mutant CAM cRNA along with the channel cRNA exhibited about 1/6th of the current magnitude observed in cells microinjected with channel cRNA and WT CAM alone. When the ratio of mutant to WT CAM was varied, we observed that the current relative to WT-CAM-microinjected cells agreed with the equation for disruption of channel activity by binding of a single mutant CAM (Fig. 9). Furthermore, even 1 mM Ca\(^{2+}\) in the patch pipette, a concentration sufficient to elicit the conformational changes seen in B1234Q, did not rescue conductance when this mutant CAM was co-expressed with hIKCa1. These results imply that Ca\(^{2+}\)-induced conformational changes must occur involving each prebound CAM in order to open the channel (Fig. 10). The requirement for four CAM molecules provides a structural basis for the previously determined steeply cooperative Ca\(^{2+}\) dependence for activation of the lymphocyte K<sub>Ca</sub> channel encoded by hIKCa1 (Hill coefficients of 3–4) (20, 42).

Our results point naturally toward a kinetic model for gating of the IK<sub>Ca</sub> channel. In the following scheme,

\[
\begin{align*}
\text{C} & \leftrightarrow \text{C-Ca}^{2+} \\
\text{C-Ca}^{2+} & \leftrightarrow \text{C-2Ca}^{2+} \\
\text{C-2Ca}^{2+} & \leftrightarrow \text{C-3Ca}^{2+} \\
\text{C-3Ca}^{2+} & \leftrightarrow \text{C-4Ca}^{2+} \\
\text{C-4Ca}^{2+} & \leftrightarrow \text{C-Ca}^{2+} \\
\text{C-Ca}^{2+} & \leftrightarrow \text{C-2Ca}^{2+} \\
\text{C-2Ca}^{2+} & \leftrightarrow \text{C-3Ca}^{2+} \\
\text{C-3Ca}^{2+} & \leftrightarrow \text{C-4Ca}^{2+} \\
\text{C-4Ca}^{2+} & \leftrightarrow \text{C-Ca}^{2+} \\
\text{C-Ca}^{2+} & \leftrightarrow \text{C-2Ca}^{2+} \\
\text{C-2Ca}^{2+} & \leftrightarrow \text{C-3Ca}^{2+} \\
\text{C-3Ca}^{2+} & \leftrightarrow \text{C-4Ca}^{2+} \\
\text{C-4Ca}^{2+} & \leftrightarrow \text{C-Ca}^{2+} \\
\text{C-Ca}^{2+} & \leftrightarrow \text{C-2Ca}^{2+} \\
\text{C-2Ca}^{2+} & \leftrightarrow \text{C-3Ca}^{2+} \\
\text{C-3Ca}^{2+} & \leftrightarrow \text{C-4Ca}^{2+} \\
\text{C-4Ca}^{2+} & \leftrightarrow \text{C-Ca}^{2+} \\
\text{OPEN} & \leftrightarrow \text{C-Ca}^{2+} \\
\end{align*}
\]

**Scheme 1**

C indicates closed channel conformations, and asterisks represent activated subunit conformations. Horizontal transitions represent Ca\(^{2+}\) binding to CAM on the channel, and vertical transitions represent Ca\(^{2+}\)-CAM-induced conformational changes in the channel subunit, with the number of asterisks symbolizing the number of activated subunits. In the above scheme, each subunit of a tetrameric channel is associated with a single CAM, which can bind up to four Ca\(^{2+}\) ions to induce a conformational change in the channel subunit. For each of four independent subunits, we envision a sequential two-step activation process: first Ca\(^{2+}\) binding by preassociated CAM, and then a conformational change in the IK<sub>Ca</sub> channel subunit to an activated conformation. In this scheme, it is imagined that conformational changes in the absence of Ca\(^{2+}\) binding to CAM are so energetically unfavorable that the states with more activated subunits than Ca\(^{2+}\)-CAM moieties do not exist, consistent with the fact that no IK<sub>Ca</sub> conductance is seen at low [Ca\(^{2+}\)]. Such states, if they existed, would fill in the lower left-hand portion of the scheme. All four subunits must be activated before the channel opens. Although speculative, the kinetic diagram is similar to previous proposals for a variety of K<sub>Ca</sub> channels based upon single-channel data (43–45). These schemes predict that the steep Hill coefficient determined in functional measurements of the Ca\(^{2+}\) sensitivity of channel opening arises from the requirement for Ca\(^{2+}\)-induced conformational changes by each of four subunits in BK<sub>Ca</sub>, SK<sub>Ca</sub>, and, as proposed here, IK<sub>Ca</sub> channels.

How might a conformational change in the C-tail of hIKCa1 result in opening of the pore? A comparison of the sequence of this region with that of the structurally defined bacterial potassium channel, KcsA, from *Streptomyces lividans* (46) suggests that the first 6–10 residues of the hIKCa1 C-tail correspond to part of the inner helix that includes S6 (Fig. 10). More specifically, these residues represent the stretch of the inner helix lying below the “bundle crossing” (Fig. 10), and any Ca\(^{2+}\)-CAM-induced conformational change in this segment could conceivably be transmitted along the helical rod, resulting in channel opening. Interestingly, recent studies on the voltage-gated K<sup>+</sup> channel, *Shaker*, suggest that gating occurs at the bundle crossing possibly due to conformational changes in this region (47). Two different algorithms (Chou/Fasman and Robson) predict that the remainder of the 1–62 segment has a high helical propensity, suggesting that the inner helix might extend further cytoplasmically (Fig. 10). Coupling of this segment with the inner helix might underlie calcium gating of hIKCa1. This heuristic model requires direct structural verification.

Although second messenger cascades involving CAM are known to modulate many ion channels (48), there is growing evidence of regulation by Ca\(^{2+}\)-CAM through direct binding (49). These phenomena have been documented for the *Paramecium* Ca\(^{2+}\)-activated sodium channels (50), the *Drosophila* Ca\(^{2+}\)-permeable channels *trp* and *trpl* (51, 52), the vertebrate photoreceptors and olfactory receptors involving cyclic nucleotide gated channels (53), the ryanodine receptor Ca\(^{2+}\)-release channels (54), and the N-methyl-D-aspartate receptors (55). However, the region of the C-tail of hIKCa1 and hSKCa3 implicated in Ca\(^{2+}\)-free CAM shows no obvious similarity to sequences with a comparable function in *trpl* (51) or the ryanodine receptor (56). In these examples, channel modulation involves either activation or deactivation by CAM. In contrast, the high affinity for Ca\(^{2+}\) and the rapid activation kinetics of SK<sub>Ca</sub> and IK<sub>Ca</sub> channels demands a fast gating mechanism (45). This “near-intrinsic” requirement is provided by preassociated CAM molecules in a tight multimeric complex with the channel tetramer, converting a modest change in intracellular Ca\(^{2+}\) to a quick, robust physiological response. Further biochemical, biophysical, and direct structural studies will help elucidate the mechanisms by which CAM-induced channel conformational changes in the C-tail translate into opening of IK<sub>Ca</sub>.
and SK$_{Ca}$ channels, leading to hyperpolarization and down-stream signaling events.

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