hSK4/hIK1, a Calmodulin-binding K\textsubscript{Ca} Channel in Human T Lymphocytes

ROLES IN PROLIFERATION AND VOLUME REGULATION*

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Human T lymphocytes express a Ca\textsuperscript{2+}-activated K\textsuperscript{+} current (IK), whose roles and regulation are poorly understood. We amplified hSK4 cDNA from human T lymphoblasts, and we showed that its biophysical and pharmacological properties when stably expressed in Chinese hamster ovary cells were essentially identical to the native IK current. In activated lymphoblasts, hSK4 mRNA increased 14.6-fold (Kv1.3 mRNA increased 1.3-fold), with functional consequences. Proliferation was inhibited when Kv1.3 and IK were blocked in naive T cells, but IK block alone inhibited re-stimulated lymphoblasts. IK and Kv1.3 were involved in volume regulation, but IK was more important, particularly in lymphoblasts. hSK4 lacks known Ca\textsuperscript{2+}-binding sites; however, we mapped a Ca\textsuperscript{2+}-dependent calmodulin (CaM)-binding site to the proximal C terminus (Ct1) of hSK4. Full-length hSK4 produced a highly negative membrane potential (V\textsubscript{m}) in Chinese hamster ovary cells, whereas the channels did not function when either Ct1 or the distal C terminus was deleted (V\textsubscript{m} = 0 mV). Native IK (but not expressed hSK4) current was inhibited by CaM and CaM kinase antagonists at physiological V\textsubscript{m} values, suggesting modulation by an accessory molecule in native cells. Our results provide evidence for increased roles for IK/hSK4 in activated T cell functions; thus hSK4 may be a promising therapeutic target for disorders involving the secondary immune response.

Both voltage-gated and Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels are widely expressed in immune cells, including human T lymphocytes. Drugs that block voltage-gated Kv1.3 channels inhibit T lymphocyte activation and proliferation, volume regulation, and cell-mediated cytotoxicity (for review, see Ref. 1). Inasmuch as these functions involve Ca\textsuperscript{2+} influx through channels activated by depletion of Ca\textsuperscript{2+} stores, one widely proposed role for K\textsuperscript{+} channels is to maintain a negative membrane potential and large driving force for Ca\textsuperscript{2+} entry. However, the relative roles of K\textsubscript{Ca} versus Kv1.3 channels in these cell functions are not known, partly owing to the previous lack of potent K\textsubscript{Ca} blockers that do not also block Kv1.3 channels.

Two K\textsubscript{Ca} channels have been found in lymphocytes and lymphocytic cell lines. They differ in biophysical and pharmacological properties (2–6). An ampin-sensitive, small conductance channel (7–8 pS) is the prevalent K\textsubscript{Ca} channel in the commonly used Jurkat T cell line (4) and is also present in rat T and human B lymphocytes (2, 6). However, a corresponding ampin-sensitive whole-cell current has not been identified in normal human T cells, perhaps a result of channel rundown we observed after cell disruption (2). Instead, a K\textsubscript{Ca} channel we first described (2, 3) is the prevalent K\textsubscript{Ca} channel in resting and activated human T lymphocytes. It is a charybdotoxin-sensitive, inwardly rectifying channel (15–35 pS in symmetrical K\textsuperscript{+} solutions (5, 6)) that is commonly called "IK," for intermediate conductance K\textsubscript{Ca}. Recently, a molecular candidate for IK was cloned from a human placental cDNA library (hSK4 (7)) and subsequently from human pancreas (hIK1 (8)) and a human lymph node library (hKCa4 (9)).

IK current increases in the 3–4 days following activation of human T cells (5). Thus, it is anticipated that this K\textsubscript{Ca} current will be especially important for secondary immune responses of activated T cells (lymphoblasts), including proliferation and volume regulation. The regulatory volume decrease (RVD) that follows T cell swelling is known to depend on K\textsuperscript{+} (and Cl\textsuperscript{−}) channels (10–12). Although Kv1.3 is involved in RVD in some resting T cells (13), the relative contribution of IK versus Kv1.3 channels is not known, either in resting human T cells or in lymphoblasts. We previously reported that intracellular Ca\textsuperscript{2+} rises immediately after human T cells are exposed to a hypotonic shock (14); thus, we predicted that K\textsubscript{Ca} currents would also subserve RVD.

In the present study we cloned hSK4 from human T lymphoblasts, expressed the channels stably in CHO cells, and compared the salient biophysical and pharmacological properties of the native and cloned channels. All intrinsic properties examined were indistinguishable, supporting the view that hSK4 homotetramer forms the \( \alpha \) subunit of the IK channel of lymphoblasts. We found that hSK4 mRNA expression is strongly up-regulated after T cell activation; thus we predicted (and observed) an increased role for IK current in lymphoblasts compared with resting T cells. Although hSK4 is functionally a K\textsubscript{Ca} channel, that is activated by a rise in intracellular Ca\textsuperscript{2+}, it...
was recently reported that brain SK channels are not gated directly by Ca\(^{2+}\) but rather by Ca\(^{2+}\) interacting with calmodulin that is irreversibly bound to the channel (15). We have presented preliminary data showing that the lymphoblast IK current is inhibited by antagonists of calmodulin and CaM kinase (16, 17). We now show details of this inhibition of native IK current and that calmodulin binds directly to the hSK4 channel protein in a Ca\(^{2+}\)-dependent manner. The CaM binding domain resides in the proximal part of the C terminus, since binding to this region occurs in the absence of flanking sequence and is eliminated in constructs lacking this region. Unlike the study of heterologously expressed brain SK channels (15), we provide evidence for additional modulation of hSK4 in lymphocytes, results that have important implications for the existence of accessory molecules and cell-specific KCa channel regulation.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human peripheral blood mononuclear cells were isolated on a Ficoll-Paque (Amersham Pharmacia Biotech, Baie D’Urfé, Quebec, Canada) density gradient. To purify resting T cells, monocytes and B lymphocytes were removed by adhering them to a nylon wool column for ~1 h and then eluting the T cells, which were then placed in culture medium containing RPMI 1640 with l-glutamine (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum, HT supplement, antibiotic/antimycotic, and 1 mg/ml Geneticin (all reagents from Life Technologies, Inc.). The monocytes were removed on a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Baie D’Urfé, Quebec, Canada) and subsequently separated into clonal populations for the existence of accessory molecules and cell-specific KCa channel regulation.

**Preparing RNA Probes**—Total RNA was isolated from resting and activated human T lymphocytes, rat lung, and human placenta using the guanidinium isothiocyanate method (18) and subjected to DNase I digestion (0.1 units/ml, 15 min, 37 °C; Amersham Pharmacia Biotech, Piscataway, New Jersey, MA). After incubating the mixture at 85 °C for 1 min, 1.25 units of RNase digestion, the samples were electrophoresed in polyacrylamide gels, dried, and exposed overnight to x-ray film (X-Omat, Eastman Kodak Co.). Specific signals were quantified by densitometric analysis of the developed film using a Bio-Rad model Gs-670 densitometer, and results are expressed as mean ± S.D. with statistical analyses using the Student’s t test.

**hSK4/Flag DNA Constructs**—An Xhol site was added by PCR to the C terminus of the full-length hSK4 and then a pair of Flag-encoding oligonucleotides was inserted into this site. This construct was used to create a second proto-construct in which all transmembrane domains were eliminated, and a consensus Kozak sequence was added to the beginning of the cytoplasmic C terminus. Morph mutagenesis (5 Prime → 3 Prime, Inc., Arapaho, CO) was used to add a second, silent EcoRV site and a second Xhol site to the C termini of each proto-construct. Derivatives of these proto-constructs (for details, see text accompanying Fig. 7) were created by cutting out the fragments flanked by either EcoRV or Xhol and religating the larger fragment.

**Calmodulin Affinity Chromatography**—CHO cells stably expressing Flag-tagged hSK4 constructs were grown to confluency in 100-mm Petri dishes. To assess binding of hSK4 protein to calmodulin-Sepharose, we used methods modified from Chapin et al. (19). The dishes were washed three times in cold phosphate-buffered saline containing calcium and magnesium. Then, 1 ml of ice-cold solubilization buffer was added, which contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1% Triton X-114 (Sigma), and protease inhibitors (5 mM pepstatin, 10 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 μg/ml antipain, 500 μg/ml benzamidine, 0.1% Trasylol), and the dishes were rotated for 15 min in a refrigerated rotator at 4 °C. The lysate was triturated with a 23-gauge needle, and after removing insoluble material by centrifugation (15 min, 4 °C) the supernatant was transferred to a new tube to which 6 μl of 0.5 mM EGTA was added (final concentration, 3 mM) to chelate the calcium. To promote Triton X-114 phase partitioning, this solution was warmed to 37 °C for 3 min and then centrifuged (5 min, room temperature) at full speed in a microcentrifuge. The detergent phase (bottom) was resuspended in 25 μl of 100 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), and 1% Triton X-114 (Sigma), and then a pair of Flag-encoding oligonucleotides was inserted into this site. This construct was used to create a second proto-construct in which all transmembrane domains were eliminated, and a consensus Kozak sequence was added to the beginning of the cytoplasmic C terminus. Morph mutagenesis (5 Prime → 3 Prime, Inc., Arapaho, CO) was used to add a second, silent EcoRV site and a second Xhol site to the C termini of each proto-construct. Derivatives of these proto-constructs (for details, see text accompanying Fig. 7) were created by cutting out the fragments flanked by either EcoRV or Xhol and religating the larger fragment.

**Ribonuclease Protection Assays**—hSK4/hIK1/hKCa4 (7–9).

**Patch Clamp Electrophysiology, T Cells**—Activated T lymphoblasts were used 3–4 days after PHA stimulation, at which time 1K current
amplitudes are much larger in resting T cells at the same cytoplasmic Ca 2+
concentration (5). Whole-cell currents were measured using an Axopatch 200 amplifier, with 8–12 MΩ pipettes. During data acquisition, capacitive currents were canceled by analogue subtraction, 50–70% series resistance compensation was used, and all currents were filtered at 10–15 Hz. Voltage clamps were set to +70 mV solution (or 140 mM potassium aspartate solution). To activate fully the IK channels (5), we used a high Ca 2+ (1.1
µM) pipette solution, consisting of the following (in mM): 140 potassium aspartate, 1 K 2BAPTA, 2 K 2ATP, 0.9 CaCl 2, 1 MgCl 2, 5 HEPES, adjusted with NaOH to pH 7.4, 270–283 mosmol. The K
selectivity was verified and current rectification examined by replacing the sodium aspartate with 80 mM sodium aspartate, 65 mM potassium aspartate (or 140 mM potassium aspartate solution), or 140 mM potassium aspartate solution. To maintain the cell volume-sensitive Cl
concentrations (K
21). In principle, CaM can reduce free Ca 2+
in cytosol by occupying its Ca 2+–binding sites (E-F hands) for a total of four binding sites (20) were small since aspartate was used in the bath and pipette, and the internal solution was slightly hypo-osmotic. Fresh K 2ATP (Sigma) was always added to pipette solutions just before use to help maintain channel and second-messenger activity.

Solutions—Stably transfected CHO cells were passaged every 3–4 days using trypsin/EDTA (Life Technologies, Inc.). Recordings were made 1–2 days after replating, using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), and Origin (version 4.1, Microcal, Northampton, MA). Where appropriate, data are presented as mean ± S.E., with paired Student’s t tests used to determine the statistical significance of differences (95% confidence interval). All recordings were made at room temperature (19–22°C), except when KN-62 and KN-04 were used at 37°C.

**RESULTS**

**Up-regulation of hSK4 and Kv1.3 mRNA Expression in Activated T Lymphocytes**—We amplified DNA corresponding to full-length transcripts of hSK4 and Kv1.3 channels from activated human T lymphocytes. The open reading frame of the full-length hSK4 clone was 1284 bp, which encodes a protein of 428 amino acids that is 100% identical to the recently cloned hSK4 (7), hIK1 (8), and hKaC4 (9). RNase protection assays (Fig. 1, A–C) on total RNA from resting and activated human T cells were used to determine if changes in mRNA expression correlate with previously observed increases in amplitude of the two K
channels.

**Biophysical and Pharmacological Properties of the Lymphoblast IK Are the Same as hSK4 Expressed in CHO Cells**—Charybdotoxin (ChTx) is an effective blocker of native IK currents in lymphocytes (3, 5) and of expressed hSK4/hIK1/hKaC4 channels (7–9). In lymphoblasts, with free Ca 2+ in the pipette buffered to 1.1 µM to maximally activate IK, typical current responses to voltage steps or ramps were resolved into the following three components: Kv1.3, IK, and an anion current (Fig. 1D). Separation of the currents was achieved by first blocking Kv1.3 with margatoxin (MgTx, 2.5 mM) and then add-

Changes in cell volume were measured using a flow cytometer (FACS- can, Becton Dickinson, CA) by monitoring changes in right angle light scattering (side scatter) as an index of cell volume (23). To indicate percent change from the initial volume, we calculated a swelling index as [(VSSCs/v/vSSC) × 100], where SSCs is the average side scatter of untreated control cells, and VSSCs is the average volume in isotonic medium. The flow cytometer was set to exclude dead cells and debris by omitting cells that stained with propidium iodide (0.04 µg/ml), a nuclear marker excluded by live cells.

For control volume measurements, resting or activated T cells were suspended in isotonic medium which contained the following (in mM): 140 NaCl, 5 KCl, 1 MgCl 2, 10 HEPES, pH 7.4, 285 mosmol. Initial measurements were performed in the presence of 500 live cells/ml, after which aliquots of the same cells were exposed to a hypotonic medium (56% of normal osmolality) with or without K
channel blockers. The hypotonic medium contained the following (in mM): 70 NaCl, 5 KCl, 1 MgCl 2, 10 HEPES, pH 7.4, 159 mosmol. Side scatter was recorded for 5000 live cells every 30 s for the first 3 min and then at 6 min after the hypotonic shock. Whenever K
channel blockers were used, the initial volume was measured in the presence of drug before exposing the cells to hypotonic medium. Data were analyzed using CellQuest software (version 3.0.1f, Becton Dickinson). Values are presented as mean ± S.D. of at least 4 experiments per treatment, and a Bonferroni multiple comparison test was used for statistical analysis. Experiments were performed at room temperature (21–23°C), except when KN-62 and KN-04 were used at 37°C. The K
channel blockers, charybdotoxin and iberiotoxin (purchased from Peptides International, Louisville, Kentucky, KY, or a gift from V. Gribkoff and S. Dworetzky, Bristol-Myers Squibb Co.), margatoxin and agitoxin-2 (Alomone Laboratories, Jerusalem, Israel), and apamin, d-tubocurarine, and clotrimazole (Sigma). The calmodulin antagonists, trifluoperazine and W-7 (N-6-aminoheptyl)-5-chloro-1-sulfonylanilide) were from Sigma. Bovine brain calmodulin, rat brain CaMKII, calmidazolium (compound R24571), and KN-62 (1-[(N,O-bis(5-isooquinolinesulfonyl))-N-methyl-L-tyrosyl]-4-phenylpiperazine) were from Calbiochem. KN-04 (N-[1-(N-methyl-p-5-isooquinolinesulfonyl)-benzyl]-2-(4-phenylpiperazine)ethyl]-5-isooquinolinesulfonamide), an analogue of KN-62 without CaMK kinase inhibitor activity, was purchased from Seikagaku America (Rockville, Maryland). These reagents (except KN-62 and KN-04) were prepared in bath saline, frozen in aliquots, and thawed just before use. KN-62 and KN-04 stock solutions were prepared in Me 2SO and diluted in bath saline before use. The Me 2SO concentration in the final bathing solution was <0.5% for T cells and ≤1% for CHO cells. Whenever cells were treated with KN-62, control cells were incubated in the same concentration of Me 2SO for the same duration.

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Results are expressed as the mean increase in fluorescence
± S.D. (n = 4 experiments, 4 replicates/experiment), and a Bonferroni multiple comparison test was used for statistical significance.

Flow Cytometric Analysis of Regulatory Volume Decrease (RVD)—
ing ChTx (15 nM) to block IK, leaving the anion current (Cl\(^-\) Nernst potential about \(-22\) mV) which we have previously characterized (20). For subsequent IK measurements we blocked Kv1.3 with 2.5–5 nM MgTx, measured the remaining current, and then blocked IK with 10–20 nM ChTx and subtracted the remaining anion current. (This procedure was not necessary for stably transfected CHO cells, wherein endogenous currents were negligible compared with the large hSK4 currents.)

The ChTx-sensitive currents (Fig. 2A) were K\(^+\)-selective, as seen from the intersection of their current versus voltage (I-V) curves with the anion current (at \(-78\) mV; Nernst potential, \(E_{K} = -86\) mV). We further confirmed their K\(^+\) selectivity using high K\(^+\) bathing solutions, wherein the reversal potentials of both Kv1.3 and IK were commensurate with changes in the external K\(^+\) concentration: at 70 mM, reversal potential, \(E_{rev} = -15\) to \(-17\) mV, and at 150 mM \(E_{rev} = -2\) to 0 mV (after junction potential corrections). Expressed hSK4 and native IK currents were not time- or voltage-dependent, even during long voltage clamp steps (Figs. 1D and 2, B and C) (5). Their whole-cell I-V relations (Fig. 2A) were nearly linear over a wide voltage range under physiological Na\(^+\)/K\(^+\) gradients (also Fig. 1D) but rectified inwardly with high K\(^+\) concentrations in the bath and pipette. These biophysical features are consistent with previous studies of native IK in lymphocytes (5, 24), and inward rectification in high K\(^+\) was observed for the channels expressed in Xenopus oocytes (hIK1 (8)) and in HEK cells (hKCa4 (9)). For the remaining experiments, whole-cell recordings from both cell types were made with high concentrations of external Na\(^+\) and internal K\(^+\).

For comparison with the literature we tested ChTx (Fig. 1D and 2, B and C) and found it to be a potent blocker of hSK4 in CHO cells (IC\(_{50}\) = 1.7 nM, \(n = 7\)) and of IK in T lymphoblasts (IC\(_{50}\) = 6 nM, \(n = 3\)). For expressed hSK4 the dose dependence was calculated from the initial block during each voltage clamp step, to avoid the time- and voltage-dependent relief of block at depolarized potentials (Fig. 2B). Clotrimazole blocks Ca\(^2+\)-activated K\(^+\) fluxes in thymocytes and red blood cells (IC\(_{50}\) 50 nM (25)).
and hIK1/hKCa4 channels expressed in Xenopus oocytes (Kd ~25 nM). Clotrimazole effectively blocked IK in lymphoblasts (IC50 = 40 nM) and hSK4 in CHO cells (IC50 = 56 nM), with no time or voltage dependence (Fig. 2, C and D). Although some brain K+ channels are potently blocked by apamin and d-tubocurarine (26, 27), neither hSK4 nor the lymphoblast IK current was sensitive to these drugs (apamin, IC50 >>100 nM; d-tubocurarine, IC50 >>250 μM). Iberitoxin, a potent inhibitor of large conductance KCa channels, did not block hSK4 or IK (IC50 >200 nM, data not shown).

For functional studies of proliferation and volume regulation in lymphocytes, we avoided ChTx since it blocks both IK and Kv1.3 currents with similar potencies. Margatoxin is much more selective for Kv1.3 but can reduce IK at high concentrations (>10 nM, data not shown); thus we used agitoxin-2, which is more potent (Kd ~200 pM) and does not block IK (28). IK was selectively blocked with clotrimazole. Drug concentrations were chosen to block different amounts of the two currents. To eliminate essentially all Kv1.3, we used 5 nM AgTx-2 (~25 Kd), whereas to allow us to test for additive effects of IK block, we used 250 nM clotrimazole (~6 Kd for IK). Peptide toxins are especially useful since they are not membrane-permeant, so are unlikely to affect other intracellular processes. Although clotrimazole is membrane-permeant and also inhibits cytochrome P-450 (25), there is no evidence for P-450 involvement especially useful since they are not membrane-permeant, so are unlikely to affect other intracellular processes. Although clotrimazole is membrane-permeant and also inhibits cytochrome P-450 (25), there is no evidence for P-450 involvement.

Proliferation Is Inhibited by Combined IK and Kv1.3 Channel Block—We first stimulated freshly isolated (naive) human T cells with the mitogen, phytohemagglutinin (PHA-P), and then measured proliferation after 3 days. A Bonferroni multiple comparison test was used to assess each combination of treatments. Values that differ significantly from controls are indicated (***, p < 0.001), as are significant differences between drug treatments (†††, p < 0.001).

![Image](53x354 to 293x729)

**Fig. 2.** Comparison of hSK4 stably expressed in CHO cells, with native IK current in activated T lymphocytes. For this, and all subsequent figures showing IK currents from lymphoblasts, 5 nM MgTx was present to block Kv1.3 current. A, hSK4 whole-cell I-V relations were plotted from currents in response to voltage steps, whereas native IK currents were from voltage ramps (as in Fig. 1D). The bath K+ concentrations were 5 or 145 mM for CHO cells, 5 or 150 mM for lymphoblasts, yielding calculated Kd values of ~86 mV and ~0 mV. B, charybdotoxin blocks hSK4 and IK currents. Currents in response to voltage clamp steps between -120 and +20 mV show profound block by 20 nM ChTx, with a slight relief over time at positive potentials in CHO cells. C, clotrimazole blocks both hSK4 and IK in a voltage- and time-independent manner. Currents in response to voltage clamp steps as in B, D, dose-response curves show fraction of current remaining (mean ± S.E.).

**Fig. 3.** Comparison of the ability of K+ channel blockers to inhibit proliferation of naive T cells (A) and previously activated T lymphoblasts (B). The term, naive, is used to indicate cells that were stimulated from an initial resting state. Each well of a 96-well plate was seeded with 2 x 10⁶ resting cells or lymphoblasts and incubated with or without channel blockers for 10 min (5 nM agitoxin-2, 300 nM clotrimazole, CLT). Then PHA-P (7 μg/ml) was added to initiate or restimulate proliferation. After 72 h, the CyQUANT assay was used to measure a change in fluorescence that is proportional to the change in cell number (see “Experimental Procedures”). Data are expressed as mean ± S.D. of four independent experiments (four replicates each). A Bonferroni multiple comparison test was used to assess each combination of treatments. Values that differ significantly from controls are indicated (***, p < 0.001), as are significant differences between drug treatments (†††, p < 0.001).

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We determined the effects of channel blockers on RVD within the first 6 min after the hypotonic shock (Fig. 4B). By this time, in control hypotonic solution both cell types had almost fully recovered from swelling; by 81.1 ± 6.8% (n = 6) in resting cells and 88.5 ± 5.5% (n = 6) in lymphoblasts. K⁺ channel blockers attenuated this recovery. Blocking Kv1.3 reduced volume recovery to 62.3 ± 9.0% (n = 4) in resting cells and 80.5 ± 5.9% (n = 4) in activated lymphoblasts, a significantly greater effect in resting cells. IK block was more effective than Kv1.3 block for both cell types: recovery was 39.6 ± 6.5% (n = 5) in resting cells and 28.8 ± 5.3% (n = 6) in lymphoblasts, a significantly greater effect in lymphoblasts. When both channels were blocked, RVD was further decreased in resting cells (30.7 ± 6.1% recovery, n = 5) but not in lymphoblasts (29.8 ± 6.8% recovery, n = 5). Our data show that both K⁺ channels contribute to RVD; IK is generally more important and, as predicted from its up-regulated expression, IK plays a greater role than Kv1.3 in lymphoblasts.

Calmodulin Antagonists Inhibit Native IK and Expressed hSK4 Currents—Three structurally unrelated drugs were used to inhibit the Ca²⁺-calmodulin complex: trifluoperazine (TFP, \(K_d \approx 1 \mu M\) (29)), W-7 (\(K_d \approx 12 \mu M\) (30)), and calmidazolium (\(K_d \approx 50 \mu M\) (31)). None of the drugs were toxic at the concentrations used; i.e. the cells did not become leaky even after a 30–60-min preincubation. Representative current traces are shown in Fig. 5, A–C, and the voltage dependence is summarized in Fig. 5D by plotting the current amplitude at the end of each step as a function of membrane potential. For hSK4 current in stably transfected CHO cells, TFP and W-7 effects were steeply voltage-dependent, with increased current at negative potentials (36% for TFP and 11% for W-7 at −100 mV). The inhibition at positive potentials (75% for TFP and 71% for W-7 at +40 mV) showed a pronounced time dependence (Fig. 5, A and B). Calmidazolium was not voltage-dependent; i.e. 500 nM inhibited hSK4 by ~35% at all voltages. For native IK current we were particularly interested in drug effects at negative membrane potentials typical of a non-excitatory lymphocyte. In contrast to hSK4, the lymphoblast IK current was significantly decreased at negative potentials by all three calmodulin antagonists. IK inhibition by 10 \(\mu M\) TFP was mildly voltage-dependent (a 55% decrease at −100 mV versus 75% at +40 mV), and this was due to a time-dependent reduction at depolarized potentials (Fig. 5A). Effects of 25 \(\mu M\) W-7 were qualitatively similar, with 42% inhibition at −100 mV and 65% at +40 mV. Again, inhibition by calmidazolium was not voltage-dependent and 500 nM inhibited IK by ~70% (Fig. 5, C and D). Thus, calmidazolium more effectively inhibited native IK than hSK4, and at negative potentials TFP and W-7 were only effective in reducing the native IK. This suggests two mechanisms of action, a physiologically relevant reduction of native IK at negative potentials and a time- and voltage-dependent reduction of both IK and expressed hSK4 at positive potentials.

To investigate further IK inhibition by CaM antagonists in lymphoblasts, we used voltage ramps or steps (see Figs. 1D and 2) and measured IK at a sufficiently negative potential to elicit large IK currents without contamination by Kv1.3. The current amplitude was measured at −120 mV from each cell before (control) and after adding a calmodulin antagonist to the bath. At the end of each experiment the anion current was recorded (after blocking IK and Kv1.3 with 20 nM ChTx) and subtracted from each total current at −120 mV to calculate the IK amplitude. Both ramp and step protocols yielded the same results. As summarized in Fig. 6, all three CaM antagonists dose-dependently inhibited IK (n = 4–6 cells unless otherwise indicated). When bath-applied during a recording, TFP reduced IK by...
Calmodulin antagonists reduce hSK4 stably expressed in CHO cells and native IK current in activated lymphoblasts. 

**A-C**, representative currents in control saline or with 10 μM trifluoperazine (TFP), 25 μM W-7, or 500 nM calmidazolium added to the bath. **D**, average current in CHO cells (●) and lymphoblasts (□) expressed as current at the end of each voltage clamp step as a fraction of control current (Icurrent/Icontrol) at each membrane potential (mean ± S.E., 2–6 cells). 

Lymphoblast IK currents were omitted near the reversal potential (about −86 mV) when they were too small to construct accurate ratios. Some error bars are smaller than the symbol. All values marked * are significantly lower for IK than respective values for hSK4 currents (p < 0.05).

61.8 ± 11.9% at 5 μM (p < 0.025) and by 46.1 ± 2.0% at 10 μM (p < 0.03) compared with control currents in the same cells. W-7 inhibited IK by 70.1 ± 8.5% (p < 0.05) at 5 μM, by 84.2 ± 5.2% at 10 μM (p < 0.01, n = 10), and by 96.6 ± 3.6% (p < 0.01) at 25 μM.

We used a competition experiment in which excess CaM (50 μM with 1.1 μM free Ca2+) was added to the pipette solution, followed by 10 μM W-7 addition to the bath, in the expectation that CaM would bind to internal W-7 and relieve inhibition. CaM addition did not significantly increase IK compared with the current in the same cells during the first 2 min of recording; however, the variability between cells was high (IK increased by 47 ± 26%, p > 0.34, n = 6). Nevertheless, as expected, excess CaM reduced the W-7-induced IK inhibition (from 84.2 ± 5.2% to 11.3 ± 29.1%, n = 12, p < 0.05), consistent with CaM-antagonist competition at an intracellular site. This result also rules out drug effects at external sites on the channel at negative potentials (see “Discussion”).

When calmidazolium was bath-applied after a recording was begun, it dose-dependently inhibited native IK current within 5–10 min, by 54.2 ± 4.5% at 100 nM (p < 0.05) and by 69.0 ± 3.1% (p < 0.01) at 500 nM. Inhibition was more effective when lymphoblasts were preincubated for 10–15 min before recording (94.1 ± 5.8% inhibition, p < 0.01). In contrast, a 1–2-h preincubation with 500 nM calmidazolium did increase the inhibition of hSK4 in CHO cells (34%, n = 8). Thus, slow drug permeation, which is a potential limiting factor, does not explain the difference in sensitivities between native IK and expressed hSK4 currents.

**Calmodulin Binds to a Proximal Portion of the C Terminus of hSK4**—One possibility is that CaM antagonists affect this K\textsubscript{Ca} current by interfering with interactions between CaM and the channel protein. If so, by analogy with properties of CaM binding to CaMK, one might expect CaM-hSK4 channel binding to be Ca\textsuperscript{2+}-dependent and competitively inhibited by CaM antagonists. We tagged hSK4 with the Flag epitope and expressed it stably in CHO cells. After pre-clearing the samples and washing the beads to remove nonspecific binding, Ponceau-stained Western blots indicated that several proteins bound to the beads (data not shown).

However, in samples containing the expressed full-length Flag-tagged hSK4 protein, only a single band was labeled by the anti-Flag antibody. Fig. 7A shows that binding of the wild-type full-length hSK4 protein was Ca\textsuperscript{2+}-dependent (i.e., greatly reduced by EGTA) but not competed by 1,000 nM of the CaM antagonist, calmidazolium. To map the region of the
channel that binds to CaM, we made several constructs by deleting the following: the distal C terminus (leucine zipper region, which we call “N-M-Ct1” since it contains the N terminus, membrane-spanning, and C terminus 1 regions), the entire N terminus and transmembrane domains (Ct1–Ct2), all but the proximal C terminus (Ct1), or the proximal C-terminal tail (N-M-Ct2). All but one channel construct bound to CaM in a Ca²⁺-dependent manner that was not competitive with calmodiazolium, i.e. the construct lacking the proximal C terminus (Ct1) did not detectably bind to CaM.

**C-terminal Deletions Have Functional Consequences**—We used the membrane potential ($V_m$) to monitor the expression of functional hSK4 channels of various constructs in transfected CHO cells (Fig. 7B). Whole-cell patch clamp recordings were made with 1 μM free Ca²⁺ in the pipette (as for all recordings of currents), and the membrane potential was recorded in the current clamp mode. The $V_m$ of CHO cells transfected with wild-type hSK4 channels was $-67 ± 4$ mV ($n = 5$) indicating a significant K⁺ permeability (Nernst potential for K⁺, $-82$ mV), and hSK4 with a C-terminal Flag tag produced the same $V_m$ ($-68 ± 4$ mV, $n = 5$). The $V_m$ of control cells transfected with vector alone (pcDNA3) was $-10 ± 8$ mV, indicating a very low background K⁺ permeability. The hSK4 construct lacking the CaM-binding region (N-M-Ct2) did not produce a negative membrane potential despite the high intracellular Ca²⁺; $V_m$ was significantly less negative than for the wild-type construct ($-2 ± 1$ mV, $n = 5$, $p < 0.001$). Interestingly, $V_m$ was also close to $0$ ($-5 ± 2$ mV, $n = 6$) for the construct lacking the distal C terminus (N-M-Ct1).

**The Lymphoblast IK Current Is Reduced by a CaM Kinase Antagonist**—KN-62 is a membrane-permeant CaM kinase antagonist, whereas KN-04 is an inactive analogue used as a negative control. KN-62 reduced IK in a time- and voltage-independent manner (Fig. 8A). Fig. 8B summarizes effects of KN-62 alone or in combination with the CaM antagonist, W-7. IK was measured in lymphoblasts at $-120$ mV after subtracting the small anion current (as for Fig. 6). We first tested intact cells at $37$ °C in an attempt to maintain kinase and phosphatase activity and turnover of protein phosphorylation. Preincubation with $10$ μM KN-62 alone (30 min, $37$ °C) decreased the current by $55.5 ± 11.2$% ($p < 0.02$, $n = 4$), whereas KN-04 had no effect ($97 ± 11$% of the control value, $n = 4$, $p > 0.8$). After KN-62 preincubation and with the drug present throughout whole-cell recordings, adding W-7 to the bath further reduced the current, i.e. by $80.2 ± 10.1$% reduction ($n = 4$, $p < 0.05$) with the combined drugs. Acute effects of bath-applied KN-62 were tested during whole-cell recordings at $37$ °C. IK was substantially reduced by KN-62 (67.9 ± 11.7%; $n = 4$, $p < 0.02$) but not by KN-04. Under drug-free conditions, IK in lymphoblasts was not obviously temperature-dependent; the specific conductance was $606 ± 210$ pS/picofarads at room temperature and $552 ± 13$ pS/picofarads when the same cells were warmed to $33$ °C ($n = 6$, $p > 0.7$). Interestingly, the variability in current amplitude was greatly reduced at the higher temperature. Unlike the native channels in lymphoblasts, hSK4 stably expressed in CHO cells at $37$ °C. IK was substantially reduced by KN-62 (67.9 ± 11.7%; $n = 4$, $p < 0.02$) but not by KN-04. Under drug-free conditions, IK in lymphoblasts was not obviously temperature-dependent; the specific conductance was $606 ± 210$ pS/picofarads at room temperature and $552 ± 13$ pS/picofarads when the same cells were warmed to $33$ °C ($n = 6$, $p > 0.7$). Interestingly, the variability in current amplitude was greatly reduced at the higher temperature. Unlike the native channels in lymphoblasts, hSK4 stably expressed in CHO cells was not inhibited by $10$ μM KN-62 and remained at $94.3 ± 2.5$% ($n = 6$, $p > 0.2$) of the control value measured with solvent alone (1% Me₂SO).

**DISCUSSION**

**Comparison of the Cloned hSK4 with IK Current in Activated T Cells**

The present results are entirely consistent with the IK current in T lymphocytes being the product of the hSK4/hIK1/hKCa4 gene, which was recently cloned from cDNA libraries from human placenta (7), pancreas (8), and lymph node (9). Since the product we cloned is 100% identical to hSK4/hIK1/hKCa4, differences in properties of the native lymphocyte IK
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The pharmacological profiles of native IK and hSK4/hIK1/hKCa4 are also similar. Native IK in lymphoblasts and hSK4 expressed in CHO cells were blocked by ChTx (IC_{50} 2–10 nM) but very poorly by iberiotoxin (IC_{50} >200 nM), margatoxin (IC_{50} >100 nM), or tetraethylammonium (IC_{50} 30–40 nM). Clostrimazole showed a similar potency for blocking IK in lymphoblasts (present study) and for heterologously expressed hSK4 (IC_{50} 25–60 nM (Ref. 8 and present study)). The hSK4/hIK1/hKCa4 channel is expected to be insensitive to both apamin and d-tubocurarine since it lacks two necessary amino acids in the putative pore (27) and, as expected, neither the lymphoblast IK nor the expressed hSK4 current were significantly inhibited by apamin (IC_{50} >100 nM (Refs. 5 and 7–9 and present study) or d-tubocurarine (IC_{50} >250 µM; present study)).

**Increased Role for hSK4 in Lymphoblast Proliferation**

K^{+} channel activity is important during the early activation phase of naive T cells, especially for maintaining a hyperpolarized membrane potential, promoting a rise in intracellular Ca^{2+}, and permitting a cascade of events that culminates in interleukin-2 production (1, 32, 33). In the first few hours after mitogenic stimulation, precisely when Ca^{2+} elevation is necessary (34, 35), K^{+} channel blockers, or other means of depolarizing T cells (high external K^{+}, voltage clamp), inhibit T cell activation (36) by compromising Ca^{2+} influx and the resulting rise in Ca^{2+}. Early studies using non-selective K^{+} channel blockers (e.g. quinidine, 4-aminopyridine) were later substantiated by more selective peptide toxins including charybdotoxin, which blocks both IK (K_{d} 2–6 nM (Refs. 3 and 5 and present study)) and Kv1.3 channels (K_{d} 1–10 nM (1, 5, 16, 17, 24)), and margatoxin or noxistoxin which block Kv1.3 but not IK channels (1, 16, 17, 28, 37). From the limited functional studies using blockers that discriminate between Kv1.3 and other K^{+} channels, Kv1.3 appears to be important for activation of naive T cells through pathways that are Ca^{2+}-dependent (1, 32).

The contribution of K_{Ca} channels to T cell activation and proliferation is still poorly understood. Since we previously found that mitogens activate K_{Ca} channels in cell-attached patches from naive human T cells, we proposed that they also play a role in T cell activation (14). In the present study we observed a 14.6-fold increase in hSK4 transcripts by 3–4 days after mitogenic stimulation. During the same period, mRNA levels for Kv1.3 increased only 1.3-fold. These changes are consistent with previous patch clamp studies showing an approximate doubling in Kv1.3 current (see Ref. 1), a 30-fold increase in the ChTx-sensitive KCa current than in naive T cells (38); however, ChTx does not discriminate between IK and Kv1.3 channels. To separate better the contributions of Kv1.3 and IK to T cell function, we used AgTx-2 to block Kv1.3 and clotrimazole to block IK. Consistent with our expectations, IK block more effectively inhibited proliferation of lymphoblasts than naive T cells. Furthermore, despite the greater Kv1.3 channel block (AgTx-2 at −25 K_{p}) than IK block (clotrimazole at −6 K_{p}), IK block was more effective in inhibiting lymphoblast proliferation, i.e. by 65.0% compared with 18.4% for Kv1.3 block. Blocking both channels (AgTx-2 + clotrimazole) was approximately additive, reducing lymphoblast proliferation by 86.8%. Proliferation of naive T cells was also sensitive to blocking both channels

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**Fig. 8. Native IK current in T cells is inhibited by the CaM kinase inhibitor, KN-62.** A, representative current traces during 800-ms voltage clamp steps between −120 and +20 mV before and 10 min after adding 10 µM KN-62. All recordings were at 37 °C and included 5 nM MgTx to block Kv1.3. B, average current amplitudes (+S.E., n = 4–5 cells from 2 to 3 batches) calculated at −120 mV (as in Fig. 4) as a percent of control values. For each batch of cells, 1 aliquot was used to measure control IK currents at room temperature. A 2nd aliquot was preincubated with KN-62 (10 µM, 30 min at 37 °C), and IK was measured at room temperature 10–15 min after beginning a recording. For some cells from the same KN-62-preincubated aliquots, 10 µM W-7 was added 15–20 min into a recording. Values that differ from control (room temperature) currents are indicated (*, p < 0.05; **, p < 0.01). A 3rd aliquot of cells was used at 37 °C for control recordings (>10 min), followed by KN-62 or KN-04 addition for 10–20 min. A significant inhibition was seen with KN-62 only (†, p < 0.05). All control cells were treated with 0.5% Me_{2}SO, the maximum solvent concentration used for KN-62 and KN-04.

and exogenously expressed hSK4 channels are not expected unless such properties are determined by something other than the α subunit of the channel. In principle, differences could arise if the channel forms heteromultimers with another protein, if alternative splice variants exist, or if the channel interacts with accessory molecules. It is intriguing that multiple transcript sizes are commonly seen for this channel, i.e. 2.6 and 3.8 kb (7), −2.1 kb, and at least one larger band (8), 2.2 kb, with two larger bands (9), and a prominent 2.2-kb band with a weaker 2.6-kb band (present study, data not shown).

Biophysical properties of the lymphocyte IK current have been described at the single channel (2, 5, 24) and whole-cell level (5). Whereas channel gating is independent of voltage, it is highly sensitive to intracellular free Ca^{2+}, activating at <200 nM in T and B cells, reaching half-maximal activation at about 450 nM, and maximal activation at −1 µM (2, 5). Both the single channel and whole-cell current versus voltage (I-V) relations are inwardly rectifying with symmetrical K^{+} concentrations on both sides of the membrane (5, 6, 24). The single channel I-V relation is linear under physiological Na^{+}/K^{+} gradients, which, together with the voltage-independent gating, results in a whole-cell current that is nearly linear (5, 24). Expressed hSK4/hIK1/hKCa4 currents (Refs. 7–9 and present study) have the following features in common with the lymphocyte IK current (Refs. 2, 3, 5, 7, and 24 and present study); activation by sub-micromolar free Ca^{2+}, time- and voltage-independent gating, inwardly rectified single channel I-V relations in symmetrical K^{+} (10–35 pS), and nearly linear I-Vs in physiological Na^{+}/K^{+} gradients (~10 pS).
How might both Kv1.3 and IK channels contribute to T cell proliferation? Within seconds after stimulating the T cell receptor, tyrosine-kinase mediated activation of phospholipase C produces inositol 1,4,5-trisphosphate and quickly triggers Ca$^{2+}$ release from internal stores. A plasma membrane channel (the Ca$^{2+}$ release-activated Ca$^{2+}$ channel) then opens to allow Ca$^{2+}$ influx, which is required for several hours. Ca$^{2+}$-release-activated Ca$^{2+}$ channel opening is not voltage-dependent, but Ca$^{2+}$ influx is strongly driven by the membrane potential. Thus, any means of increasing the K$^{+}$ conductance and hyperpolarizing the cell will facilitate Ca$^{2+}$ entry. Kv1.3 is voltage-gated, activated by depolarization, and its steady-state activity is maximal between −50 and −30 mV in resting T cells, depending on post-translational modulation (37). Hence it is likely to play a role only when the membrane is moderately depolarized. In contrast, gating of the IK/hSK4 channel is voltage-independent but exquisitely sensitive to internal Ca$^{2+}$; thus, it is well designed to open whenever Ca$^{2+}$ rises marginally above the resting level. Rather than Ca$^{2+}$ changing in a sustained manner after T cell receptor stimulation, Ca$^{2+}$ and membrane potential can oscillate (1, 38, 39). Collectively, these complementary properties would allow the cell alternately to use IK channels when Ca$^{2+}$ is high, even if the membrane is hyperpolarized, and Kv1.3 channels during periods of low Ca$^{2+}$ and/or depolarization.

**Role of hSK4 and Kv1.3 in Volume Regulation**

Volume regulation in leukocytes and other mammalian cells has been extensively reviewed (10–12). The RVD involves ion efflux through separate K$^{+}$ and anion channels. Its rate and extent depend on the combined ion conductances; thus if either the K$^{+}$ or Cl$^{-}$ current is small (or blocked pharmacologically) it will limit volume recovery. Identifying the particular K$^{+}$ channel(s) that underlie RVD has been problematic, largely due to the lack of selective blockers and uncertainty over whether swelling evokes a rise in intracellular Ca$^{2+}$. For instance, early evidence of a role for Kv1.3 was not convincing since it first relied on nonspecific K$^{+}$ channels, if they exist), or by directly interfering with the membrane potential since these channels require moderate depolarization to be tonically active (37, 42, 43). A simple model is that Ca$^{2+}$ signaling during T cell activation. Thus, we proposed (14) that both K$_{Ca}$ and Kv1.3 channels will contribute to RVD, with Ca$^{2+}$ and voltage oscillations alternately opening each type of K$^{+}$ channel.

Previous studies of RVD in T cells have been restricted to resting cells and show a stereotypical response (10, 11, 13); within 1–2 min after a hypotonic shock, T cells swelled to ~120% of their original volume and then returned to their original volume within 5–15 min. Our results, using right angle light scattering to measure RVD, are in excellent agreement both in the extent of swelling (~120%) and in the time course, with maximal swelling within 1–2 min and nearly full recovery within 6 min for both resting T cells and lymphoblasts. We have now assessed the relative contributions of IK and Kv1.3 channels to RVD in resting T cells compared with activated lymphoblasts. Owing to the dramatic increase in IK and small increase in Kv1.3 in lymphoblasts, we expected RVD to depend more on IK than Kv1.3 channels in lymphoblasts.

We examined the role of each channel type in the initial swelling to maximal volume and in the degree of recovery by 6 min after a 56% hypotonic shock. For resting T cells, blocking Kv1.3 or IK, or both channels simultaneously, increased the maximal volume, implying that K$^{+}$ efflux through both channels occurs even during the initial swelling phase. Consistent with this conclusion, in previous studies T cells swelled less than predicted for a passive osmometer (10–12). We had anticipated a role for IK channels in resting cells, since IK is expressed (Refs. 6 and 39 and present study), and intracellular Ca$^{2+}$ rises after a hypotonic shock (14). For lymphoblasts, IK block was very effective in increasing the maximal volume, whereas Kv1.3 block had no effect. Thus, in lymphoblasts volume regulation also proceeds during the swelling phase but IK plays a much greater role than Kv1.3 current at this time.

The extent of recovery after the maximal volume is reached reflects both the K$^{+}$ and Cl$^{-}$ conductances during the RVD phase. Substantial recovery occurred within 6 min in both cell types, and the slightly greater recovery in lymphoblasts is consistent with up-regulation of IK/hSK4 expression. RVD was significantly inhibited by blocking Kv1.3 channels in both resting T cells and lymphoblasts but was more effective in resting cells. For both cell types IK block was more effective than Kv1.3 block; moreover, inhibition of RVD was greater in lymphoblasts. Thus, both K$^{+}$ channels contribute to RVD, but their relative importance is opposite as follows: Kv1.3 plays a greater role in resting cells, and IK is more important in lymphoblasts. Not only are these results predicted from the up-regulated expression of IK/hSK4 in lymphoblasts but IK activation implies that hypotonic shock elicits an early and sustained rise in Ca$^{2+}$ in activated lymphoblasts, as we have previously shown for resting T cells (14).

Although there is insufficient information to calculate K$^{+}$ fluxes through Kv1.3 and IK channels during RVD, some predictions can be made by considering their expression and biophysical properties. Flux through each channel type is proportional to the number of channels (n), their open probability (P$_{o}$), their single channel conductance (γ), and the driving force, which is the same at a given voltage. For resting T cells, the number of Kv1.3 channels per cell is much larger than IK/hSK4 channels, and γ is similar (~10 pS in a normal Na/K gradient). So, for IK channels to play a substantial role in RVD, their P$_{o}$ must be much larger than the P$_{o}$ of Kv1.3 channels in resting cells. Kv1.3 contribution will be controlled by the membrane potential since these channels require moderate depolarization to be tonically active (37, 42, 43). A simple model is that Ca$^{2+}$ remains elevated thereby activating IK, and the membrane potential remains hyperpolarized, thereby reducing the opening of Kv1.3 channels. IK/hSK4 expression is much higher in lymphoblasts than in resting T cells, and since IK gating is voltage-independent, this channel is expected to contribute more whether or not the membrane potential fluctuates, provided Ca$^{2+}$ remains modestly elevated.

**Calmodulin-dependent Modulation, Evidence for More Than One Mechanism**

Our electrophysiological results implicate calmodulin (CaM) in regulating native IK channels in lymphoblasts. In principle, CaM antagonists could act by interfering with interactions between CaM and the channel protein, by interacting with accessory CaM-binding molecules (e.g. CaM kinases or channel β subunits, if they exist), or by directly interfering with the
channel protein. Some predictions can be made from the mechanism by which the antagonists inhibit CaM (44–46). In cell-free systems, CaM changes conformation when at least two of its four Ca\(^{2+}\)-binding domains are saturated (\(K_d \approx 2.4 \, \mu M\) Ca\(^{2+}\)). CaM antagonists can then bind reversibly to a newly exposed hydrophobic site (47) thereby preventing interactions between CaM and target proteins. Thus, it is expected that excess CaM will competitively reduce inhibition by titrating the amount of drug available for inhibition.

Direct interactions of some CaM antagonists in the pore of some K\(^+\) channels have been proposed when their potency for CaM inhibition differed from that of channel inhibition (48–50), or the drugs were effective even when Ca\(^{2+}\) was not elevated (50), or exogenously added CaM did not compete with the antagonists (49). We found that trifluoperazine and W-7 produced time- and voltage-dependent decreases in both native IK and expressed hSK4 current at positive potentials, which may reflect a direct drug interaction with the channel protein. Of greater physiological relevance is the inhibition of native IK channels we observed at negative membrane potentials. In this case the potency of inhibition by W-7 and TFP was consistent with effects on CaM, and as expected for competitive drug binding, excess internal CaM significantly relieved the inhibition by W-7. This result also rules out significant channel block by W-7 from the outside at negative potentials. CaM antagonists may affect the lymphoblast IK current through interactions between CaM or other CaM-binding molecules and the channel protein. Such interactions must differ for hSK4 channels stably expressed in CHO cells since, at negative potentials, these currents were not inhibited by TFP or W-7, and calmidazolium was less effective than on IK currents. As discussed below, differences in actions on native IK and hSK4 channels may reflect multiple sites of action.

Direct Interactions between CaM and IK/hSK4 Channels—Despite the exquisite Ca\(^{2+}\) sensitivity of IK/hSK4 gating, the primary amino acid sequence of the \(a\) subunit contains no known Ca\(^{2+}\)-binding sites, that is no E-F hands, C2 domains (51), or Ca\(^{2+}\) “bowls” (52). We found that channels made from wild-type, full-length hSK4 \(a\) subunits bind to calmodulin. Although binding was greatly inhibited when Ca\(^{2+}\) was chelated, some binding remained. Deletion mutants of several cytoplasmic regions that are relatively conserved between hSK4 and brain SK channels showed that CaM binding was restricted to the proximal C-terminal tail of hSK4 (a region we call “Ct1,” see Fig. 7). Deleting the Ct1 region prevented the expression of functional Ca\(^{2+}\)-gated hSK4 channels. When wild-type hSK4 was expressed and whole-cell membrane potential (\(V_m\)) recordings were made with micromolar intracellular Ca\(^{2+}\) to maximally activate hSK4, \(V_m\) became highly negative owing to the hyperpolarizing \(K^+\) conductance. In contrast, the Ct1-deleted channel failed to produce a hyperpolarizing \(K^+\) conductance, and \(V_m\) remained essentially at zero. There are two possibilities as follows: without CaM binding the channels did not open in response to high Ca\(^{2+}\) (as is the case for SK2 channels (15)) or the mutant channels did not assemble properly in the cell membrane. In the future it will be useful to examine the assembly and trafficking of mutant hSK4 channels, particularly since the Ct2-deletion mutant (lacking the leucine zipper region) also failed to produce a hyperpolarizing \(K^+\) conductance. The \(a\) subunits of brain SK channels (SK1–3) also bind to CaM in the proximal part of the cytoplasmic C terminus, and CaM apparently serves as the Ca\(^{2+}\)-binding gate (15). Most of the C terminus of SK2 channels (4 \(a\) helices, A–D) bound to CaM, whereas a “post-D” C-terminal tail (corresponding to our Ct2, leucine-zipper region) did not. If helices A–D were all present, binding was independent of Ca\(^{2+}\), whereas helices B and C and B–D conferred Ca\(^{2+}\)-dependent binding to CaM.

Our results on hSK4 share several features with SK2 (15), but they also differ in ways that are consistent with additional sites of interaction or modulation of the native channel in lymphocytes. Although brain SK channels have only modest overall homology (\(-40%\)) to hSK4 (7), some regions are more homologous. The 95-amino acid CaM-binding domain (Ct1) that we identified in hSK4 is the same channel region as helices A–C in SK2. Helix A in SK2 is highly homologous to the corresponding region of hSK4 (79% identical), whereas regions B and C have much lower homology (20% identical). For SK2, the Ca\(^{2+}\)-independent CaM binding and patch clamp studies in which calmidazolium failed to inhibit the expressed channels were taken as evidence that CaM binds constitutively and irreversibly to brain SK channels (15). We found that most of the CaM binding to hSK4 was Ca\(^{2+}\)-dependent; however, as explained earlier, the binding assay we used would not detect channels irreversibly bound to CaM. Several possible explanations for these differences will require further study. For instance, there may be more than one CaM-binding site with different affinities, as is the case for cation channels in retinal rods (53), with a lower affinity site that is reversible and Ca\(^{2+}\)-dependent, perhaps in Ct1 of hSK4 (helices B–D in SK2). CaM antagonists inhibited the native current in lymphoblasts, with little or no inhibition of expressed hSK4 currents, a result that is consistent with the failure of 1000 nM calmidazolium to prevent CaM binding to the expressed hSK4 protein. A further possibility is that weaker CaM channel binding in lymphoblasts, perhaps as a result of other protein-channel interactions (see below), allows more effective competition by CaM antagonists.

Evidence for Accessory Molecules in Lymphocytes—The striking differences in inhibition at negative potentials of native IK versus expressed hSK4 channels provide the first evidence that accessory molecules (other than CaM) modulate a member of the SK channel family. Candidate molecules include CaMK, calcineurin, and \(\beta\) subunits analogous to those interacting with voltage-gated K\(^+\) channels. Although \(\beta\) subunits have not been identified for SK channels, there is evidence that apamin-sensitive SK channels form hetero-oligomers. In a variety of cells expressing SK channels, apamin binds to both high (59 or 86 kDa) and low (30 or 33 kDa) molecular mass polypeptides that are integral membrane proteins (54). It is unlikely that such accessory molecules are essential for channel activity since all known members of the SK family, with the exception of rSK1 (7), are functional in expression systems, including Xenopus oocytes, HEK, and CHO cells. This observation also implies that the Ca\(^{2+}\)-binding site, which is thought to be CaM bound to the channel (15), functions normally in these cells.

Since the CaM kinase antagonist, KN-62, inhibited native IK current but had no effect on hSK4, it is necessary to consider how CaM kinase might selectively modulate the current in lymphocytes. hSK4 contains a potential phosphorylation site in the C-terminal domain that should accommodate either CaM kinase II (or protein kinase A) (45); however, this site is not necessarily phosphorylated. It may be that CHO cells have insufficient CaM kinase (T cells express high levels of CaM kinase II and IV (55)) or that the site of phosphorylation is not on the channel protein itself, but rather on a \(\beta\) subunit or other unknown accessory molecule. Interestingly, in lymphoblasts the increased inhibition by W-7 in the presence of KN-62 is consistent with dual modulation by CaM binding and CaM kinase.

Potential modulation of lymphocyte IK/hSK4 channels by CaM and CaM kinase is of broader importance. Early in T cell
activation or lymphoblast re-activation, there is a rise in intracellular Ca$^{2+}$ that activates CaM-dependent enzymes. These include CaM kinases II and IV (55) and calcineurin (protein phosphatase 2B), which is highly expressed in lymphocytes and crucial for T cell proliferation (34). CaM antagonists can inhibit some lymphocyte functions that either trigger conductive K$^+$ fluxes or are sensitive to the membrane potential of the cell (which depends on K$^+$ channels). T cell activation (56), cell-mediated cytotoxicity (57), and volume regulation (40) are inhibited both by K$^+$ channel blockers and by CaM antagonists. Our present results provide new evidence that a specific K$^+$ channel (IK/hSK4) that is important for at least two of these functions (proliferation and volume regulation) is susceptible to CaM antagonists, thus providing a link between CaM and K$^+$ channels in regulating lymphocyte function.

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REFERENCES

1. Lewis, R. S., and Cahalan, M. D. (1995) Annu. Rev. Immunol. 13, 623–653
2. Mahaut-Smith, M., and Schlichter, L. C. (1989) J. Physiol. (Lond.) 415, 69–83
3. Mahaut-Smith, M., and Schlichter, L. C. (1989) Pflugers Arch. 414, S164–S165
4. Grissmer, S., Lewis, R. S., and Cahalan, M. D. (1992) J. Gen. Physiol. 99, 63–84
5. Grissmer, S., Nguyen, A. N., and Cahalan, M. D. (1993) J. Gen. Physiol. 102, 601–620
6. Schlichter, L. C., Papapill, P., and Schumacher, P. A. (1993) Receptors Channels 1, 201–215
7. Joiner, W. J., Wang, L.-Y., Tang, M. D., and Kazmackre, L. K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11013–11018
8. Ishii, T. M., Silvia, C., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1996) Pflugers Arch. 431, 415–425
9. Hait, W. N., and Lazo, J. S. (1986) J. Clin. Oncol. 4, 353–359
10. Schumacher, P. A., Sakellaropoulos, G., Phipps, D. J., and Schlichter, L. C. (1995) J. Membr. Biol. 156, 73–85
11. Deuchts, C., and Chen, L.-Q. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10384–10400
12. Schlichter, L. C., and Sakellaropoulou, G. (1994) Exp. Cell Res. 215, 211–222
13. Xia, X.-M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 563–567
14. Chang, M. C., and Schlichter, L. C. (1996) Soc. Neurosci. Abstr. 22, 1444
15. Khanna, R., Chang, M. C., Joiner, W., Kazmackre, L. K., and Schlichter, L. C. (1996) Biophys. J. 74, 35
16. Chomzynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
17. Koo, G. C., Blake, J. T., Talento, A., Nguyen, M., Lin, S., Sirotina, A., Shah, K., Mullvany, K., Hora, D., Cunningham, P., Wunderler, D. L., McNamara, O. B., Slaughter, R., Bugianesi, E., Feltz, J., Garcia, M. J., Mulvany, G. J., and Koo, G. C. (1993) J. Exp. Med. 177, 673–674
18. Price, M., Lee, S. C., and Deutsch, C. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1017–10175
19. Crabtree, G. R., and Chipstone, N. A. (1994) Annu. Rev. Biochem. 63, 1045–1083
20. Timmerman, L. A., Chipstone, N. A., Ho, S. N., Northrup, J. P., and Crabtree, G. R. (1996) Nature 383, 837–840
21. Cheung, R. K., Grinstein, S., Gelfand, E. W. (1983) J. Immunol. 131, 529–529
22. Linse, S., Helmersson, A., and Forsen, S. (1991) J. Biol. Chem. 266, 8050–8054
23. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 31, 156–159
24. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 89, 248–251
25. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 89, 248–251
26. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 89, 248–251
27. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 89, 248–251
28. McCann, J. D., and Welsh, M. J. (1987) J. Gen. Physiol. 89, 248–251