Up-regulation of the \( \text{IKCa1} \) Potassium Channel during T-cell Activation

MOLECULAR MECHANISM AND FUNCTIONAL CONSEQUENCES*

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We used whole cell recording to evaluate functional expression of the intermediate conductance \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) channel, \( \text{IKCa1} \), in response to various mitogenic stimuli. One to two days following engagement of T-cell receptors to trigger both PKC- and \( \text{Ca}^{2+} \)-dependent events, \( \text{IKCa1} \) expression increased from an average of 8 to 300–800 channels/cell. Selective stimulation of the PKC pathway resulted in equivalent up-regulation, whereas a calcium ionophore was relatively ineffective. Enhancement in \( \text{IKCa1} \) mRNA levels paralleled the increased channel number. The genomic organization of \( \text{IKCa1} \), \( \text{SKCa2} \), and \( \text{SKCa3} \) were defined, and \( \text{IKCa} \) and \( \text{SKCa} \) genes were found to have a remarkably similar intron-exon structure. Mitogens enhanced \( \text{IKCa1} \) promoter activity proportional to the increase in \( \text{IKCa1} \) mRNA, suggesting that transcriptional mechanisms underlie channel up-regulation. Mutation of motifs for AP1 and Ikaros-2 in the promoter abolished this induction. Selective \( \text{K1.3} \) inhibitors Shk-Dap\(^{22} \), margatoxin, and correolide suppressed mitogenesis of resting T-cells but not preactivated T-cells with up-regulated \( \text{IKCa1} \) channel expression. Selectively blocking \( \text{IKCa1} \) channels with clotrimazole or TRAM-34 suppressed mitogenesis of preactivated lymphocytes, whereas resting T-cells were less sensitive. Thus, \( \text{K1.3} \) channels are essential for activation of quiescent cells, but signaling through the PKC pathway enhances expression of \( \text{IKCa1} \) channels that are required for continued proliferation.

Lymphocyte activation involves two key intracellular signaling pathways, the calcium-signaling cascade and protein kinase C (PKC)\(^1 \)-dependent events. Stimulation of either of these pathways is capable of triggering different gene transcription events, and both are required for complete lymphocyte activation. A recent gene-chip survey of T-cells stimulated with a variety of mitogens, detected the induction of hundreds of genes (1). The rise in intracellular calcium ([\( \text{Ca}^{2+} \)]\(_i\)) activates calcineurin, a phosphatase that dephosphorylates the cytoplasmic transcription factor NFAT (nuclear factor of activated T-cells), enabling it to translocate to the nucleus and bind to NFAT-response elements of several genes, including the T-cell growth factor interleukin-2 (IL-2) (2, 3). The immunosuppressive drug, cyclosporin A (CsA), blocks this pathway by interacting with calcineurin and thereby suppresses activation (3, 4). In a separate pathway, activation of PKC leads to phosphorylation of numerous substrates and results in assembly of Fos/Jun heterodimers that bind to activation protein-1 (AP1) elements on an overlapping set of genes via activation of the Ras and JNK (c-Jun N-terminal kinase) pathways. The functional significance of PKC\(_\gamma\), in particular, has been recently demonstrated (5, 6). Cross-talk between these signaling pathways integrates the activation response. For example, the JNK pathway is co-activated by increases in cytoplasmic calcium (7). Sustained [\( \text{Ca}^{2+} \)]\(_i\) signaling, mediated by calcium entry through calcium release-activated \( \text{Ca}^{2+} \) (CRAC) channels, and PKC activation are both essential for complete activation.

Two potassium channels, the voltage-gated \( \text{K}^{+} \) channel \( \text{Kt.1.3} \) and the calcium-activated \( \text{K}^{+} \) channel \( \text{IKCa1} \) (also known as \( \text{CkCN4} \), \( \text{IK1} \), \( \text{hKCa4} \), and \( \text{hSK4} \) (8–10), modulate calcium influx through CRAC channels by regulating the membrane potential and hence the driving force for calcium entry (11). Freshly isolated resting human T-cells functionally express on average –300 \( \text{Kt.1.3} \) channels (11–13) along with –10 \( \text{IKCa1} \) channels (14). During activation with phytohemagglutinin (PHA), expression of \( \text{IKCa1} \) channels is strongly enhanced, while levels of \( \text{Kt.1.3} \) exhibit a modest enhancement (13, 14). Changes in expression levels of \( \text{K}^{+} \) channels during activation have also been noted in murine T-cells (15) and in human and murine B cells (16, 17). A recent gene chip survey (1) revealed a reduction in \( \text{Kt.1.3} \) mRNA levels in activated compared to resting T-cells, suggesting that post-transcriptional mechanisms contribute to the up-regulation of this channel.

In this study, we define the pathway leading to \( \text{IKCa1} \) up-regulation using phorbol myristate acetate (PMA) to trigger PKC selectively or ionomycin to stimulate the calcium-dependent cascade, and other mitogens (anti-CD3 Ab or PHA) that

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‡‡Abbreviations used are: PKC, protein kinase C; NFAT, nuclear factor of activated T-cells; IL-2, interleukin-2; CsA, cyclosporin; AP1, activation protein-1; JNK, c-Jun N-terminal kinase; CRAC, calcium-release activated calcium; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; Ik-2, Ikaros-2; ChTX, charybdotoxin; ShK, Stichophyllum helianthus toxin; LEF, lymphoid enhancing factor; MNC, mononuclear cells; INF-\(\gamma\), interferon-\(\gamma\); NCR, non-coding region; bp, base pair(s); kb, kilobase pair(s); [\( \text{Ca}^{2+} \)]\(_i\), intracellular [\( \text{Ca}^{2+} \)]; RBL, rat basophilic leukemia.
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stimulate both pathways. By combining electrophysiological and molecular methods we show that stimulation of the PKC pathway alone is sufficient to enhance IKCa1 channel expression via transcriptional activation of the IKCa1 promoter. A reporter gene assay combined with mutational analysis defined the minimally active promoter region of the IKCa1 gene, demonstrating the importance of AP1, the PKC-dependent site of binding. Using selective K1.3 and IKCa1 inhibitors we demonstrate an important functional role of K1.3 channels in resting T-cells and IKCa1 channels in activated T-cells.

EXPERIMENTAL PROCEDURES

Reagents—CaCl2, clonitazene, econazole, ketocanazole, and tetaethylammonium chloride were from Sigma, nifedipine, nimodipine, and nitrendipine were from RBI (Natick, MA), PHA was from DIFCO (Detroit, MI), PMA was from Calbiochem (La Jolla, CA), monoclonal mouse anti-human CD3 Ab was from Biomedica (Foster City, CA), charybdotoxin (CLTX), CLTX-Glu, ShK (Stichodactyla helianthus) toxin, ShK-Dap2, and margatoxin were from BACHEM (King of Prussia, PA). TRAM-34 ([1-(2-chlorophenyldiphenyl)methyl]1H-pyrazole) was described previously (18). Correolide was a gift from Dr. Maria L. Garcia (Merck, Kirkland, WA).

Reporter Constructions—Luciferase reporter gene plasmids, pGL2-enhancer (pGL2-e) and pGL2-basic (pGL2-b), were purchased from Promega (Madison, WI). Parent luciferase constructs correspond to a 5'-flanking subfragment (−1877/+395) in pGL2-e and pGL2-b vectors in both orientations. Deletion fragments, generated by polymerase chain reaction, were engineered in both orientations into pGL2-e or pGL2-b. Ik-2 (5′-TTCTGGCTGGACCTT, AP1 (5′-GTGAGTCAC), and Ik-2/AP1 (5′-TTGCTGGGAGTT) sites in the −117/+34 fragment (Fig. 8) were mutated to the following sequences: Ik-2 mutant, ACCCGTTTTCG; AP1 mutant, TTAGGCGGGGG; and Ik-2/AP1 mutant, TTGGGCGGGGG, and Ik-2/AP1, ACGCCGAAAAAATCCACACC. The orientation and integrity of all constructs were confirmed by sequencing. The IL-2-pGL2-e construct (19) was a gift from Dr. C. Hughes (University of California, Irvine, CA).

Genomic Organization of IKCa1—1.1 × 106 plaques from a human EMFLS genomic λ library (CLONTECH, Palo Alto, CA) were screened with a human IKCa1 (accession number AF033021) coding region probe to a final stringency of 1.0 × SSC and 0.1% SDS at 65 °C for 45 min. Five clones were isolated and two of these, KCNN4-9 and KCNN4-16 (which hybridized to both the 5′ and 3′ fragments of the probe) were further characterized. Precise localization of the exon/intron boundaries was established by sequencing across the junctions in genomic DNA with primers derived from the cDNA sequence.

Northern Blot Analysis—Northern blots (CLONTECH) were hybridized to an IKCa1-specific probe in Expresshyb solution (CLONTECH), washed at a final stringency of 0.1 × SSC, 0.1% SDS for 40 min at 55 °C, and exposed to x-ray film at −80 °C with an intensifying screen for 3–5 days. The IKCa1-specific probe corresponds to residues 380–427 and includes −490 bp of 3′ noncoding sequence. Blots were stripped and re-probed with the control β-actin probe (CLONTECH). For Northern blots experiments on peripheral blood lymphocytes, poly(A) RNA was isolated from resting (2 × 106 cells) and mitogen-activated human MNC (9 × 106 cells) using the Ambion Pure mRNA Isolation kit (Ambion, Austin, TX). Cells were activated for 24 h with PHA (0.5 μg/ml) or PMA (40 nM). A Northern blot containing 2 μg of mRNA was probed and washed as described. Northern blots were scanned and the intensity of bands determined by densitometry. The IKCa1 mRNA levels were normalized against the control probe, LEF (lymphoid enhancing factor).

Primer Extension—The Primer Extension System (Promega) was used to define the transcription initiation site. Briefly, an antisense primer (5′-ATCGGCTTTTGACTCAACAATGGG-3′) located 52 bases downstream of the 5′-end of the previously reported IKCa1 cDNA (accession number AF022797) was end-labeled using T4 polynucleotide kinase. In parallel reactions, 0.4 pmol labeled primer was annealed to either 20 μg of human placental total RNA (Ambion) or 10 μg of yeast tRNA at 68 °C for 20 min and cooled for 10 min at room temperature. The annealed primer was next extended at 48 °C for 30 min in the presence of AMV Primer Extension Buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM each of four dNTPs, and 0.5 mM spermine), 3 mM sodium pyrophosphate, and 20 units of AMV reverse transcriptase. Extension products were concentrated, and loaded onto a 6% polyacrylamide gel adjacent to a sequencing reaction of genomic DNA primed with the same oligonucleotide. In vitro transcribed kanamycin RNA and the control primer (Promega) produced an extension product that served as a positive control for the reaction.

Transfection of Human Peripheral Blood T Lymphocytes—In order to transfect primary T-cells, we stimulated them with a subminiscule dose of PHA (1 μg/ml) which induces these cells to pass through a “window” of 20–35 μV caused by an aspartate-based internal solution, 340–427 nmol KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM each of four dNTPs, and 0.5 mM spermine), 3 mM sodium pyrophosphate, and 20 units of AMV reverse transcriptase. Extension products were concentrated, and loaded onto a 6% polyacrylamide gel adjacent to a sequencing reaction of genomic DNA primed with the same oligonucleotide. In vitro transcribed kanamycin RNA and the control primer (Promega) produced a sequencing reaction of genomic DNA primed with the same oligonucleotide. In vitro transcribed kanamycin RNA and the control primer (Promega) produced an extension product that served as a positive control for the reaction.

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Human MNCs were either nylon-wool purified and then activated with 5 μg/ml PHA, 40 nM PMA, 10 nM PMA + 175 nM ionomycin, or 175 nM ionomycin; or activated with 5 ng/ml anti-CD3 Ab and then nylon-wool purified directly before the experiments. The same aspartate-based pipette solution as above was used with Na<sup>+</sup>-aspartate Ringer as an external solution (mM: 160 Na<sup>+</sup>, 4.5 KCl, 1 MgCl<sub>2</sub>, 5 HEPES, pH 7.4). Voltage ramps from −120 to +40 mV over 200 ms were applied every 30 s. Kv1.3 currents in activated T lymphocytes were measured in normal Ringer with an internal pipette solution containing (in mM) 134 KF, 2 MgCl<sub>2</sub>, 10 HEPES, 10 EGTA. 200-ms depolarizing pulses to 40 mV were applied every 30 s and K<sub>v</sub> values were determined by fitting the Hill equation to the reduction of peak current.

**[3H]Thymidine Incorporation Assay:** Resting or 2-day activated (5 ng/ml anti-CD3 Ab) cells were washed 3 times, re-suspended and seeded at 2 × 10<sup>5</sup> cells/well in culture medium in flat-bottom 96-well plates (final volume 200 μl). These cells were preincubated with drug (60 min), and then stimulated with mitogen (5 ng/ml anti-CD3 Ab) for 48 h. [3H]Thymidine (1 μCi/well) was added for the last 6 h. Cells were harvested onto glass fiber filters and radioactivity measured in a scintillation counter.

**Intracellular Fluorescence Activated Cell Sorter Analysis for IL-2 and Interferon-γ (IFN-γ) N-MHCs:** Washes were done 3 times in complete RPMI medium, re-suspended at a concentration of 3 × 10<sup>6</sup> cells/ml and allowed to rest overnight in an upright costar T-75 tissue culture flask. Cells were placed in small Falcon tubes (1 × 10<sup>6</sup>/ml) and stimulated with 10 nM PMA, 10 nM PMA + 175 nM ionomycin, PMA + ionomycin + 25 nM Ca<sup>2+</sup>, or PMA + ionomycin + 1 μM TRAM-34. After 48 h stimulation, cells were treated with brefeldin A (Golgi Plug, Pharmingen BD) for 2 h to inhibit intracellular transport. Cells were pelleted at 1200 × g, vortexed, fixed, and permeabilized with Cytofix/Cytoperm solution (Pharmingen BD) and washed 2 times in Perm/Wash solution (Pharmingen BD). The cells were then stained with anti-CD4-FITC antibody, Cy5, and anti-CD8-PE antibody and then fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen BD) and washed 2 times in Perm/Wash solution (Pharmingen BD). The cells were then stained with anti-CD3-PE antibody and anti-IFN-γ-PE antibody and washed 3 times and then analyzed using a Becton Dickinson FACScan flow cytometer. The number of CD4+ T-cells (red channel) that expressed intracellular IL-2 or IFN-γ (green channel) was determined. The green/red channel compensation and gain were set using singly stained samples and isotype matched controls.

**RESULTS**

**Pharmacological Profile of the Cloned IKCa1 Channel Matches the IK<sub>Ca1</sub> Channel in Human T Cells:**—We initially compared characteristics of the cloned intermediate-conductance calcium-activated channel, IKCa1, expressed in COS or RBL cells, with native IKCa<sub>1</sub> currents in resting and activated human T lymphocytes. Cloned IKCa1 channels and native IKCa<sub>1</sub> current both exhibit a P<sub>H</sub>/P<sub>K</sub> permeability ratio of 1.2 (Fig. 1A) and are blocked in a voltage-dependent manner by 10 mM Ba<sup>2+</sup> (Fig. 1B) and 16 mM Ca<sup>2+</sup> (data not shown) (14). In Fig. 1C we show that the cloned IKCa1 channel is also blocked by peptides ChTX and ShK, and by a ChTX analog, ChTX-Glu<sub>32</sub>, designed to target the IKCa1 channel specifically (25). Several structurally diverse small molecules also block the cloned channel (Fig. 1C), including clotrimazole, TRAM-34, nifedipine, nimodipine, nifedipine, econazolone, ketoconazolone, and tetraethylammonium chloride, with potencies similar to that of the endogenous channel in T-cells. The close similarity in ion selectivity and pharmacological characteristics, here using 11 channel blockers spanning 7 log units of potency, strongly suggests that the native channel is a homotetramer of IKCa1 subunits, in agreement with previous reports (10, 14, 22, 24).

**Mitogen-induced Up-regulation of IKCa1 Channels in Human T-Cells:**—To determine the effect of mitogen stimulation on IKCa1 expression, whole cell patch clamp measurements were performed on lymphocytes pre-stimulated to activate either the calcium signaling cascade, PKC-dependent events, or both. As an example, Fig. 2 illustrates up-regulation of IKCa1 currents in T-cells pre-stimulated through the T-cell receptor by the anti-CD3 Ab to trigger both calcium signaling and PKC. Two components of K<sup>+</sup> current can be observed during voltage ramps in T-cells dialyzed with 1 μM free Ca<sup>2+</sup> in the pipette. At potentials more negative than −40 mV, IKCa1 currents are induced rapidly upon break-in to achieve whole cell dialysis with 1 μM free Ca<sup>2+</sup> in the pipette, as illustrated by changes in slope conductance with a reversal potential of −80 mV (Fig. 2A). At depolarized potentials, K<sup>+</sup> currents are carried by a combination of IKCa1 and Kv1.3 channels. With 50 nM Ca<sup>2+</sup> in the pipette, only Kv1.3 currents are observed (Fig. 2B). Clotrimazole selectively blocks the IKCa1 current, while ShK-Dap<sup>22</sup> (25) selectively blocks the residual Kv1.3 current (Fig.

**FIG. 1. Expression of the IKCa1 channel in mammalian cells. A, selectivity sequence of monovalent cations for the IKCa1 channel expressed in RBL cells. B, Ba<sup>2+</sup> block of IKCa1. IKCa1 channels were activated as in A and ramp currents recorded with the bath solution changed from K<sup>+</sup>-Ringer to a K<sup>+</sup>-Ringer solution containing 10 mM Ba<sup>2+</sup>. C, dose-dependent block of IKCa1 current in RBL or COS-7 cells by inhibitors: ChTX (Δ, K<sub>v</sub> = 3 ± 2 nM), TRAM-34 (△, K<sub>v</sub> = 20 ± 3 nM; COS-7), ShK toxin (■, K<sub>v</sub> = 30 ± 7 nM; RBL), ChTX-Glu<sub>32</sub> (Δ, K<sub>v</sub> = 33 ± 8 nM), clotrimazole (○, K<sub>v</sub> = 70 ± 10 nM; COS-7), nifedipine (▼, K<sub>v</sub> = 0.9 ± 0.1 μM; COS-7), nimodipine (▲, K<sub>v</sub> = 4 ± 0.3 μM; COS-7), econazolone (●, K<sub>v</sub> = 12 ± 1 μM; COS-7), ketoconazolone (×, K<sub>v</sub> = 30 ± 4 μM; COS-7), and tetraethylammonium chloride (□, K<sub>v</sub> = 24 mM; RBL). IKCa1 currents were activated as in A and ramp currents were elicited every 10 s in normal Ringer solution and then in the presence of varying amounts of each blocker. K<sub>v</sub> values for each blocker (n = 3, mean ± S.D.) were determined from the reduction of slope conductance at −80 mV.
pharmacologically confirming the channels' identity in resting and activated T-cells. In cells activated with anti-CD3 Ab for 2 days, the increased slope conductance near 280 mV indicates a dramatic enhancement in \( \text{IKCa1} \) conductance, compared with resting cells (Fig. 2D). A similar enhancement of \( \text{IKCa1} \) current is observed in cells pretreated with PMA for 48 h (Figs. 2, E and F). \( \text{Kv1.3} \) currents are also enhanced in both anti-CD3 Ab- and PMA-activated cells (Fig. 2, D and F) in agreement with previous results (13, 14). Acute treatment of resting T-cells with PMA (1–4 h) did not augment \( \text{IKCa1} \) conductance (0.022 ± 0.029 nS; 0.009 ± 0.009 nS/pF; mean ± S.D.) compared with resting T-cells (Fig. 3), suggesting that the enhanced \( \text{IKCa1} \) conductance is most likely due to an increase in channel number induced by the activation stimulus, rather than modulation of existing channels.

Fig. 3 summarizes experiments with a variety of stimuli, assaying the expression of \( \text{IKCa1} \) channels. The number of channels per cell was computed by dividing the whole cell conductance by the measured single-channel conductance of 11 pS (14). Resting T-cells have an average \( \text{IKCa1} \) conductance of ~0.1 nS, corresponding to an average of 8 channels/cell. As described previously (14), the mitogenic lectin PHA augments \( \text{IKCa1} \) expression dramatically (second and third columns). The average \( \text{IKCa1} \) conductance 24 h after PHA stimulation is 0.37 nS (34 channels/cell), representing a 4-fold increase that is statistically significant (\( p = 0.006 \)). By day 2, the \( \text{IKCa1} \) conductance increases substantially to ~5.7 nS, corresponding to 520 channels/cell (a 65-fold increase). In comparison, following stimulation with anti-CD3 Ab, the conductance increases more rapidly, to ~1.25 nS (113 channels/cell) on day 1 and to ~5.69 nS (516 channels/cell) on day 2 (fourth and fifth columns, Fig. 3). Since lymphocytes enlarge during activation, we measured membrane capacitance to determine each cell's surface area and surface density of \( \text{IKCa1} \) channels. The surface area of T-cells increases 3-fold following PHA or anti-CD3 Ab stimulation (Fig. 3). When normalized for membrane capacitance, the normalized \( \text{IKCa1} \) conductance in resting cells is 0.05 nS/pF, representing a very low channel density of 0.04 channels/
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The selective enhancement of IKCa1 induced up-regulation of membrane capacitance (Fig. 3). Two days after activation with ionomycin, the average number of IKCa1 channels increases 10-fold to 135 channels/cell; normalized conductance 0.40 nS/pF; column 10, Fig. 3), but not to the level in resting cells. This concentration of CsA suppresses mitogen-induced [3H]thymidine incorporation (Fig. 3) and intracellular expression of the cytokines IL-2 (quiescent cells: 10% IFN-γ; 2 day PMA + ionomycin-treated cells: 47% IL-2; PMA + ionomycin + CsA-treated cells: 21% IL-2γ) and IFN-γ (resting: 10% IFN-γ; 2 day PMA + ionomycin: 63% IFN-γ; 2 day PMA + ionomycin + CsA: 29% IFN-γ). Collectively, these data indicate that IKCa1 channel up-regulation is mediated primarily through the PKC pathway, with calcium signaling events potentiating the PMA-induced channel up-regulation.

New Synthesis Contributes to the Up-regulation of IKCa1 in Mitogen-activated Lymphocytes—Mitogen up-regulation of IKCa1 might be a consequence of new synthesis of the IKCa1 mRNA and/or protein, or due to the recruitment and activation of pre-existing IKCa1 molecules in the cell. In earlier studies, IKCa1 mRNAs were isolated from Northern blot analysis or RNase protection were found to be increased 10-fold 24 h after activation with PMA (10, 26), suggesting that new synthesis of IKCa1 proteins may underlie the up-regulation of functional

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FIG. 3. Increased IKCa1 conductance following T-cell activation. IKCa1 conductance on days 1 and 2 in cells stimulated with anti-CD3 Ab (5 ng/ml), PHA (5 µg/ml), PMA (40 nM), or ionomycin (175 nM), or a combination of PMA and ionomycin compared with the IKCa1 conductance in quiescent cells. Also shown is the effect of 100 nM CsA on IKCa1 up-regulation by PMA and ionomycin. The mean ± S.D. for each stimulus (n = 24). 1-day PMA-activated cells (■, n = 19), 1-day anti-CD3 antibody-stimulated cells (+, n = 19).

FIG. 4. IKCa1 conductance versus membrane capacitance. Resting cells (■, n = 24), 1-day PMA-activated cells (■, n = 19), 1-day anti-CD3 antibody-stimulated cells (+, n = 19).
IKCa1 channels. To investigate this issue in more detail, we examined the distribution of IKCa1 mRNAs in six different human lymphoid tissues and discovered three IKCa1 mRNA species (2.2, 2.5, 4.5 kb). IKCa1 mRNAs are expressed abundantly in the spleen, lymph node, bone marrow, and fetal liver, while the thymus and peripheral blood leukocytes have lower levels (Fig. 5A). The 2.2-kb mRNA is the major band in the spleen, whereas the 4.5-kb transcript predominates in lymph nodes. Bone marrow and fetal liver, tissues containing immature hematopoietic cells, express roughly equivalent levels of the 2.2- and 2.5-kb mRNAs and very little of the large transcript. All three transcripts are expressed at roughly equivalent levels in the 2.2- and 2.5-kb mRNAs and very little of the large transcript. The larger 4.5-kb transcript is detected in some tissues. Analysis of other human tissues reveals abundant expression of the 2.2-kb transcript in placenta and smaller amounts in lung and pancreas. Human heart, brain, liver, and skeletal muscle do not exhibit this transcript in any appreciable amount (Fig. 5B). The larger 4.5-kb IKCa1 transcript is detected in some tissues. Several transformed cell lines also express IKCa1 transcripts (Fig. 5C). Thus, IKCa1 has a wide tissue distribution.

In keeping with earlier reports (10, 26), IKCa1 transcripts are usually undetectable in resting peripheral blood leukocytes, while cells stimulated with PHA for 48 h enhance expression of the 2.2-kb IKCa1 mRNA (Fig. 5D). Although equal amounts of mRNA (2 μg/lane) were loaded in both lanes, as an additional control, the blot was probed with LEF, a T-cell specific transcription factor that is not significantly up-regulated following T-cell activation (27). Since we observed a ~3-fold increase in the LEF signal by densitometric scanning in activated versus resting cells (Fig. 5D, bottom), we normalized the LEF signal to be the same in both lanes and obtained a corrected estimate of the IKCa1 mRNA levels. PHA activation for 48 h augments IKCa1 mRNA levels ~10-fold compared with resting cells. In separate experiments, cells stimulated with PHA for 24 h had ~4-fold more IKCa1 mRNAs than resting cells, while PMA enhanced IKCa1 expression ~18-fold (data not shown). These results, in combination with earlier published data (10, 26), indicate that new synthesis of IKCa1 transcripts is a consequence of enhanced transcription of the gene and/or mRNA stability. The presence of ATTTA motifs in 3′ non-coding regions (NCR) destabilize many transcripts, including those of T-cell cytokine genes (28, 29) and the potassium channel Kv1.4 (30), and their removal enhances mRNA stability. The 3′ NCR of IKCa1 lacks ATTTA motifs indicating that this mechanism does not underlie the mitogen-stimulated increase in IKCa1 mRNA expression. If transcriptional mechanisms are responsible for the up-regulation, both mitogens might be expected to enhance IKCa1 promoter activity to roughly the same extent as the increase in IKCa1 mRNAs and currents. To address this possibility, we determined the genomic organization of the major 2.2-kb IKCa1 transcript (the mRNA that is increased during T-cell activation) and characterized the promoter. Since the known IKCa1 cDNAs (AF033021 and AF022797) are...
The transcriptional start site lies three nucleotides upstream of this tissue primarily expresses the 2.2-kb transcript (Fig. 5B). We used mRNA from the placenta for this purpose since this tissue primarily expresses the 2.2-kb transcript (Fig. 5B). The transcriptional start site lies three nucleotides upstream of the first nucleotide in the published cDNA sequences. An identical start site was found (data not shown) using mRNA from MOLT-4 and HL-60 cells that predominantly express the 2.2-kb mRNA (Fig. 5C). From the transcription start site to the polyadenylation signal the IKCa1 mRNA is 2229 bp long and is composed of 399 bp of 5' non-coding sequence, 1284 bp of coding region, and 546 bp of 3' NCR.

We next screened a human genomic λ library with a human IKCa1-specific probe and isolated two overlapping genomic clones. Analysis of these clones shows that IKCa1 is encoded by nine exons (Fig. 6). We also determined the genomic organization of the related human small conductance calcium-activated K⁺ channels, SKCa2/KCNN2 and SKCa3/KCNN3, by BLAST analysis and sequence alignments of known cDNAs with genomic contigs (Fig. 6). The intron-exon structure of SKCa2/KCNN2 was ascertained by comparing the sequences of the chromosome 5 contigs, AC021415 and AC0121085 with the rat cDNA U69882, while the genomic organization of SKCa3/KCNN3 was discerned by the sequence alignment of human cDNA AY767647 and AA731772 (31). The chromosomal locations of IKCa1 (19q13.2) (32), SKCa1 (19p13.1) (31), SKCa2 (5q23.1–23.2), personal communication Dr. Jan-Fang Cheng; Lawrence Berkeley laboratory human Genome Sequencing Center), and SKCa3 (1q21) (53) are shown to the right of each figure. The additional exon in SKCa1 encoding the sequence AQK is also shown. The exonic sequences have been submitted to GenBank (accession number AF305731-AF305735 and AH009923).

2226-bp long, roughly the length of the 2.2-kb transcript, the transcription start site for this message must lie at or close to the beginning of the known cDNA sequence. To test this idea, primers close to the 5' end of the cDNA were used in primer extension assays to map the IKCa1 transcription start site (Fig. 5E). We used mRNA from the placenta for this purpose since this tissue primarily expresses the 2.2-kb transcript (Fig. 5B). The transcriptional start site lies three nucleotides upstream of the first nucleotide in the published cDNA sequences. An identical start site was found (data not shown) using mRNA from MOLT-4 and HL-60 cells that predominantly express the 2.2-kb mRNA (Fig. 5C). From the transcription start site to the polyadenylation signal the IKCa1 mRNA is 2229 bp long and is composed of 399 bp of 5' non-coding sequence, 1284 bp of coding region, and 546 bp of 3' NCR.

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Transcriptional Up-regulation of IKCa1 Channels in Activated T Cells

IKCa1 mRNA expression, and IKCa1 promoter activity by both mitogens strongly suggest that transcriptional mechanisms contribute to the channel up-regulation that accompanies T-cell activation.

We next analyzed AP1 and Ik-2 mutants to determine whether they are required for mitogen-dependent up-regulation. As shown in Fig. 10C, the AP1 mutant exhibits substantially diminished PMA responsiveness relative to the wild-type fragment (~117/~34) and to its activity in resting T-cells. Although the Ik-2 mutant is less effective in reducing PMA inducibility of the promoter than the AP1 mutant, a double AP1/Ik-2 knockout decreases PMA responsiveness to a greater extent than either mutant alone. These results suggest that AP1, and to a lesser extent Ik-2, is essential for PMA inducibility of the IKCa1 promoter. Either mutant alone attenuates the PHA-stimulated enhancement of promoter responsiveness to PMA (Fig. 10C), indicating that the AP1 and Ik-2 sites are required for this effect. Thus, AP1 and Ik-2-dependent transcriptional mechanisms contribute to the IKCa1 up-regulation during human T-cell activation.

Since the putative AP1 site is critical for IKCa1 promoter activity, we examined whether this site could bind AP1 protein. HeLa cell extracts (Promega, Madison, WI), previously characterized for AP1 binding, interact with a 32P-labeled commercially available AP1 oligonucleotide probe in gel-shift assays (Fig. 11, lane 2). This binding is competed by 100-fold excess unlabeled AP1 probe (lane 3) and by a 24-bp IKCa1 probe spanning the AP1 site (lane 4), but not by an IKCa1 probe in which the AP1 site is mutated (lane 5). HeLa cell extracts also bind to the IKCa1 AP1 site (lane 7), but not to the mutated site (lane 11). This interaction is specific since it can be competed by 100-fold excess of the AP1 probe (lane 8) and by the IKCa1/AP1 wild-type probe (lane 9), but not by the IKCa1/AP1 mutant probe (lane 10).

CsA (100 nM) partially suppresses mitogen-induced up-regulation of IKCa1 currents (Fig. 3). Is this suppression due to direct inhibition of the IKCa1 promoter activity via a NFAT-dependent step? Two NFAT consensus motifs are present in the 5'-flanking region of IKCa1 (Fig. 8). However, simultaneous deletion of both these NFAT motifs does not diminish basal promoter activity (e.g. −300/+34 sense fragment) or the promoter’s responsiveness to mitogens (Figs. 9 and 10). To test the effect of CsA on IKCa1 promoter activity more directly, human T-cells were transfected with the IKCa1 promoter constructs, then activated with PMA in the presence or absence of CsA (100 nM) for 24 h, and luciferase activity measured. As a control, cells were transfected with the CsA-sensitive IL-2-promoter and subjected to the same activation protocol. CsA does not suppress mitogen induction of the IKCa1 promoter (mitogen, 7,738 ± 1008 light units; mitogen + CsA, 6,722 ± 406 light units) while potently inhibiting mitogen-stimulated up-regulation of the IL-2 promoter (mitogen, 45,061 ± 7,180 light units; mitogen + CsA, 2728 ± 302 light units). These results suggest that the observed partial suppression of mitogen-induced channel up-regulation by 100 nM CsA (Fig. 3) is not mediated by direct inhibition of the IKCa1 promoter, and may involve a post-transcriptional mechanism.

Kv1.3 Blockers Suppress Mitogen-stimulated [3H]Thymidine Incorporation by Human Lymphocytes, whereas IKCa1 Blockers Suppress Mitogen-stimulated [3H]Thymidine Incorporation by Pre-activated Cells—To examine the relative functional roles of Kv1.3 and IKCa1 channels in resting and activated lymphocytes, we compared the effects of potent and selective Kv1.3 and IKCa1 inhibitors of anti-CD3 Ab-induced [3H]thymidine incorporation. ShK-Dap22, margatoxin, and correolide are potent inhibitors of the Kv1.3 channel (Fig. 12A), while TRAM-34 and
clotrimazole block the \textit{IKCa1} channel at low nanomolar concentrations (Fig. 12B). In confirmation of several published studies (12, 25, 36–41), all three \textit{Kv1.3} blockers, at concentrations that block the channel (Fig. 12A), potently suppress \[^{3}H\]thymidine incorporation in freshly isolated T-cells stimulated for 48 h with anti-CD3 Ab (Fig. 12C). On the contrary, blockade of \textit{IKCa1} suppresses T-cell proliferation (Fig. 12C) only at concentrations (IC\(_{50}\) = 3–5 \(\mu\)M) that are \(\sim\)70–250 times the dose required for 50% block of the channel, possibly via nonspecific mechanisms (18, 26, 42). Thus, resting T-cells containing \(\sim\)300 \textit{Kv1.3} channels and \(\sim\)8 \textit{IKCa1} channels are dependent on \textit{Kv1.3} and not \textit{IKCa1} for activation.
The situation is reversed in mitogen-activated lymphocytes that express 300–800 IKCa1 channels along with 400–500 Kv1.3 channels (Refs. 10, 13, 14, 18, and 26, this paper). Cells were preactivated with anti-CD3 Ab for 48 h (to up-regulate IKCa1) and then reactivated for a further 48 h with the same mitogen in the presence or absence of channel blockers. Both IKCa1 inhibitors suppress [3H]thymidine incorporation at concentrations (IC50 = ~300 nM) that block 80–90% of the IKCa1 channels, whereas Kv1.3 inhibitors are ineffective under these circumstances (compare Fig. 12, A and B with D). At a concentration (1 μM) that suppresses proliferation of preactivated lymphocytes, TRAM-34 also significantly inhibits the intracellular expression of IL-2 (resting: 1.8 ± 0.7% IL-2+ cells; mean ± S.D. from 3 donors; PMA + ionomycin: 23.1 ± 16.6% IL-2+ cells; PMA + ionomycin + TRAM-34: 7.9 ± 2.9% IL-2+ cells) and IFN-γ (resting: 1.1 ± 0.9% IFN-γ+ cells, mean ± S.D. from 3 donors; PMA + ionomycin: 17.6 ± 7.1% IFN-γ+ cells; PMA + ionomycin + TRAM-34: 9.5 ± 5.1% IFN-γ+ cells). These results suggest that activated cells require IKCa1 but not Kv1.3 channels for the re-activation response.

**DISCUSSION**

To investigate the molecular mechanism of IKCa1 channel up-regulation in T lymphocytes, we determined the genomic organizations of IKCa1, SKCa2, and SKCa3, and functionally mapped the promoter of IKCa1. The striking similarity in intron-exon boundaries suggests a common evolutionary origin of IKCa1 and SKCa1–3 genes. IKCa1 functional expression is enhanced by treatment with PHA, anti-CD3 Ab, PMA, or PMA + ionomycin (Fig. 3). This increase is in direct proportion to the increase in IKCa1 transcripts (Fig. 5) and to the enhanced activity of the IKCa1 promoter. The PMA-triggered IKCa1 up-regulation is an early event in the T-cell activation cascade. Enhanced IKCa1 promoter activity is detected as early as 3 h after activation (Fig. 10), and augmented channel expression is observed prior to increase in cell size, onset of DNA synthesis, or cytokine production (Figs. 3 and 4). Thus, transcriptional mechanisms are likely to underlie the increased IKCa1 expression in activated lymphocytes, although post-transcriptional mechanisms (including increased channel trafficking) may also contribute. Within the promoter region of the IKCa1 gene, several potential transcription factor-binding sites were identified and functionally probed by deletion and mutational analysis. Mutagenesis and gel-shift studies suggest that the AP1 and IκB-2 transcription factors, but not NFAT, are required for basal transcription of the IKCa1 gene and mediate the transcriptional augmentation of IKCa1 expression during the T-cell activation response. These results may be relevant to B lymphocytes (16) and T-cell subsets (43) in which levels of IKCa1 channels are up-regulated during mitogenesis. Recent studies have reported Ras-induced up-regulation of IKCa1 in fibro-
blasts (44, 45), which may be mediated via the AP1-dependent pathway described below in human T lymphocytes (Fig. 13).

Fig. 13 summarizes the signaling pathways that likely contribute to \( \text{IKCa1} \) up-regulation in T lymphocytes. Anti-CD3 Ab or PMA augment \( \text{IKCa1} \) transcription in an AP1-dependent manner via stimulation of the PKC and downstream Ras and JNK pathways. The resulting AP1 (c-Fos/c-Jun heterodimer) complex binds to the \( \text{IKCa1} \) promoter (as shown in Fig. 11) and initiates transcription of the \( \text{IKCa1} \) message in conjunction with the transcription factor, Ik-2. Ik-2 is a nuclear factor that sets a threshold for T-cell mitogenesis; in activated T-cells, Ik-2 co-localizes with the DNA replication machinery and modulates cell entry into the S-phase (46). Increased \( \text{IKCa1} \) mRNA levels lead to enhanced expression of functional \( \text{IKCa1} \) channels on the cell membrane tightly complexed to calmodulin, which serves as the calcium sensor for these channels (22). Interestingly, calmodulin expression is also augmented during human T-cell activation, especially the CAM-III mRNA and protein (47). CsA partially suppresses the mitogen-stimulated increase in \( \text{IKCa1} \) expression (Fig. 3), but this is not due to inhibition of the \( \text{IKCa1} \) promoter, and may instead result from blockade of a post-transcriptional step. Ionomycin by itself fails to increase \( \text{IKCa1} \) expression significantly, most likely due to its inability to stimulate AP1 production, whereas its enhancement of PMA-induced up-regulation of \( \text{IKCa1} \) (Figs. 2, 3, and 12) may be due to co-activation of the JNK pathway via an increase in cytoplasmic calcium (Fig. 13). The up-regulation of \( \text{IKCa1} \) channels during human T-cell activation parallels the recently described 10-fold increase in numbers of the CRAC channels induced by PHA and PMA, but not ionomycin (48), raising the possibility that these two channels involved in calcium signaling could be coordinately regulated.

Calcium-entry through CRAC channels is promoted by membrane hyperpolarization due to the opening of \( \text{IKCa1} \) and \( \text{Kr} \) channels (11). Since quiescent human T lymphocytes contain on average roughly \(-300–400 \text{Kr} \) channels/cell and only \(-8\)

**Fig. 12. Suppression of T-cell proliferation by K+ channel blockers.** A, dose-dependent inhibition of \( \text{Kv1.3} \) currents in activated human T-cells by ShK-Dap 22 (\( \bullet \), \( K_d = 52 \pm 10 \text{ pM} \)), margatoxin (\( \bullet \), \( K_d = 110 \pm 16 \text{ pM} \)), and correolide (\( \blacktriangle \), \( K_d = 90 \pm 15 \text{ nM} \)). B, dose-dependent inhibition of \( \text{IKCa1} \) in activated T-cells by TRAM-34 (\( \blacktriangle \), \( K_d = 25 \pm 5 \text{ nM} \)) and in COS-7 cells by clotrimazole (\( \square \), \( K_d = 70 \pm 10 \text{ nM} \)). C, [\( ^{3} \text{H} \)]thymidine incorporation into T-cells activated with anti-CD3 Ab (5 ng/ml) for 48 h in the presence or absence of \( \text{Kr} \) blockers (solid lines) (margatoxin (\( \bullet \), \( EC_{50} = 300 \pm 42 \text{ pM} \)), ShK-Dap 22 (\( \bullet \), \( EC_{50} = 4.0 \pm 0.5 \text{ nM} \)), correolide (\( \blacktriangle \), \( EC_{50} = 400 \pm 72 \text{ nM} \)) or \( \text{IKCa1} \) blockers (dashed lines) (clotrimazole (\( \square \), \( EC_{50} = 3 \pm 0.5 \text{ pM} \)) or TRAM-34 (\( \blacktriangle \), \( 5.5 \pm 1 \text{ pM} \))). Four donors were used for these studies. Means ± S.D. are shown. Our results with margatoxin and correolide are consistent with those previously reported by Koo et al. (41) for human T-cells (margatoxin: \( EC_{50} = 290 \text{ pM} \); correolide: \( EC_{50} = 307 \text{ nM} \)). D, [\( ^{3} \text{H} \)]thymidine incorporation into anti-CD3 Ab (5 ng/ml) preactivated T-cells that were reactivated with anti-CD3 Ab for a further 48 h in the presence or absence of channel blockers. The \( EC_{50} \) values for \( \text{IKCa1} \) blockers are 250 ± 40 nM for TRAM-34 (\( \blacktriangle \)) and 320 ± 60 nM for clotrimazole (\( \square \)) (mean ± S.D. from six donors). \( \text{Kv1.3} \) blockers produced little or no suppression.
IKCa1 channels, the membrane potential of quiescent cells is thought to be mainly dependent on the voltage-gated channel with the IKCa1 channels playing a minimal role (11). In keeping with this idea, blockade of Kv1.3 by specific and potent inhibitors attenuates the calcium signaling response and suppresses the activation response of resting human T-cells both in vitro and in vivo (25, 38, 39, 41) (Fig. 12C). In contrast, clotrimazole and TRAM-34, both potent IKCa1 channel blockers, suppress the activation of resting human T-cells (Fig. 12, B and C) (18, 26, 42) only at concentrations (~5 μM) that are 70–250 times the channel-blocking dose, perhaps through nonspecific mechanisms.

The relative numbers of the two K⁺ channels change in activated human T-cells. T-cells stimulated for 48–72 h with mitogens have 300–800 IKCa1 channels along with 400–500 Kv1.3 channels (Figs. 2 and 3) (13, 14). Khanna and colleagues (26), reported that PHA-induced proliferation of PHA preactivated T-cells was potently suppressed by clotrimazole tested at a single dose of 250 nM. We have extended these studies by using a complete range of concentrations of clotrimazole and TRAM-34 and showing that these inhibitors potently suppress reactivation of anti-CD3 Ab- or PMA preactivated lymphocytes at submicromolar concentrations consistent with their channel-blocking dose (Fig. 12, B and D (18)). Our results taken together with earlier studies (10, 14, 26) suggest that different mitogens (PHA, anti-CD3 Ab, PMA, PMA + ionomycin) augment IKCa1 channel expression in lymphocytes and this induction is functionally important. The parallel enhancement of IKCa1 and CRAC channels might allow the activated T-cell to fine-tune its regulation of membrane potential in response to subtle changes in cytoplasmic calcium, which in turn would modulate calcium entry. Consistent with this notion, previous studies on activated T-cells have shown coupling between IKCa1 channels and membrane potential (49, 50). More recently, we have found that the IKCa1 peptide inhibitor, ChTX-Glu³² (23), suppresses thapsigargin-induced calcium entry into activated human T-cells (51). Thus, the concerted action of the two potassium channels regulates the entry of calcium through CRAC channels in quiescent and activated T-cells and thereby modulates the immune response.

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