Communication

A Novel Gene, \textit{hKCa4}, Encodes the Calcium-activated Potassium Channel in Human T Lymphocytes

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We have isolated a novel gene, \textit{hKCa4}, encoding an intermediate conductance, calcium-activated potassium channel from a human lymph node library. The translated protein comprises 427 amino acids, has six transmembrane segments, S1–S6, and a pore motif between S5 and S6. \textit{hKCa4} shares 41–42% similarity at the amino acid level with three small conductance calcium-activated potassium channels cloned from brain. Northern blot analysis of primary human T lymphocytes reveals a 2.2-kilobase transcript that is highly up-regulated in activated compared with resting cells, concomitant with an increase in KCa current. \textit{hKCa4} transcript is also detected by Northern blots or by polymerase chain reaction in placenta, prostate, thymus, spleen, colon, and many cell lines of hematopoietic origin. Patch-clamp recordings of \textit{hKCa4}-transfected HEK 293 cells reveal a large voltage-independent, inwardly rectifying potassium current that is blocked by externally applied tetraethylammonium (K\textsubscript{e} = 30 ± 7 mM), charybdotoxin (K\textsubscript{e} = 10 ± 1 mM), and clotrimazole (K\textsubscript{e} = 387 ± 34 mM), but is resistant to apamin, iberiotoxin, kalitoxin, scyllatoxin (K\textsubscript{e} > 1 μM), and margatoxin (K\textsubscript{e} > 100 mM). Single \textit{hKCa4} channels have a conductance of 33 ± 2 picosiemens in symmetrical potassium solutions. The channel is activated by intracellular calcium (K\textsubscript{i} = 270 ± 8 mM) with a highly cooperative interaction of approximately three calcium ions per channel. These properties of the cloned channel are very similar to those reported for the native KCa channel in activated human T lymphocytes, indicating that \textit{hKCa4} encodes this channel type.

Potassium channels play a critical role in modulating calcium signaling of lymphocytes (1). Human T lymphocytes express at least two types of potassium channels (2); those that open in response to changes in membrane potential (Kv channels)\(^*\) and those that are activated following elevations of intracellular calcium levels (KCa channels). The predominant Kv channel in human T cells is encoded by \textit{Kv1.3}, a Shaker-related voltage-gated potassium channel gene. Kv1.3 has been characterized extensively at the molecular and physiological level and plays a vital role in controlling T cell proliferation, mainly by maintaining the resting membrane potential of the cell (3). Upon T cell activation, there is at most a 2-fold increase in Kv1.3 currents. The predominant KCa channel on human T lymphocytes is of intermediate conductance, is voltage-insensitive, and is potently blocked by the scorpion venom peptide, charybdotoxin (CTX; Ref. 4). Unlike Kv1.3, KCa currents are up-regulated dramatically (10–25-fold) following mitogenic or antigenic stimulation and are thought to play a significant role in post-activation and secondary immune phenomena (4, 5). KCa channels with biophysical and pharmacological properties very similar to the T lymphocyte channel also have been identified in red blood cells (Gardos channel; Ref. 6), macrophages (7), neutrophils (8), and B lymphocytes (9), as well as in other peripheral tissues. However, the molecular identity of this channel type was hitherto unknown. We report the cloning and characterization of an intermediate conductance, CTX-sensitive KCa channel, which we call \textit{hKCa4}, from a human lymph node cDNA library. We present convergent molecular, biophysical, and pharmacological evidence that \textit{hKCa4} encodes the predominant KCa channel in human T cells.

EXPERIMENTAL PROCEDURES

Data Base Search—We performed a BLAST search of a proprietary EST data base (licensed from Incyte Pharmaceuticals, Palo Alto, CA) for unannotated potassium channel sequences using the pore sequence of \textit{hKv2.1}, a Shab-related Kv channel (PASFWBATTMTTVYGDIYP; Ref. 10). Two overlapping clones of interest were identified, and their sequences were determined (Applied Biosystems PRISM®377 automated sequencer). Both of these clones were from a cDNA library of adherent mononuclear cells, which came from a pool of male and female donors.

Library Screening and Computer Analysis—A \(^{32}\)P-labeled DNA fragment from one of the above clones, corresponding to nucleotides 262–1265 in Fig. 1A, was used as a probe to screen ~600,000 recombinant plagues from a human lymph node \(\beta\)-gal10 cDNA library (CLONTECH). Hybridizations were at 42 °C overnight. Filters were washed twice in 1 × SSC (150 mM NaCl, 15 mM Na\textsubscript{2} citrate, pH 7.0) and 0.5% SDS at 65 °C for 1 h and exposed to x-ray film overnight. Of 38 doubly positive clones, 10 were selected to two rounds of plaque purification and rescreening. Inserts were amplified using \(\lambda\)-specific primers, and amplicons were sequenced directly by automated sequencing as above. Six clones had \textit{hKCa4} sequence information, and three were full length. One full-length clone was subcloned and sequenced entirely on both strands and used for subsequent expression constructs.

Computer analysis of the \textit{hKCa4} sequence was done using Lasergene software (DNaster, Inc., Madison, WI). Alignments with other potassium channel sequences were performed using the CLUSTAL algorithm, and these were used to create a dendrogram. The gap penalty and the gap length penalty were 10 each. Hydrophathy plots were according to Kyte-Doolittle criteria, averaging over a nine-residue window. Post-translational modification sites were identified using pattern searches within the Protean program. Patterns were derived from the Protis data base, and the threshold for matching was 100%.

Isolation and Activation of Human T Lymphocytes—Mononuclear cells were isolated from whole blood (obtained from healthy donors) on Ficoll-Hypaque density gradients. Contaminating red blood cells were removed by hypotonic lysis for 45 s in 0.2% saline followed by an equal volume of 1.6% saline to bring saline back to physiological concentration. Monocytes and B cells were removed using M450 Pan-B (CD19) green fluorescent protein; S, siemens.

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and M450 (CD14) Dynabeads according to the Dynal protocol (Lake Success, NY). Remaining cells were assumed to be mainly T cells (typically 80–90% as assessed by fluorescence-activated cell sorter analysis). T cells were cultured overnight at 37 °C, 5% CO₂ in complete RPMI 1640 medium containing 10% fetal bovine serum and 1 × penicillin/streptomycin (Life Technologies, Inc.). The cells then were activated with 10 μg/ml final concentration phytohemagglutinin (Sigma) in complete RPMI for 48–72 h.

RNA Isolation and Northern Blot Analysis—T cell poly(A)+ RNA was isolated using Invitrogen’s FastTrack 2.0 kit (Carlsbad, CA). 0.9 μg each of resting and activated T cell RNA (equivalent to 12 × 10⁶ resting and 2.5 × 10⁵ activated T cells) was resolved by electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde, with 3 μg of RNA ladder as a size marker (Life Technologies, Inc.). RNA was transferred for 6 h to positively charged nylon membrane (Hybond-N+; Amersham Life Science, Inc.), followed by UV cross-linking.

Twenty-five to forty ng (9–15 × 10⁶ cpm) of ³²P-labeled polymerase chain reaction fragment corresponding to nucleotides 262–702 of hKCa4 (Fig. 1A) was used to probe multiple tissue Northern blots (2–4 μg RNA/lane, CLONTECH) and a T cell blot. Blots were hybridized for 1.5 h at 68 °C and washed two times in 0.1 × SSC + 0.1% SDS at 50 °C, then exposed to film 3–14 days using two intensifying screens. Laser densitometry was used to quantitate relative band intensities on the T cell blot. The same blot, when probed with β-actin, revealed 2.0-kb bands of differing intensity confirming the integrity of the RNA.

Transient Transfections—A 1.3-kb Smal/Scal fragment containing the coding region of hKCa4 was cloned into pcDNA3 vector (Invitrogen) at the EcoRV site. This cloning strategy introduced an additional methionine and two amino acids (G, A) upstream and in-frame with the authentic initiator methionine. Approximately 3 × 10⁵ HEK 293 cells (ATCC, Rockville, MD) were transfected with 5 μg of hKCa4 gene in pcDNA3 vector along with 1 μg of green fluorescent protein (GFP) in pEGFP-C1 vector (CLONTECH) using the LipoTAXI transfection kit (Stratagene, La Jolla, CA) as per the manufacturer’s instructions. Currents were recorded 24–72 h later.

Patch-Clamp Recording—Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) using the whole cell, cell-attached, and inside-out configurations (12). Transfected HEK cells were selected for recording by the presence of GFP fluorescence (excitation: 485/22 nm; emission: 505 nm). Thin-wall borosilicate glass pipettes were fabricated, syngardened, and fire-polished to a DC resistance of 2–8 MΩ. The resistance of patch seals was >10 GΩ. Liquid junction potentials were corrected for in all experiments, and series resistance compensation of 70% was used where maximal current was >0.5 nA. Voltage clamp protocols were implemented and data acquisition performed with pClamp 6.0 software (Axon Instruments.). Currents were low pass-filtered (~3 dB at 1 kHz) and then digitized at 3–8 kHz as computer files with a TL-1 interface (Scientific Solutions, Solon, OH). Currents were measured with p-Clamp software, and iterative curve fittings were performed with either p-Clamp or Origin software (Version 3.73; Microcal Inc., Northampton, MA).

For the cell-attached recording, the pipette solution contained (in mM) 160 potassium aspartate, 2 MgCl₂, 5 HEPES, and 16 EGTA with either 0.8 CaCl₂ (calculated [Ca²⁺]^1/2 = 100 nM; Eqcal software, Biosoft Corp., Cambridge, United Kingdom) or 1.6 CaCl₂ (calculated [Ca²⁺]^1/2 = 1 μM) at pH 7.2 (by KOH) and osmolality ~315 mOsm (by sucrose). Cells were perfused locally with a solution containing 160 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, and 5 glucose at pH 7.4 (by KOH) and osmolality ~325 mOsm. In K⁺ selectivity experiments, equimolar Na⁺ was substituted for K⁺. For cell-attached patches, the pipette solution contained 160 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES at pH 7.4 (by NaOH) and osmolality ~325 mOsm. Cells were perfused locally with Ringer solution (160 NaCl, 4.5 KCl, 5 MgCl₂, 5 HEPES, and 2 CaCl₂ at pH 7.4) ± 1 μM ionomycin. For excised inside-out patches, the pipette solution contained 160 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES at pH 7.2 (by KOH) and osmolality ~325 mOsm. The local perfusion solution (cytoplasmic face) contained 160 K aspartate, 2 MgCl₂, 5 HEPES, and 10 EGTA at pH 7.2 (by KOH) and osmolality ~329 mOsm with CaCl₂ added at concentrations that yielded the calculated [Ca²⁺]^1/2 specified. Perfusion solutions were delivered locally using a solenoid valve system (13). Toxins were purchased from Sigma and were diluted in solution containing 0.01% bovine serum albumin.

RESULTS AND DISCUSSION

We obtained a 2.2-kb cDNA clone from a human lymph node λ library using a probe derived from an EST sequence that was identified from a data base search for novel potassium channel proteins. The clone has 400 bp of 5'-UTR, 1.3 kb of coding region, and 540 bp of 3'-UTR (Fig. 1A). We have mapped the entire transcript of the predominant 2.2-kb band seen in Northern blots from various tissues (see Fig. 2), because four independent λ clones started with the same 5'-UTR sequence (±15 bp), and the 3'-UTR ended with a poly(A) tail. We also detected an in-frame stop codon upstream of the initiator ATG in all four clones, ruling out the existence of alternate upstream initiator ATG codons. The translated protein comprises 427 amino acids. Hydrophathy plot analysis reveals a short intracellular N terminus, followed by six transmembrane segments (S1–S6) and a long intracellular C terminus (Fig. 1B). The loop between S5 and S6 contains the highly conserved GYG sequence characteristic of all cloned potassium channel pores (10). The hKCa4 protein has one consensus N-linked glycosylation site between S5 and the pore and several sites for serine-threonine phosphorylation. There are no consensus EF hand motifs in hKCa4. A comparison of the amino acid sequence of hKCa4 with representative members of the K⁺ channel superfamily reveals that it is most similar to the small conductance KCa channels hSK1, rSK2, and rSK3 recently cloned from brain (14). However, it shares only 41–42% amino acid identity with them, warranting placement within a distinct subfamily (Fig. 1C). The amino acid sequence of hKCa4 is 11–14% similar to the other cloned six-transmembrane K⁺ channels (Kv, SLO, HERG, and KVLQT1; Ref. 10). In a recent abstract, J. P. Adelman reported cloning an interme-
diolate-conductance KCa channel from a human pancreatic cDNA library; sequence information was not published (15).

Northern blot analysis of hKCa4 revealed a strong signal at approximately 2.2 kb in activated human T lymphocytes, placenta, prostate, and colon (Fig. 2A and B). Moderate signal was observed in spleen, thymus, and peripheral blood leukocytes (Fig. 2A); there was no detectable signal in brain. Minor transcripts of larger sizes also were seen in some tissues (Fig. 2A).

The hKCa4 gene, when co-transfected with a reporter gene for GFP into HEK 293 cells, produced a calcium-dependent K+ current with strong inward rectification in symmetrical K+ solutions upon perfusion of 1 μM ionomycin (Fig. 3A). Current induced by ionomycin was blocked completely by 100 nM CTX, but unaffected (i.e. <10% change in amplitude) by 1 μM amin (n = 2). Internal dialysis with a solution containing 1 μM [Ca2+][free] also activated a stable large current (15.4 ± 2.4 nS slope conductance between −100 and −30 mV; n = 11) with similar characteristics. Control current during ramps in symmetrical 160 mM K+ converged with current elicited during 100 mM CTX perfusion close to 0 mV (e.g. Fig. 3A), supporting K+ selectivity of the channel. K+ selectivity was evaluated further by examining reversal potentials of control versus CTX currents over a range of different K+ [out] concentrations (Fig. 3C). Reversal potential shifted 57 mV per 10-fold increase in K+ [out] in close agreement with the predicted Nernstian value for a K+-selective channel. Pharmacological evaluation (Fig. 3B) revealed that this current was inhibited by CTX (Kd = 10 ± 1 nM) and tetraethylammonium (TEA; Kd = 30 ± 7 μM), but insensitive to margatoxin (Kd > 100 nM), apamin, iberiotoxin, kalimotoxin, and scyllatoxin (Kd > 1 μM, n = 2–5 each). Clotrimazole, a drug that is reported to block KCa channels in erythrocytes and thymocytes in the low micromolar range (16), blocked hKCa4 currents with a Kd of 387 ± 54 nM. Untransfected HEK 293 cells or cells transfected with GFP alone showed very small CTX-resistant currents in response to voltage ramps during dialysis with 1 μM [Ca2+][free] (conductance < 0.1 nS; n = 8).

Cell-attached recordings revealed single channel openings during perfusion with Ringer solution containing 1 μM ionomycin. Channels showed a unitary conductance of 33 ± 2 pS (n = 3; Fig. 4A) measured during voltage ramps between −120 and −30 mV with pipettes containing 160 mM K+ and a unitary conductance of 9 ± 1 pS (n = 3) with pipettes containing 4.5 mM K+. Pipette potentials between −100 and +20 mV had no apparent effect on the probability of channel openings (P o) during ramps (e.g. Fig. 4A) or steps. The inside-out configuration...
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Fig. 4. Unitary conductance and calcium sensitivity of hKCa4 channels. A, single-channel current (openings are down) from a cell-attached patch containing two channels with 160 mM K⁺ in the pipette. The bath contained Ringer with 1 μM ionomycin. The patch was ramped over 100 ms from −120 to +60 mV. A blank trace (i.e. no channel openings) was digitally subtracted. The dashed line represents a slope conductance of 31 pS. B, representative traces obtained from an inside-out patch excised from an hKCa4-transfected cell (E₉₀₋₀ = −80 mV). The pipette contained 160 mM K⁺, and the patch was perfused with the [Ca²⁺]ₗₚ indicated. C, relationship between open probability and cytoplasmic [Ca²⁺]ₗₚ determined using the experimental protocol in B. Single channel open probabilities were determined by the relative areas of peaks in second-order Gaussian fits to amplitude frequency histograms (patches contained one apparent channel). Points are the mean ± S.E. from the designated number of experiments. Solid line is a fit to the Hill equation described in Fig. 3B for the range of 10 nM to 600 nM [Ca²⁺]ₗₚ. D, summary table of data obtained from experiments shown in Figs. 3 and 4 for hKCa4 expressed in HEK cells compared with published data for the human T cell KCa channel.

The significant up-regulation of hKCa4 transcript levels during T cell activation highlights the importance of these channels in early activation and post-activation immune responses. Engagement of the T cell receptor by mitogen or antigen evokes an increase in intracellular calcium concentration leading to membrane hyperpolarization (rather than depolarization) caused by opening of KCa channels (1). This event, in turn, maximizes the electrical driving force for calcium influx through calcium release-activated channels, facilitating sustained calcium oscillations required for cell proliferation, cytokine production, and the expression of immune function (1, 4, 5). Blockade of both KCa and Kv channels has been reported to cause profound inhibition of T cell proliferation (17), whereas blockade of KCa channels is sufficient to prevent secondary immune responses (5). With the identification and characterization of hKCa4, it is now possible to dissect the molecular mechanisms by which calcium-activated potassium channels control leukocyte development and activation.

Note Added in Proof—During review of this manuscript, two papers were published describing a gene similar to hKCa4. The clone of Joiner et al. (Joiner, W. J., Wang, L. Y., Tang, M. D., and Kaczmarek, L. K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11013–11018) had functional properties that differed significantly from ours. The sequence of Ishii et al. (Ishii, T. M., Silvia, C., Hirschberg, B., Bond, C. T., Adelman, J. P., and Maylie, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11651–11656) differed by two amino acids in critical regions of the protein. Neither paper established a connection to lymphocytes.

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