cDNA Cloning and Functional Characterization of the Mouse Ca^{2+}-gated K^{+} Channel, mIK1

ROLES IN REGULATORY VOLUME DECREASE AND ERYTHROID DIFFERENTIATION*

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We have cloned from murine erythroleukemia (MEL) cells, thymus, and stomach the cDNA encoding the Ca^{2+}-gated K^{+} (KCa) channel, mIK1, the mouse homolog of hIK1 (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). mIK1 mRNA was detected at varied levels in many tissue types. mIK1 KCa channel activity expressed in Xenopus oocytes closely resembled the KCa of red cells (Gardos channel) and MEL cells in its single channel conductance, lack of voltage-sensitivity of activation, inward rectification, and Ca^{2+} concentration dependence. mIK1 also resembled the erythroid channel in its pharmacological properties, mediating whole cell and unitary currents sensitive to low nM concentrations of both clotrimazole (CLT) and its des-imidazoyl metabolite, 2-chlorophenyl-bisphenyl-methanol, and to low nM concentrations of charybdotoxin. Whereas control oocytes subjected to hypotonic swelling remained swollen, mIK1 expression conferred on oocytes a novel, Ca^{2+}-dependent, CLT-sensitive regulatory volume decrease response. Hypotonic swelling of voltage-clamped mIK1-expressing oocytes increased outward currents that were Ca^{2+}-dependent, CLT-sensitive, and reversed near the K^{+} equilibrium potential. mIK1 mRNA levels in ES cells increased steadily during erythroid differentiation in culture, in contrast to other KCa mRNAs examined. Low nanomolar concentrations of CLT inhibited proliferation and erythroid differentiation of peripheral blood stem cells in liquid culture.

Terminal differentiation of erythroid precursor cells is marked by enucleation and reduction in cell volume. A major component of cell volume reduction is achieved by reduction of cell K^{+} content. Mature, circulating erythrocytes retain two major ion transport pathways mediating K^{+} efflux (1). These are: 1) electroneutral K-Cl cotransport and 2) a voltage-insensitive, Ca^{2+}-activated potassium (K^{+}) channel of intermediate conductance (2–4), also known as the Gardos channel (5). The Gardos channel is thought to play a major role in volume regulation in normal (6) and sickle (SS) human erythrocytes (7, 8).

Especially in the chronically hypoxic environment of adherent or trapped sickle cells, the Gardos channel appears to mediate the major component of K^{+} loss from the erythrocyte (9), leading to an increased concentration of intracellular hemoglobin S, and exponentially decreasing the lag time for accelerated hemoglobin S polymerization (10). The Gardos channel’s biophysical and pharmacological properties have been characterized in excised inside-out human red cell membrane patches, in which Ca^{2+}-activated K (KCa) currents show inwardly rectifying properties with a unitary slope conductance ranging from 15 to 40 picosiemens, depending on the ionic conditions used (11–13). The channel is sensitive to block by charybdotoxin (14–16), but insensitive to the SK channel blocker, apamin, and to the K_{ATP} channel blockers, the antihypoglycemic drugs (17).

Sickle cell disease is a lifelong illness in which severity varies for poorly understood reasons not only among but within families, and even during an individual patient’s clinical course. It is very likely that the pathological consequences of the autosomal recessive hemoglobin S mutation underlying sickle cell disease (18) are influenced by the polypeptide products of many yet undefined modifier genes. The central role of the erythroid

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† The abbreviations used are: SS, pertaining to or describing homozygosity for the hemoglobin S mutation causing sickle cell disease; ChTX, charybdotoxin; CLT, clotrimazole; MEL cell, murine erythroleukemia cell; ES cell, murine embryonic stem cell; IC50, 50% inhibitory concentration; [Ca^{2+}], intracellular calcium concentration; V_m, transmembrane potential of the erythro plasma membrane; E_{50}, the equilibrium transmembrane potential for the ion “N”; RT, reverse transcriptase; ANOVA, analysis of variance; RVD, regulatory volume decrease; bp, base pair(s).

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K\(_{\text{Ca}}\) channel in SS red cell dehydration has suggested it as a strong candidate modifier gene in sickle cell disease. We have hypothesized that K\(_{\text{Ca}}\) channel blockade could serve as a useful adjunct therapy of sickle cell disease (7, 8, 16, 19).

A subset of antifungal imidazole drugs was found potently to inhibit K\(^+\) and Rb\(^+\) flux in normal and SS human red blood cells. Clotrimazole was the most potent of those tested (7, 20), blocking calcium ionophore A23187-induced \(86\)Rb\(^+\) influx and displacing \(125\)I-ChTX binding to red cells with equivalent ID\(_{50}\) values of \(\sim 30\) nM. The combined results of \(86\)Rb flux and \(125\)I-ChTX binding studies led to the proposal that CLT inhibited Ca\(^{2+}\)-activated K transport by direct binding to the external surface of the Gardos channel (7), in contrast to earlier (20) and continuing suggestions (21) that CLT blocks K\(^+\) conductance via its inhibitory effects on cytochrome P450 enzymes. We subsequently showed that an inwardly rectifying K\(_{\text{Ca}}\) channel from murine erythroleukemia (MEL) cells (22) was inhibited directly not only by charybdotoxin and CLT, but also by the major des-imidazolyl metabolite of CLT, 2-chlorophenyl-bisphenyl-methanol, incapable of inhibiting cytochromes P-450 (16).

The potency of CLT blockade of the erythroid K\(_{\text{Ca}}\) channel and the status of CLT as a drug already long in clinical use for other indications recommended consideration of CLT as an erythroid K\(_{\text{Ca}}\) channel blocker for adjunct therapy of sickle cell disease (19). Indeed, oral administration of CLT was shown to inhibit the erythroid K\(_{\text{Ca}}\) channel in vivo, and to diminish formation of dehydrated dense cells in a mouse model of sickle cell disease (23) and in a phase I clinical trial with sickle cell disease patients (8). More recently, a complementary approach to prevention of red cell dehydration in patients with sickle cell disease via inhibition of K-Cl cotransport (24) provided the first preliminary evidence for long term clinical benefit in a group of disease patients (8). More recently, a complementary approach to inhibition of the erythroid K\(_{\text{Ca}}\) channel might yield similar (and possibly additive) clinical benefit.

Early attempts to clone the cDNA encoding the erythroid K\(_{\text{Ca}}\) channel based on then-hypothesized homology to the maxi-K (slo) family of K\(_{\text{Ca}}\) channels (26) were unsuccessful. The subsequently cloned SK family of apamin-sensitive K\(_{\text{Ca}}\) channels (27) also differed biophysically and pharmacologically from the erythroid K\(_{\text{Ca}}\) channel. However, the SK-related human IK1 channel more recently cloned by Ishii et al. (28) and others (29, 30) has displayed many characteristics expected of the erythroid K\(_{\text{Ca}}\) channel (2–4, 7, 11–17, 22).

In the present study, we have cloned the cDNA encoding mIK1, the mouse homolog of hIK1 (28–30), and report the tissue distribution of mIK1 mRNA, and the lack of N-glycosylation of in vitro translated mIK1 polypeptide. We have functionally expressed mIK1 in Xenopus oocytes, and studied its biophysical and pharmacological properties at the levels of whole cell and unitary currents. We have shown that hypotonic swelling of oocytes leads to activation of mIK1 currents and to the novel volume regulatory response of regulatory volume decrease (RVD). Both the currents and the volume regulation are inhibited by CLT and require Ca\(^{2+}\). We have also investigated the developmental profiles of mIK1 and other K\(^+\) channel mRNAs in ES cells undergoing hematopoietic differentiation along the erythroid lineage. Finally, we have demonstrated that 10 nM CLT retards erythroid differentiation of human peripheral blood stem cells, consistent with a role of IK1 in this process.

**MATERIALS AND METHODS**

**Inhibitors and Chemicals**—CLT was purchased from Sigma. The CLT metabolite, 2-chlorophenyl-bisphenyl-methanol, was the kind gift of R. Lombardy (Pharm-Eco Laboratories, Lexington, MA). Z1-Iodo-ChTX was synthesized by the MIT Biopolymers Facility, using N-Butytoxycarbonyl-O-bromobenzyl-3-mono-iodo-t-tyrosine (Peninsula Laboratories, Belmont, CA) in place of tyrosine in position 36. The final product used was purified by high performance liquid chromatography, confirmed by amino acid analysis, and by inhibition of A22187-induced 86Rb\(^+\) flux into normal human red cells (15, 17). All salts were of analytical grade.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Total RNA from MEL cells and mouse tissues (freshly resected kidney, stomach, spleen, distal colon, proximal colon, and epididymis) was prepared using the RNeasy kit (Qiagen, Chatsworth, CA). Total RNA from ES cells was prepared using the RNeasy kit (Qiagen) and the Qiagen RNeasy kit. A mouse tissue total RNA panel (liver, brain, thymus, heart, testis, ovary, and embryo) was purchased from Ambion (Austin, TX).

Reverse transcription was performed with the First Strand DNA Synthesis Kit (Ambion) using 1 \(\mu\)g of total RNA. 5% of the reaction volume was used for hot start PCR in a total reaction volume of 50 \(\mu\)l, using either Taq DNA polymerase (Qiagen), Expand High Fidelity PCR system (Boehringer Mannheim), or Taq DNA polymerase (Promega, Madison, WI) in the suppliers’ recommended buffers.

PCR mixes lacking only primers were preheated at 82 \(^\circ\)C for 1 min, after which appropriate primers (Table I) were injected into the mix through mineral oil. The complete reaction mixes were denatured for 3 min at 95 \(^\circ\)C, then cycled through these conditions: denaturation for 45 s at 94 \(^\circ\)C, annealing for 2 min at 60 \(^\circ\)C (or, as indicated, in the presence of Q-solution (Qiagen), 52 \(^\circ\)C), and elongation for 2–3 min at 72 \(^\circ\)C. Final extension of 10 min at 72 \(^\circ\)C was terminated by rapid cooling to 4 \(^\circ\)C after the indicated number of cycles. PCR products were separated in 1% agarose gels for analysis and purified as necessary with the QiAquick Gel Extraction Kit (Qiagen). Control amplification experiments were performed on RNA samples in which reverse transcriptase was omitted.

DNA sequence analysis and data base searches were carried out with the GGCG suite of programs (University of Wisconsin Genetics Computing Group, Madison, WI).

**cDNA Cloning, Reconstruction, Transcription, and Translation of mIK1**—With hIK1 sequence information (GenBank AF022150), four degenerate oligonucleotides were designed (Table I), two corresponding to N- and C-terminal peptides (IK1.F, IK1.R) and two to internal peptide sequences of low degeneracy (IK1.IP, IK1.IR). With the combined use of the Expand High Fidelity PCR system and of Q-solution (Qiagen), 32 amplification cycles produced sufficient mIK1 cDNA for sequencing and cloning. Overlapping PCR products for mIK1 were sequenced directly in both directions (CTP prim, Ambion). Search of the EST data base identified 12 anonymous clones from mouse embryo, lymph node, melanoma, and cultured myotubes that encoded parts of mIK1: W30402, W45910, W79984, W82293, W999685, AA033013, AA402002, AA142988, AA185916, AA185547, AA265296, and AA592555. These clones helped verify nucleotide sequences encoding the N and C terminal of mIK1 polypeptide. They also assisted in design of the primers IK1.5UTRF and IK1.3UTRR (Table I), used for PCR amplification of cDNAs from MEL cells and mouse stomach that encompassed mIK1 initiator and terminator codon sequences. All PCR products and their subclones were sequenced on both strands.

Subclones without mutations were selected to reconstruct the full-length mIK1 polypeptide coding sequence between the Xenopus \(\beta\)-globin 5'- and 3'-untranslated regions in the Xenopus oocyte expression vector, pX7T (31). EcoRl/BglII-cut pX7T underwent a four-way ligation with the following mIK1 cDNA fragments: the N-terminal fragment ((vector-derived) EcoRl/MluI), the central fragment (MluI/NcoI), and the C-terminal fragment (NeoI/BamHI). The resulting recombinant plasmid, pXmIK1, was linearized with XbaI to generate transcription template for synthesis of capped cRNA from the T7 promoter (MEGAScript, Ambion). In vitro translation of mIK1 from capped cRNA in the presence of

\(^3\)Not only these and other degenerate primers, but also perfectly matched hIK1 primers failed to amplify hIK1 cDNA from a positive control tissue, human placenta (28), under a range of standard conditions. The same was true for many mIK1 primer pairs used with mouse cDNA. However, the addition of Q-solution (Qiagen) to the PCR reaction mix allowed successful amplification of mIK1 fragments with multiple sets of primer pairs from many mouse tissue sources. The unusually high GC content of mIK1 (62% overall, with clusters up to 82% of 79 nucleotides) is likely related to these observations.
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**Table I**

| Name     | Sequence          | Position | GenBank | Footnotes |
|----------|-------------------|----------|---------|-----------|
| IK1.F    | 5′-CATGGGCCGGAAGTCTGGTGC-3′ | 1–22     | AF022150 | a         |
| IK1.R    | 5′-CTACTGGGCCGGAAGTCTGGTGC-3′ | 1284–1261| AF022150 | a         |
| IK1.F    | 5′-CAGCTTTTGTGKCAAGCTKTYATG-3′ | 574–600  | AF022150 | a         |
| IK1.R    | 5′-ACCKCCGCTGTTGCKACCA-3′ | 665–646  | AF022150 | a         |
| IK1.5UTRF| 5′-GAAGCTCGTCTGAGCAGAC-3′ | 9–30     | AF042457 | b         |
| IK1.3UTRR| 5′-CCAGAGATCCAGCTACCCAGACAC-3′ | 1469–1447| AF042457 | b         |
| AE1.F    | 5′-GGCAAGCAGACAGCCACCTGTTGAC-3′ | 1219–1249| X02677   |           |
| AE1.R1   | 5′-GTTTTGGCTTCTAACAACAGC-3′ | 1865–1847| X02677   |           |
| GLO.F    | 5′-ACAGACATATGGCAGCCCTGAC-3′ | 2708–2731| J00413   |           |
| GLO.R    | 5′-GGCAAAGGGTCTAGGAGTCA-3′ | 3094–3078| J00413   |           |
| oSlc.F   | 5′-ATGAGCGTGCTACATACGC-3′ | 82–101   | L16912   |           |
| oSlc.R1  | 5′-AAXGTCAGCCACAGCTAACTGTC-3′ | 1134–1110| L16912   |           |
| oSlc.R2  | 5′-GCSATTCCTTCTAGCCTGAGTC-3′ | 1395–1373| L16912   |           |
| SK1.F    | 5′-AYGTACACGCAACACTGCT-3′ | 1097–1116| U69885   | d         |
| SK1.R    | 5′-TACCACTACAGCTGATGCTGCT-3′ | 1377–1357| U69885   | d         |
| SK2.F    | 5′-CTATGGACTGTCGCCAAGACCT-3′ | 1438–1458| U69882   | d         |
| SK2.R    | 5′-CTATGCTCTCTGACAGGATG-3′ | 1745–1721| U69882   | d         |
| SK3.F    | 5′-CTGGTGGACCTTTCAAGATG-3′ | 1360–1380| U69884   | d         |
| SK3.R    | 5′-CTAGATATAGGAAATCCGGGAGGATG-3′ | 1662–1632| U69884   | d         |
| IRK1–3.3F| 5′-GCMSAGCGCCTGGTCTGAAAGA-3′ | 465–486  | X73052   |           |
| IRK1.R    | 5′-GGAGTGGATCTGCTGATGCT-3′ | 1551–1528| X73052   |           |
| IRK2.R    | 5′-GTCTGGCTGAGGGGCCCTGAGCA-3′ | 1284–1271| X02677   |           |
| IRK3.R    | 5′-CCCTCTTTGAGAATCTGCTCA-3′ | 1321–1298| S71382   |           |
| ROMK.F    | 5′-CTATCCCTCTGCCAAGGACCT-3′ | 484–507   | X72341   | d         |
| ROMK.R    | 5′-CTGGATATTTCTCTTCTGGTAC-3′ | 1164–1142| X72341   | d         |

* Designed from hIK1 amino acid sequence, found to amplify mouse cDNA.

† From EST clones allowing extension in both 5′- and 3′-untranslated regions of miK1.

‡ Degenerate oligonucleotide designed to amplify miRK1, miRK2, and miRK3.

§ Designed from rat sequences, found to amplify mouse cDNA.

III EST clone AA28411 contains C terminus and part of 3′-untranslated region of mSK2.

![Fig. 1. Alignment of deduced amino acid sequences of miK1 and hIK1. Transmembrane spans (S1—S6) and the pore (P) region are indicated above the sequences. Underlined residues are those assigned to transmembrane regions by all reports (28–30); boldface residues not underlined are those so assigned by at least one report. Boldface italics show putative leucine zipper sequences.](image-url)

Tran35S-Label (ICN, Costa Mesa, CA) was performed with the rabbit reticulocyte lysate (nuclease-treated) system (Promega) in the presence of canine pancreatic microsomal membranes (Promega), according to the manufacturer’s protocol. N-Deglycosylation of in vitro-translated polypeptide with peptidyl-N-glycosidase F (PNGase F, Promega), SDS-polyacrylamide gel electrophoresis, and autoradiography were as described previously (32, 33).

Expression of miK1 in Xenopus Oocytes—Female Xenopus laevis were purchased from NASCO (Madison, WI), maintained at room temperature in running distilled water, and fed with frog bristle (Nasco). Oocytes were manually defolliculated after collagenase digestion of ovarian segments (34), then were microinjected with water or with 10 ng miK1 cRNA and incubated at 19 °C for 2–7 days in ND-96 with 2.5 mM sodium pyruvate and 500 μg/ml gentamicin. ND-96 contained (in mM): 96 NaCl, 2 KCl, 1.8 MgCl2, 1 CaCl2, and 5 HEPES hemisodium, pH 7.40. All experiments with oocytes were conducted at 21 °C. Two-microelectrode Studies—Oocytes injected 2–7 days previously with cRNA or with water were placed in a 1-ml chamber (Model RC-11, Warner Instrument Corp, Hamden, CT) on the stage of a dissecting microscope and impaled with microelectrodes under direct view. Current-injecting and potential-sensing microelectrodes were pulled from borosilicate glass with a Narishige puller, filled with 3M KCl, and had resistances of 2–3 megohms. They were used with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) interfaced to a Phoenix 386 computer with a TL-1 AD/DA board (Axon Instruments). PCLAMP 6.0.3 software was used to control data acquisition and to analyze data (Axon Instruments, Burlingame, CA). The isotonic ND-96 solution was 212 mosM; hypotonic bath solution (ND-70) was made by reducing [NaCl] from 96 to 70 mM, yielding an osmolarity of 160 mosM. Membrane potentials and currents were expressed as means ± S.E.

Single-channel currents were recorded using standard techniques (35). All voltages refer to the cell interior referenced to the patch...
pipette. Currents were measured with a 10-gigohm headstage, low pass-filtered at 1 kHz (Axopatch 1-D, Axon Instruments, Burlingame, CA), digitized at 5 kHz, and stored on the hard drive of an Hewlett Packard 486-IX computer. PCLAMP 6.0.3 software was used to control data acquisition via a Digitdata 1200 interface and to analyze data (Axon Instruments, Burlingame, CA). Data were acquired continuously using FETCHEX subroutines.

The pipette and bath solution compositions used in studies of outside-out patches were as described (27). Inside-out or outside-out recordings were made with symmetrical solutions containing 116 mM potassium gluconate, 4 mM KCl, 10 mM HEPES, adjusted to pH 7.2 with KOH. The intracellular solution was supplemented with CaCl_2 to give a free calcium concentration of 5 μM (assuming a stability constant of 15.9 M^{-1} for calcium gluconate). To obtain intracellular calcium concentrations below 1 μM, 1 mM EGTA was added to the bath solution and CaCl_2 was added as calculated using published stability constants. After a cell-attached gigaseal patch was established, whole cell configuration was attained by gentle suction. The outside-out patch configuration was attained subsequently by slow withdrawal of the micropipette from the cell body. Cell-attached patches were excited to attain the inside-out configuration. A voltage ramp protocol (≥100 mV to ~100 mV, duration 2600 ms) was used to test sensitivity to activation by calcium ([Ca^{2+}]) in inside-out patch configuration and sensitivity to inhibition by extracellular antagonists in the outside-out patch configuration. Relative effects of these agents (I/I0 inhibition by extracellular antagonists in the outside-out patch configuration) were determined from 30-s records during control and experimental periods, and corrected by Bonferroni post-test.

Fluorescence Measurements of Oocyte Volume and Intracellular Ions—Oocytes were loaded in ND-96 medium containing 2–5 mM BCCF-AM (Molecular Probes, Eugene, OR) for 45 min, then mounted in a superfusion chamber (36). 530 nm fluorescence emission images resulting from timed excitations at 495 nm were recorded at the equatorial plane of the oocyte. Acquired images were loaded into Image-1 software and replayed with enhanced intensity and contrast in contour display mode for measurement of oocyte diameter. Acquired images were calibrated to the cross hatch lines of a standard hemocytometer as described previously (36). Oocyte volume was calculated from measured diameter, assuming spherical geometry, and was expressed as mean % S.D. of original volume in isotonic medium. Oocyte volume so calculated differed by <0.5% from that computed by the Image-1 software from the equatorial spheroid area.

Global estimates of oocyte intracellular calcium concentration ([Ca^{2+}]) were obtained with two ion-sensitive dyes. Oocytes loaded for 45 min with 5–10 μM Fura-2/AM (Molecular Probes) were excited alternately at 340 and 380 nm (37, 38). Excitation ratio images were collected at 15–90 s intervals at an emission wavelength of 510 nm. In vitro calibration of the Fura-2 free acid fluorescence ratio was performed (38) using a value for the K_d of Ca^{2+} binding to Fura-2 of 224 nM, and with R_{min} and R_{max} determined at 10 nM and 40 μM free Ca^{2+}, respectively.

Alternatively, oocytes injected with Calcium Green-Dextran 70 kDa (Molecular Probes) to a final intracellular concentration of 3.5–7 μM were excited at 490 nm and imaged at 530 nm. Oocyte [Ca^{2+}], was determined from relative fluorescence intensity, and calculated from the equation below.

\[
[Ca^{2+}] = K_{app} \times (F - F_{min})/(F_{max} - F) \quad (Eq. 2)
\]

K_{app} is the apparent Ca^{2+} dissociation constant for the the dye, and values for F_{min} and F_{max} were determined in vitro. For Calcium Green,

![Fig. 2. A, mIki1 mRNA expression in mouse tissues as detected by RT-PCR (38 cycles) using primers Iki1.1F and Iki1.R (Table 1). Equal RNA loading was confirmed by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase cDNA (25 cycles, not shown). B, [35S]-labeled mIki1 polypeptide translated from cDNA by rabbit reticulocyte lysate in the absence and presence of dog pancreatic microsomes. The resting oocyte [Ca^{2+}], was assumed to be 80 nM, and the limiting value for the ratio F_{max}/F_{min} was assumed to be ~14 (39–41). Bleaching averaged ~0.5% during 30 min of intermittent 490 nm irradiation. Oocyte [Ca^{2+}], was measured as relative changes from the fluorescence intensity in isotonic medium.

With both methods, the transition from isotonic to hypotonic medium was marked by small fluctuations in fluorescence ratio (Fura-2) or intensity (Calcium Green), consistent with previously reported spatial and temporal oscillations of [Ca^{2+}] (39, 40). However, these oscillations could not be clearly resolved over the large sampled areas within the nonconfocal equatorial plane. The reported values of [Ca^{2+}], therefore represent averages of global (putatively cytosolic) free Ca^{2+} concentrations, and were expressed as means ± S.D.

**Culture and Erythroid Differentiation of ES Cells—**CCE murine erybryonal stem cells maintained on primary mouse embryo fibroblasts were transferred onto gelatin-coated dishes in the presence of 100 U/ml leukemia inhibitory factor (Genetics Institute, Cambridge, MA) one day before a differentiation experiment. Two ES subclones, B2 and A20, were used (42). In vitro differentiation of ES cells at a density of 3000 cells/ml was performed as described previously (42–45) in 0.9% methyl cellulose, 400 units/ml interleukin-1, 100 units/ml interleukin-3, 2 units/ml erythropoietin, 50 ng/ml Kit ligand, 5 × 10^{-4} M monothioglycollate, and 20% fetal calf serum. The cell suspension was cultured in bacterial plates and incubated at 37 °C in a humidified chamber with 5% CO_{2}. After the indicated times of differentiation in culture, embryoid bodies were pooled from several dishes, rinsed several times in 150 mM NaCl, 20 mM sodium phosphate, pH 7.4, and harvested for RNA extraction.

**Culture and Differentiation of Human Peripheral Blood Stem Cells**—Two-stage erythroid cultures derived from normal human peripheral blood were set up as described by Fibauch et al. (46, 47). CLT was added at the beginning of the second phase of culture (upon addition of erythropoietin), on culture day 5. Cells were harvested and counted at the indicated times between culture days 9 and 19. Cell viability was determined by Trypan Blue exclusion. Cytocentrifuge preparations from the experiment of Fig. 10 were stained with May Grunwald’s Giemsa and benzidine-HCl for erythroid developmental staging (46, 47). In three additional experi-
mRNAs and Polypeptide—As has been previously noted for \( \text{hIK1} \) mRNA (28), \( \text{mIK1} \) mRNA was detected in thymus, colon, and stomach (Fig. 2A). With the enhanced sensitivity of RT-PCR, \( \text{mIK1} \) mRNA was also detected in kidney, liver, testis, ovary, heart, and brain. In addition, among tissues not tested in the human, \( \text{mIK1} \) mRNA was present in whole embryo, epididymis, as well as in spleen, murine MEL cells (Fig. 2A), and murine ES cells (Fig. 9A).

\( \text{mIK1} \) cRNA in vitro translated by reticulocyte lysate yielded a homogeneous polypeptide of \( \sim 40 \) kDa (Fig. 2B), somewhat lower than predicted from the calculated apoprotein molecular mass of \( 47,783 \) Da. The presence of dog pancreatic microsomes led to no increase in mass (Fig. 2B). Incubation of the membrane-associated \( \text{mIK1} \) polypeptide with PNGase-F led to no reduction in \( M_r \) for \( \text{mIK1} \) polypeptide, under conditions in which the glycoprotein anion exchanger \( \text{AE1} \) (33) was N-deglycosylated (data not shown). Thus, in vitro translation in the presence of microsomes provided no evidence for utilization of the N-deglycosylation sequon in \( \text{mIK1} \). This apparent absence of glycosylation may result from the sequon’s distal proximity to the P-region, at most 6 amino acids in contrast to the 12 amino acids on either side required for optimal N-glycosylation (49).

Pharmacological Characterization of Recombinant \( \text{mIK1} \) Expressed in Xenopus Oocytes—Xenopus oocytes were injected with water or with \( 10 \) ng of capped cRNA encoding \( \text{mIK1} \), and examined 2–5 days later. \( \text{mIK1} \) expression hyperpolarized resting membrane potential (\( V_m \)) measured \( -3–5 \) min after impalement (\( -74 \pm 2 \) mV; \( n = 49 \)) compared with that of water-injected oocytes (\( -37 \pm 1 \) mV; \( n = 13, p < 0.0001, \) unpaired \( t \) test). This change was consistent with a shift toward \( E_k \) of the oocyte \( V_m \) normally dominated by \( \text{Cl}^- \) conductance, and as predicted for increased expression of a \( K^+ \) channel with some basal activity.

\( \text{hIK1} \) (28–30) and the murine Gardos (\( K_{Ca} \)) channel (16) were activated by \( \text{Ca}^{2+} \) and inhibited by CLT. Therefore, we assessed the response to \( \text{CaCl}_2 \) injection into oocytes at a holding potential of \( -50 \) mV chosen to discriminate \( K^+ \) from \( \text{Cl}^- \) currents in the ND-96 bath (27, 50). The peak currents elicited by \( \text{CaCl}_2 \) injection (arrows) were outward in \( \text{mIK1} \)-expressing oocytes (\( +600 \pm 139 \) nA, \( n = 6 \); Fig. 3A) and significantly differed from the inward currents (\( -216 \pm 63 \) nA, \( n = 4 \); Fig. 3B) recorded in control oocytes (\( p = 0.002, \) unpaired \( t \) test). Similar results were observed in oocytes expressing \( \text{hIK1} \) (data not shown). These results suggest that an outward \( K^+ \) current developed in response to \( \text{CaCl}_2 \) injection in \( \text{mIK1} \)-expressing oocytes, likely greater in magnitude than the inward \( \text{Cl}^- \) current stimulated in control oocytes.\(^5\)

The identity of the \( \text{mIK1} \)-associated \( K^+ \) current elicited by \( \text{Ca}^{2+} \) injection was further tested pharmacologically. The Gardos channel is potently inhibited by CLT (7, 8, 16). This property was subsequently demonstrated in outside-out patch for recombinant \( \text{hIK1} \) (28) and confirmed in two-electrode voltage clamp recordings (data not shown). We have shown that inhibition of the Gardos channel by CLT-related drugs does not require inhibition of cytochrome P450 lipid oxidases (16, 51). Therefore, 2-chlorophenyl-bisphenyl-methanol, the major in \textit{vivo} des-imidazolyl metabolite of CLT (51), was tested for its activating potential of \( V_m \) increased with time post-cRNA injection. The larger currents shown in Fig. 3A were recorded 2–3 days after cRNA injection. The larger currents shown in Fig. 8 were recorded from oocytes 2–7 days after cRNA injection. \( \text{mIK1} \) current amplitude in the oocytes increased with time post-cRNA injection.

\(^5\) The whole cell currents shown in the protocol shown in Fig. 4 were recorded 2–3 days after cRNA injection. The larger currents shown in Fig. 8 were recorded from oocytes 2–7 days after cRNA injection. \( \text{mIK1} \) current amplitude in the oocytes increased with time post-cRNA injection.
ability to inhibit mIK1. As shown in Fig. 3 (arrows c), the mIK1 current was significantly inhibited ($-781 \pm 216$ nA, $n = 6$; $p = 0.015$, paired $t$ test) whereas control currents were unaffected ($+66 \pm 50$ nA, $n = 4$; $p = 0.63$).

Inhibition of mIK1 by 2-chlorophenyl-bis-phenylmethanol was investigated further in outside-out patch recordings. As shown in Fig. 4A, inwardly rectifying currents were elicited by ramped voltages between $-100$ and $+100$ mV. These currents were progressively inhibited by increasing concentrations of 2-chlorophenyl-bis-phenylmethanol to the bath, with an ID$_{50}$ value of $14 \pm 7$ nM (Fig. 4B).

The Gardos channel is also inhibited by the scorpion venom component, charybdotoxin (ChTX, 16, 28). However, whereas the maxi-K channel of skeletal muscle (52) and whole cell K$^+$ currents of lymphocytes (53) were much less potently inhibited by $^{125}$I-ChTX than by ChTX, mIK1 was inhibited by synthetic iodo-ChTX with an ID$_{50}$ value of $4 \pm 3$ nM (Fig. 4C; ID$_{50}$ was $9 \pm 5$ nM from a Langmuir fit). This value was very similar to those with which both cold iodo-ChTX (data not shown) and $^{125}$I-ChTX inhibited $^{86}$Rb influx into red cells (15) and with which bound $^{125}$I-ChTX was displaced from intact red cells by ChTX (15). Thus, mIK1-mediated channel activity in Xenopus oocytes was sensitive to inhibition not only by CLT, but also by its major des-imidazolyl metabolite, and not only to ChTX (data not shown) but also by iodo-ChTX. These results are fully consistent with the pharmacological properties of the erythroid Gardos channel.

Detection and Regulation by Ca$^{2+}$ of mIK1 at the Single-channel Level—Single-channel events mediated by mIK1 in inside-out patches were detected in symmetrical potassium
FIG. 6. A, volume of water-injected oocytes during transition from isotonic to hypotonic medium, with no evident volume regulation. B, volume of five mIK1-expressing oocytes during transition from isotonic to hypotonic medium, exhibiting RVD. C, volume of five mIK1-expressing oocytes during transition from isotonic to hypotonic medium in the presence of clotrimazole (10 μM). RVD has been inhibited. D, volume of five mIK1-expressing oocytes loaded with BAPTA-AM and incubated in EGTA-containing medium (≤10 nM [Ca2+]i) prior to and during the transition from isotonic to hypotonic medium. Hypotonic swelling is enhanced, and RVD is absent.

**Murine Erythroid IK1 Confers Regulatory Volume Decrease**

All values expressed as mean ± S.D.

| Hypotonic V<sub>max</sub> | Recovery from Hypotonic V<sub>max</sub> | dV/dt swelling |
|--------------------------|---------------------------------|---------------|
|                           | % of isotonic value | % | % min<sup>−1</sup> of isotonic value |
| Water (n = 3)             | 106.9 ± 3.7         | 3.7 ± 3.8     | 0.38 ± 0.07   |
| mIK1 cRNA (n = 5)         | 105.8 ± 1.9         | 74.0 ± 0.6**  | 0.34 ± 0.12   |
| mIK1 cRNA + CLT (n = 5)   | 105.3 ± 1.5         | 19.5 ± 10.8***| 0.24 ± 0.05   |
| mIK1 cRNA + BAPTA/EGTA (n = 5) | 116.2 ± 2.0      | 0.0           | 0.44 ± 0.10   |
| hiIK1 cRNA (n = 3)        | 105.7 ± 1.4         | 59.0 ± 37.0   | 0.29 ± 0.12   |

*Percent recovery to isotonic value from hypotonic V<sub>max</sub>, measured 45 min after transition from isotonic to hypotonic medium. *, p < 0.05; **, p < 0.01 compared to water-injected oocytes; ***, p < 0.01 compared to mIK1 cRNA-injected oocytes studied in the absence of clotrimazole, and p > 0.1 compared to water-injected oocytes.

Glucuronate solutions (Fig. 5A). N<sub>P</sub><sub>o</sub> was 0.51 ± 0.04 at +50 mV, 0.44 ± 0.15 at +100 mV, 0.43 ± 0.08 at −25 mV, and 0.59 ± 0.18 at −50 mV. Thus, voltage dependence of N<sub>P</sub>, was not present over the range tested (p = 0.66 by ANOVA). The slope conductance (n = 3) measured between −50 to −100 mV was 35 picoSiemens, and 9 picoSiemens between 0 and +100 mV (Fig. 5B). This degree of inward rectification was slightly greater than that reported for the native Gardos channel (4), but was almost exactly as reported for hiIK1 expressed in oocytes (28).

Inside-out patches containing mIK1 were voltage-ramped from +100 to −100 mV in the presence of 0.1, 0.2, 0.5, 1.0, and 10 μM Ca<sup>2+</sup>. As shown in Fig. 5C, mIK1 was half-maximally activated by 158 ± 8 nM free Ca<sup>2+</sup>, and the Hill coefficient for activation was 0.9 ± 0.05. Thus, in voltage independence (see above) and in Ca<sup>2+</sup> sensitivity, mIK1 resembled the erythroid Gardos channel (3). This property also resembled those of hiIK1 (28) expressed in Xenopus oocytes (K<sub>H</sub> of 300 nM, Hill coefficient for activation by Ca<sup>2+</sup> of 1.7), but differed more substantially from the Hill coefficients of 2.7 and 3.2 determined for hiIK1 in HEK293 cells (29, 30).

**mIK1 Expression Confers on Oocytes CLT-sensitive Regulatory Volume Decrease—Native oocytes lack the ability to respond to mild (36) or extreme hypotonic swelling (54) with RVD. As shown in Fig. 6A, three oocytes previously injected with water swelled upon exposure to mildly hypotonic medium, and failed to return to their isotonic volumes. Whereas the resting membrane potential of native oocytes is dominated by Cl<sup>−</sup> conductances, mIK1-expressing oocytes show a large shift in membrane potential toward E<sub>K</sub>. Therefore, we hypothesized that mIK1 expression might confer on oocytes a novel RVD response. Fig. 6B shows that mIK1-injected oocytes subjected to mild hypotonic swelling indeed recovered their original isotonic volume in the continued presence of hypotonic medium, but this RVD response was prevented when exposure to hypotonicity occurred in the presence of 10 μM CLT (Fig. 6C). Moreover, mIK1-associated RVD was abrogated in BAPTA-AM-loaded oocytes exposed to hypotonic medium containing EGTA (Fig. 6D). These results and similar results for oocytes expressing hiIK1 are summarized in Table II, and demonstrate that IK1 expression conferred on oocytes a novel, Ca<sup>2+</sup>-dependent, CLT-sensitive RVD response.

**mIK1-mediated, CLT-inhibited, Ca<sup>2+</sup>-dependent K<sup>+</sup> Conduction Is Activated by Hypotonic Swelling of Xenopus Oocytes—Hypotonic swelling activates an endogenous Cl<sup>−</sup>-current in Xenopus oocytes (50). However, this activation (Fig. 7A) is not accompanied by RVD (Fig. 6A). We reasoned that RVD in IK1-expressing oocytes (Fig. 6B, Table II) likely was mediated by concomitant activation of heterologous IK1 in coordination with endogenous Cl<sup>−</sup> channels. The left panels of Fig. 7A show that hypotonic swelling of control oocytes previously injected with water led to enhanced current that displayed no inhibition by subsequently added CLT. As summarized in Fig. 7B, currents in control oocytes (n = 9) measured at +20 mV were +88 ± 14 nA in isotonic medium, increased to +276 ± 50 nA in hypotonic medium (p < 0.01), but were not reduced by addition of 10 μM CLT (317 ± 43 nA, p > 0.05, ANOVA).

In contrast, the right panels of Fig. 7A show that outward currents measured in isotonic medium in oocytes previously injected with mIK1 cRNA (n = 30) were larger than in control oocytes (p < 0.03), were also activated by hypotonicity, and...
A oocyte was subjected to sequential I-V protocols in ND-96 (isotonic), A currents. oocytes are significant at resting potential, but negligible in control minus CLT difference currents (isotonic difference currents (left control (left) and mIK1-expressing oocytes (right)). Hypotonic currents were sampled after 8-min exposure to hypotonic medium. CLT currents were sampled 2 min after exposure to CLT. Whereas control oocyte currents were mildly outwardly rectified and insensitive to CLT, mIK1-associated currents were strongly outwardly rectified and CLT-sensitive. B, current-voltage relationships for control (left, n = 9) and mIK1-expressing oocytes (right, n = 21) in isotonic ND-96 (open circles), hypotonic medium (closed circles), and hypotonic medium plus 10 µM CLT (open triangles). Expression of mIK1 is associated with a negative shift of of ~50 mV that is partially reversed by CLT. C, current-voltage relationships of difference currents in control (left) and mIK1-expressing oocytes (right). Expression of mIK1 is associated with a negative shift of for hypotonic minus isotonic difference currents (open diamonds) of ~60 mV. Hypotonic minus CLT difference currents (closed diamonds) in mIK1-expressing oocytes are significant at resting potential, but negligible in control oocytes.

Displayed sensitivity to inhibition by clotrimazole. As summarized in the right panel of Fig. 7B, currents in mIK1-measured at +20 mV increased from +496 ± 100 nA in isotonic medium to +1157 ± 269 nA in hypotonic medium (p < 0.001), then fell to +657 ± 144 nA upon exposure to CLT in hypotonic medium (p < 0.01, ANOVA). Thus, CLT inhibited (at +20 mV) 75% of the mIK1-associated oocyte current elicited by hypotonic swelling. It is also evident in the right panels of Fig. 7 (A and B) that whereas hypotonicity reduced total inward current in mIK1-expressing oocyte, inward current was restored upon addition of CLT, likely representing unmasking of the chloride current activated by hypotonic swelling.

Fig. 7C plots the potential dependence of isotonic minus hypotonic difference currents and of hypotonic minus clotrimazole difference currents. Note that, whereas of the hypotonicity-induced difference current was ~26 mV in control oocytes, was ~80 mV in mIK1-expressing oocytes. Similarly, whereas the CLT-induced difference current was negligible in control oocytes, the substantial CLT-induced difference current in mIK1-expressing oocytes (Fig. 7C) displayed an dependence of hypotonicity-activated steady-state currents in mIK1-expressing oocytes. mIK1-expressing oocytes were exposed first to isotonic EGTA-containing media (~10 nM (Ca^{2+}), open circles), then again 11 ± 1 min after the transition to hypotonic EGTA-containing media (closed circles), and again 5 ± 1 min after the subsequent transition to hypotonic medium containing 2 mM extracellular Ca^{2+} (open triangles). Values are means ± S.E. (n = 5).

Fig. 8 demonstrates that the outward current activated in mIK1-expressing oocytes by hypotonicity requires Ca^{2+}. mIK1-expressing oocytes bathed in isotonic zero-Ca^{2+} medium exhibited an of ~29 mV (n = 5). This value changed minimally to ~33 mV upon exposure to hypotonic in the continued absence of extracellular Ca^{2+}, consistent with the activation of . However, subsequent addition of 2 mM Ca^{2+} to the hypotonic extracellular medium elicited a substantial outward current characterized by an of ~88 mV (close to the predicted ) and by sensitivity to inhibition by 10 mM CLT (data not shown). Thus, the CLT-sensitive outward current activated by hypotonic swelling of mIK1-expressing oocytes was characterized by a Ca^{2+} requirement.

**Hypotonic Swelling of Oocytes Increases Intracellular [Ca^{2+}].—**The ability of hypotonic swelling to activate the Ca^{2+}-sensitive, voltage-independent mIK1 channel suggested that hypotonicity might also elevate [Ca^{2+}] in the oocyte. The resting global [Ca^{2+}], in isotonic medium as estimated by Fluo-3 fluorescence ratio did not differ significantly (p > 0.1) between oocytes previously injected with water (106 ± 12 nM) or with mIK1 cRNA (94 ± 11 nM, n = 5). Both control and mIK1-expressing oocytes responded to 25 min hypotonic swelling with gradual increases in global [Ca^{2+}], (7.4–9.0% as reported by Calcium Green Dextran and 3.9–4.5% as reported by

**FIG. 7. Hypotonic bath solutions activate CLT-sensitive mIK1 currents.** A, two-electrode voltage clamp traces of hypotonicity-activated currents in control (left) and mIK1-expressing oocytes (right). B, current-voltage relationships for control (left, n = 9) and mIK1-expressing oocytes (right, n = 21) in isotonic ND-96 (open circles), hypotonic medium (closed circles), and hypotonic medium plus 10 µM CLT (open triangles). Expression of mIK1 is associated with a negative shift of of ~50 mV that is partially reversed by CLT. C, current-voltage relationships of difference currents in control (left) and mIK1-expressing oocytes (right). Expression of mIK1 is associated with a negative shift of for hypotonic minus isotonic difference currents (open diamonds) of ~60 mV. Hypotonic minus CLT difference currents (closed diamonds) in mIK1-expressing oocytes are significant at resting potential, but negligible in control oocytes.

**FIG. 8. Ca^{2+}-dependence of hypotonicity-activated steady-state currents in mIK-expressing oocytes.** MIR-expressing oocytes were exposed first to isotonic EGTA-containing media (~10 nM (Ca^{2+}), open circles), then again 11 ± 1 min after the transition to hypotonic EGTA-containing media (closed circles), and again 5 ± 1 min after the subsequent transition to hypotonic medium containing 2 mM extracellular Ca^{2+} (open triangles). Values are means ± S.E. (n = 5).
Regulation of mRNAs Encoding mIK1 and Other K Channels during Erythroid Differentiation—mIK1 mRNA was almost as abundant in MEL cells and from murine thymus and stomach as in spleen (Fig. 2). mIK1 expressed in mouse tissues (Fig. 2). mIK1 mRNA was widely but not ubiquitously expressed in mouse tissues (Fig. 2). mIK1 expressed in Xenopus oocytes mediated whole oocyte K+ currents (Fig. 3). mIK1 mRNA was detectable at all times tested. Cellular progression along the erythroid differentiation pathway was retarded in the presence of the lowest tested concentration of CLT, 10 nM (Fig. 10B). Cell proliferation was only slightly inhibited at this concentration of CLT at the times when retardation of developmental progression was most pronounced on culture days 13 (CLT day 8) and 16 (CLT day 11).

2 DISCUSSION

We have cloned the mIK1 intermediate conductance K\textsubscript{Ca} channel from MEL cells and from murine thymus and stomach (Fig. 1). mIK1 mRNA was widely but not ubiquitously expressed in mouse tissues (Fig. 2). mIK1 expressed in Xenopus oocytes mediated whole oocyte K\textsuperscript{+} currents (Fig. 3), as well as inwardly rectifying, Ca\textsuperscript{2+}-activated, voltage-insensitive, unitary K\textsuperscript{+} currents (Fig. 5) similar to those previously reported for the erythroid K\textsubscript{Ca} (Gardos) channel (2–4, 16) and for hIK1 (28). Inhibition of mIK1-mediated currents by CLT did not require the presence of the imidazole group responsible for cytochrome P450 inhibition by CLT. Moreover, inhibition of mIK1 was not attenuated by the substitution of 3-iodotyrosine for tyrosine at residue 36 of ChTX (Fig. 4), a critical residue for inhibition of skeletal muscle

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Among K\textsubscript{Ca} channel mRNAs (data not shown), ROMK was undetectable after 38 cycles amplification, and IRK1 (36 cycles) steadily increased from very low levels at day 0 to low levels at days 10–12. In contrast, mRNA levels of IRK2 (33 cycles) and IRK3 (36 cycles) steadily decreased during the observed periods. Though reproduced in three ES lines through multiple amplifications, and with appropriate control RT-minus amplifications, these results are to be interpreted with caution. The amplified regions of these K\textsubscript{Ca} cDNAs are encoded by genomic segments uninterrupted by introns.
maxi-K channels as defined by systematic ChTX mutagenesis (55). Hypotonic swelling of oocytes activated mIK1-mediated Ca^{2+}-dependent outward currents sensitive to CLT (Figs. 7 and 8) in parallel with small but significant increases in global [Ca^{2+}]_i, and conferred on oocytes the novel property of Ca^{2+}-mediated secretagogue-stimulated transepithelial chloride secretion (59–61). The wide distribution of mIK1 mRNA suggests other physiological functions for this channel, such as that suggested in lymphocyte blast transformation (30).

Relation between IK1 and the Erythroid KCa (Gardos) Channel—The strong functional similarities between recombinant IK1 and the Gardos channel suggest that IK1 likely is or contributes to erythroid KCa channel activity. This is especially true with respect to the pharmacological profile of the recombinant and native channels. However, the differences between recombinant and native function, including the more extreme inward rectification of mIK1, demand consideration of two possibilities. It is possible that mIK1 function is influenced by native oocyte K^+ channels such as GIRK or minK, or that overexpression of mIK1 elicits atypical expression of endogenous oocyte channels (62). This possibility, as well as differences in kinase or phosphatase activities, may relate to the different apparent cooperativities of activation by Ca^{2+} in 293 cells with Hill coefficients of 2.7–3.2 (29, 30), compared with erythrocytes (3) and oocytes (28 and this work) with Hill coefficients of 0.9–1.7. It is also possible that IK1 must interact with another erythroid channel subunit (of α or β type) to reconstitute with complete fidelity the native Gardos channel activity. Whatever the reason, one consequence of the less steep [Ca^{2+}]_i activation profile in the oocyte membrane is to enhance the ability of resting values of [Ca^{2+}]_i partially to activate IK1 in basal conditions.

IK1 and RVD—RVD in many cell types has been associated with swelling-associated elevations of [Ca^{2+}]_i (63). The swelling-induced elevation in global oocyte [Ca^{2+}]_i, though small, may nonetheless contribute to the ability of hypotonic swelling further to activate IK1. This small increase in global [Ca^{2+}]_i may have reflected larger oscillatory changes in local [Ca^{2+}]_i (39–41) in the vicinity of plasmalemmal IK1 channels. The importance of juxtaplasmalemmal [Ca^{2+}]_i, in activation of IK1 is further suggested by its activation upon restoration of extracellular Ca^{2+} (Fig. 9). In parallel with hypotonic activation of IK1, several endogenous oocyte Cl^– channels may also be activated, including IC_{swell} (50) and two types of I_{Ca_{o}} (64). How might the combined activities of these channels lead to RVD? Heterologous expression in the RVD-deficient T lymphoid cell line CTLL-2 of voltage-gated K^+ channel Kv1.3 conferred the capacity for RVD (65), likely by complementing a limiting a functional tetramer, and in the current study in Xenopus oocytes a homotetramer. The N-terminal cytoplasmic tail (56), among other channel regions (57), has been proposed to contribute to subunit oligomerization of the Kv channels. However, the lack of conservation of the candidate leucine zipper sequence in this region of mIK1 (L25 in hIK1 versus V25 in mIK1) suggests some other mechanism for oligomerization possibly mediated through this portion of the polypeptide. The conserved leucine zipper near IK1 C termini may suggest a possible role for this region in protomer oligomerization or in other protein-protein interactions. The S6 helix is remarkable for conservation of four Cys residues spaced so as to allow speculation of possible disulphide bonding within or between channel protomers. The extreme C termini of mIK1 and hIK1 are not conserved, and neither displays a consensus PZD binding domain (58). Although mIK1 displayed only 88% identity to hIK1, this lower than usual degree of identity between orthologous polypeptides from different mammalian species arises almost entirely from several delimited regions.

The presence of IK1 mRNA in mouse colon (Fig. 2) and in T84 cells (data not shown), suggests that it may mediate or contribute to the ChTX- and CLT-sensitive K_{Ca} current present in T84 cells (59, 60) required for Ca^{2+}-mediated secretagogue-stimulated transepithelial chloride secretion (59–61). The presence of IK1 mRNA in mouse colon, T84 cells (data not shown), and in day 10 of culture cells (Fig. 9), the ability of 10 nM CLT to retard cell proliferation during erythroid differentiation of human peripheral blood stem cells in liquid culture. CLT exposure began on culture day 5. Cell numbers in the absence of CLT (represented as 100% in graph) were (×10^6): 1.86 ± 0.10 at culture day 10 (diamonds), 1.72 ± 0.06 at culture day 15 (squares), and 2.30 ± 0.09 at culture day 19 (triangles). Results are means ± S.D. from four identical experiments. a, inhibition by 10 nM CLT of progression through the morphological stages of erythroid differentiation: open bars, proerythroblasts; stippled bars, basophilic erythroblasts; dark gray bars, polychromatophilic erythroblasts; light gray bars, orthochromatophilic normoblasts. Numbers within bars represent percentage of total cells.

**FIG. 10.** A, inhibition by increasing concentrations of CLT of cell proliferation during erythroid differentiation of human peripheral blood stem cells in liquid culture. CLT exposure began on culture day 5. Cell numbers in the absence of CLT (represented as 100% in graph) were (×10^6): 1.86 ± 0.10 at culture day 10 (diamonds), 1.72 ± 0.06 at culture day 15 (squares), and 2.30 ± 0.09 at culture day 19 (triangles). Results are means ± S.D. from four identical experiments. B, inhibition by 10 nM CLT of progression through the morphological stages of erythroid differentiation: open bars, proerythroblasts; stippled bars, basophilic erythroblasts; dark gray bars, polychromatophilic erythroblasts; light gray bars, orthochromatophilic normoblasts. Numbers within bars represent percentage of total cells.
endogenous K+ conductance. RVD was elicited in *Xenopus* oocytes expressing mIK1 likely by similar complementation of the limiting endogenous K+ conductance. The net K+ efflux (3.77 nmol) required to achieve the measured 74% recovery from a 6% peak increase in oocyte volume (Table II) would require a time-averaged mean outward K+ current of 284–331 nA, assuming [K+]i = 91–104 mM in the maximally swollen oocyte and in the effluent, an operational K+ space = 900 nl, and 20 min recovery time (Fig. 6). As shown in Fig. 7, mean mIK1-associated outward CLT-sensitive current at −60 mV was 243 nA. Fig. 8 shows mean mIK1-associated outward Ca2+-dependent currents at −60 mV of −309 nA. Taken together with the concurrent −100 nA of inward current attributable to ICcL-mediated Cl− efflux (50), and the increased electrical driving force for Cl− efflux in hyperpolarized mIK1-expressing oocytes, we conclude that net conductive flux of K+ plus Cl− in mIK1-expressing oocytes could reasonably account for most or all of the observed RVD.

Red cell volume decrease following Gardos channel activation by prostaglandin E2 also required parallel endogenous K+ and Cl− conductances, as suggested by inhibition not only by ChTX and CLT, but also by DIDS (66). However, though activation of the Gardos channel by A23187 or by PGE2 leads to extreme hypotonicity (68). However, the ability of ClC-2 to contribute to volume regulation in the voltage-clamped *Xenopus* oocyte, attenuating or abolishing oocyte swelling induced by extreme hypotonicity (68). However, the ability of CIC-2 to confer RVD was not tested in this study.

**Possible Role of IK1 in Erythroid Differentiation**—mIK1 mRNA increased in abundance during erythroid differentiation of ES cells (Fig. 9A). Other Kca channel mRNAs tested either decreased or remained unchanged during ES cell erythroid differentiation (Fig. 9B). A possible requirement for or influence of IK1 in erythroid differentiation was tested by assessing the effect of increasing concentrations of CLT on the proliferation and erythroid differentiation of human peripheral blood CD34+/CD38+ stem cells. CLT inhibited stem cell proliferation with an ID_{50} of ~30 nM (Fig. 10A). The observed ID_{50} of ~30 nM was >10-fold more potent than previously observed for inhibition by CLT of mitogen-stimulated [3H]thymidine incorporation and intracellular Ca2+ signaling in fibroblasts and tumor cells (69), but close to that observed for inhibition of IK1 (16, 28, 30 nM) required to achieve the measured 74% recovery from a 6% peak increase in oocyte volume (Table II) would require a time-averaged mean outward K+ current of 284–331 nA, assuming [K+]i = 91–104 mM in the maximally swollen oocyte and in the effluent, a K+ space = 900 nl, and 20 min recovery time (Fig. 6). As shown in Fig. 7, mean mIK1-associated outward CLT-sensitive current at −60 mV was 243 nA. Fig. 8 shows mean mIK1-associated outward Ca2+-dependent currents at −60 mV of −309 nA. Taken together with the concurrent −100 nA of inward current attributable to ICcL-mediated Cl− efflux (50), and the increased electrical driving force for Cl− efflux in hyperpolarized mIK1-expressing oocytes, we conclude that net conductive flux of K+ plus Cl− in mIK1-expressing oocytes could reasonably account for most or all of the observed RVD.

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**Possible Role of IK1 in Erythroid Differentiation**—mIK1 mRNA increased in abundance during erythroid differentiation of ES cells (Fig. 9A). Other Kca channel mRNAs tested either decreased or remained unchanged during ES cell erythroid differentiation (Fig. 9B). A possible requirement for or influence of IK1 in erythroid differentiation was tested by assessing the effect of increasing concentrations of CLT on the proliferation and erythroid differentiation of human peripheral blood CD34+/CD38+ stem cells. CLT inhibited stem cell proliferation with an ID_{50} of ~30 nM (Fig. 10A). The observed ID_{50} of ~30 nM was >10-fold more potent than previously observed for inhibition by CLT of mitogen-stimulated [3H]thymidine incorporation and intracellular Ca2+ signaling in fibroblasts and tumor cells (69), but close to that observed for inhibition of IK1 (16, 28, 30 nM) required to achieve the measured 74% recovery from a 6% peak increase in oocyte volume (Table II) would require a time-averaged mean outward K+ current of 284–331 nA, assuming [K+]i = 91–104 mM in the maximally swollen oocyte and in the effluent, a K+ space = 900 nl, and 20 min recovery time (Fig. 6). As shown in Fig. 7, mean mIK1-associated outward CLT-sensitive current at −60 mV was 243 nA. Fig. 8 shows mean mIK1-associated outward Ca2+-dependent currents at −60 mV of −309 nA. Taken together with the concurrent −100 nA of inward current attributable to ICcL-mediated Cl− efflux (50), and the increased electrical driving force for Cl− efflux in hyperpolarized mIK1-expressing oocytes, we conclude that net conductive flux of K+ plus Cl− in mIK1-expressing oocytes could reasonably account for most or all of the observed RVD.
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