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A Novel Potassium Channel in Lymphocyte Mitochondria*

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The margatoxin-sensitive Kv1.3 is the major potassium channel in the plasma membrane of T lymphocytes. Electron microscopy, patch clamp, and immunological studies identified the potassium channel Kv1.3, thought to be localized exclusively in the cell membrane, in the inner mitochondrial membrane of T lymphocytes. Patch clamp of mitoplasts and mitochondrial membrane potential measurements disclose the functional expression of a mitochondrial margatoxin-sensitive potassium channel. To identify unambiguously the mitochondrial localization of Kv1.3, we employed a genetic model and stably transfected CTLL-2 cells, which are genetically deficient for this channel, with Kv1.3. Mitochondria isolated from Kv1.3-reconstituted CTLL-2 expressed the channel protein and displayed an activity, which was identical to that observed in Jurkat mitochondria, whereas mitochondria of mock-transfected cells lacked a channel with the characteristics of Kv1.3. Our data provide the first molecular identification of a mitochondrial potassium conductance.

Despite the vast literature on mitochondrial cation transport, basically little information is available regarding the molecular identity of these transporters (1, 2). Several antiporters and uniporters selective for sodium, potassium, and calcium have been described, based on measurements of ion fluxes in suspensions of isolated mitochondria. In energized mitochondria, the potassium flux is considerable. In fact, potassium ions are required for optimal oxidative phosphorylation (3), and electrophoretic potassium uptake plays a critical role in volume regulation (4). The application of the patch clamp technique to mitoplasts (inner membrane vesicles) permits the control of the transmembrane voltage and ion concentrations and allows the observation of activities at single channel level (5, 6). The calcium uniporter has been discovered recently to be a highly selective ion channel, whose molecular identity is unknown, however (7). Sodium-selective currents have not been recorded up to now by patch clamp, whereas an ATP-sensitive, potassium-selective channel is present in the inner membrane (8–10). The molecular identity of this latter channel is currently under debate, given that its pharmacology differs from that of the plasma membrane K_{ATP}[1] channel (11–13). The mitochondrial K_{ATP} channel does not seem to be the only potassium-selective channel in the inner membrane, because its activation requires swelling and magnesium depletion, whereas an electrophoretic potassium flux can be observed in isolated mitochondria at physiological ion concentrations (1, 14). Recently, a calcium-activated mitochondrial potassium channel has been described in glioma (15) and cardiac tissue (16), which is reminiscent of the BK-type channel in the plasma membrane. This channel seems to be activated under pathological conditions, *e.g.* ischemic reperfusion, and has been hypothesized to modulate ATP production under physiological conditions (16). The mitochondrial ATP- and calcium-dependent channels have both been proposed to play a crucial role during apoptosis (6, 17). Despite the fact that several 50–60-kDa proteins purified from mitochondria by quinine affinity column showed potassium-selective channel activity upon reconstitution in artificial membranes (18, 19), the molecular identity of the above-mentioned channels is still unknown.

Kv1.3, a member of the Shaker family of voltage-gated Kv channels, is the main potassium channel in T lymphocytes but is also expressed in other tissues, *e.g.* kidney (20), central nervous system (21), brown and white fat (22), and epithelia (23). In T lymphocytes Kv1.3 has been clearly demonstrated to represent a crucial factor for proliferation and volume regulation (24). Accordingly, inhibitors of Kv1.3 potently suppress effector memory T cell proliferation, making these blockers a promising tool for the therapy of autoimmune diseases (25).

We have shown previously that Kv1.3 is functionally inhibited and tyrosine-phosphorylated during CD95/Fas and ceramide-induced apoptosis (26, 27). By using a genetic model, we also demonstrated that Kv1.3 plays an important role in apoptosis induced by the cytostatic drug, actinomycin D. Most surprisingly, the lack of Kv1.3 significantly prevented cyto-

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[1] The abbreviations used are: K_{ATP}, ATP-sensitive potassium channel; DTT, dithiothreitol; EYFP, enhanced yellow fluorescence protein; IMM, inner mitochondrial membrane; MgTx, margatoxin; PTP, permeability transition pore; ShK, *Stichodactyla helianthus* toxin; TEACl, tetraethylammonium chloride; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; $DiOC_6(3)$, 3,3′dihexyloxacarbocyanine.

chrome *c* release and mitochondrial membrane potential ($\Delta\Psi m$) changes induced by actinomycin D, *i.e.* apoptotic events that are known to occur at the level of mitochondria (28). This finding prompted us to investigate the subcellular localization of Kv1.3. In the present work we report a previously undescribed mitochondrial localization of the potassium-selective channel Kv1.3 in genetically nonmanipulated lymphocytes. The molecular identity of the channel was assessed by comparing mitochondria isolated from the T lymphocyte cell line CTLL-2, known to lack Kv1.3 (29), with those from CTLL-2 cells stably transfected with Kv1.3.

MATERIALS AND METHODS

Cell Culture

All cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 50 μM β-mercaptoethanol. Murine interleukin-2 (4 units/ml, Roche Applied Science) was added daily to CTLL-2 cells (ATCC, Manassas, VA). CTLL-2 cells were transfected with 40 μg each of pJK/*Kv1.3* or pJK plasmids by electroporation and cultured with 800 μg/ml geneticin (G418) antibiotic to obtain stable transfectants. All experiments were performed with a mixed population obtained from several independent transfections of Kv1.3-positive or -negative cells, respectively, in order to exclude selections of clones with altered apoptotic machinery. In addition, all cultures were re-established from frozen stocks after 4 weeks of growth to prevent selection of certain clones.

Patch Clamp Experiments

Lymphocytes—Whole-cell currents were recorded with an EPC-7 amplifier (List). Leak currents were not subtracted. The bath solution was composed of 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.3). The intracellular solution contained 134 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH 7.3).

Mitochondria—To purify mitochondria, cells were incubated for 30 min at 4 °C in 0.3 M sucrose, 10 mM TES (pH 7.4), 0.5 mM EGTA and finally Dounce-homogenized. Nuclei and unbroken cells were pelleted by centrifugation for 5 min at 600 × *g* and at 4 °C. Supernatants were centrifuged at 6000 × *g* for 10 min at 4 °C, and the pellet was washed twice. The purified mitochondria were resuspended in the buffer described above. Mitoplasts were formed by osmotic shock of isolated mitochondria in 30 mM Tris/HCl (pH 7.4) and were then equilibrated in 134 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES (pH 7.3). Mitoplasts with a diameter of 2–3 μm were selected by video microscopy. Gigaohm seals were established on the membrane section opposite to the cap region. Data were sampled at 10 kHz and filtered at 200 Hz. Currents were recorded under asymmetrical [K^+] conditions with a bath solution of 134 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES/K^+ (pH 7.3) and a pipette solution consisting of 50 mM KCl, 84 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES/K^+ (pH 7.3). The theoretical E_K under these conditions, assuming permeability to K^+ only, is −25 mV. As indicated, some experiments were performed under symmetrical [K^+] conditions with 114 mM potassium gluconate, 20 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES/K^+ (pH 7.3). In the indicated patch clamp experiments, charybdotoxin was used at a concentration of 20 nM (added to the bath in whole-cell experiments), or patch pipettes were backfilled with 2 nM MgTx (in experiments on mitoplasts). The tip contained a solution without inhibitors, and the pipette was backfilled with inhibitor-containing medium. The concentration of MgTx employed in the present experiments does not inhibit other K^+ channels (30, 31). Both toxins were from Alamone Labs. Intracellular or intramitoplast voltages are reported, and outward currents are plotted upward.

Kv1.3 Western Blotting in Lymphocytes

Cells were lysed in 25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each of sodium fluoride, EDTA, and sodium pyrophosphate, and 10 μg/ml each of aprotinin and leupeptin and were centrifuged, and proteins were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were incubated with 1 μg/ml anti-Kv1.3 antiserum followed by an alkaline phosphatase-coupled anti-rabbit antibody and detection with the Tropix chemiluminescence system (TROPIX Inc., Bedford, MA). The anti-Kv1.3 antibody used in the Western blots was kindly provided by Dr. O. Pongs, University of Hamburg, Germany.

Electron Microscopy Studies

To perform EM studies, we produced a polyclonal rabbit anti-Kv1.3 antibody that is specific for the extracellularly located amino acids 204–220 (LPEFRDEKDYPASTSQD), and the results were confirmed with a second polyclonal rabbit anti-Kv1.3 antibody (kindly provided by Dr. O. Pongs, University of Hamburg, Germany). Peripheral blood lymphocytes were purified by Ficoll-Paque gradient, and the cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde, dehydrated in ethanol gradients, and embedded in LR white resin (London Resin Company Limited, Berkshire, UK). Sections were cut at 85 nm for on-grid immunochemistry, blocked, and washed with phosphate-buffered saline containing 0.1% gelatin from cold water fish skin (Sigma), 0.5 M NaCl, and 0.05% Tween 20. Sections were labeled with rabbit anti-Kv1.3 antibodies and 6 nm gold-coupled goat anti-rabbit IgG (Aurion, Wageningen, Netherlands). Samples were stained with uranyl acetate and viewed with a JEOL 1200EX-II electron microscope (JEOL, Peabody, MA).

Kv1.3 Detection in Isolated Mitochondria by Flow Cytometry and Western Blotting

For the flow cytometry studies, cells were transiently transfected with pEYFP-Mito or pEYFP-ER (Clontech) and sorted after 48 h. For Western blotting experiments, mitochondria from wild type Jurkat cells, CTLL-2/pJK, or CTLL-2/Kv1.3 cells were employed. Mitochondria isolated as described above for patch clamp experiments were further purified using a 10-min Percoll gradient centrifugation at 4 °C (60, 30, and 18% Percoll in the buffer above, 8,500 × *g*). Mitochondria at the interface between the 30 and 60% layers were collected, washed twice, and resuspended in 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 10 μg/ml aprotinin and leupeptin, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, 50 μg/ml creatine kinase (buffer 1).

Equivalents to 30 μg of total protein were used for Western blotting of Kv1.3. The Western blots were developed using anti-Kv1.3 antibodies (kindly provided by Dr. O. Pongs), an alkaline phosphatase-coupled secondary anti-rabbit antibody, and the Tropix chemiluminescence system. Flow cytometry analysis was performed with Cy3-labeled anti-Kv1.3 antibodies against an extracellular portion of the channel from Alamone Labs on intact and 0.1% Triton X-100-permeabilized mitochondria to access the IMM. To label mitochondria, we transiently transfected CTLL-2/Kv1.3 or CTLL-2/pJK cells with the plasmids pEYFP-Mito or control pEYFP-ER (Clontech) that direct expression of the EYFP protein specifically in mitochondria or the endoplasmic reticulum, respectively.

For staining with anti-Tim23 antibodies (BD Biosciences), purified mitochondria were permeabilized with 0.1% Triton X-100 on ice for 15 min, washed twice in buffer 1, blocked with 1% fetal calf serum, and stained consecutively for 45 min each with rabbit anti-Kv1.3 and mouse anti-Tim23 antibodies. The samples were washed extensively and finally stained consecutively for 45 min each with FITC anti-mouse and Cy3 anti-rabbit antibodies. The samples were washed twice and analyzed by FACS.

Membrane Potential in Isolated Mitochondria

To determine membrane potential of isolated mitochondria, mitochondria were isolated as above, washed, and resuspended in 5 mM succinate, 50 mM PIPES (pH 7.4 with KOH), 50 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 10 μg/ml aprotinin and leupeptin, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase. A slightly hyperosmotic medium (340 mosmol as measured by osmometer analysis) was used to protect the mitochondria from swelling. The membrane potential of isolated mitochondria was determined by incubation of purified mitochondria with 10 nM $DiOC_6(3)$ (32) in DTT-free buffer for 30 min at 4 °C in the presence or absence of 10 nM Shk or 20 nM MgTx. Higher concentrations of the toxins than in the patch clamp studies were employed in order to ensure their rapid diffusion across the mitochondrial outer membrane. Recombinant GST-Kv1.3 (AA1–618) was added at 400 ng/ml if indicated. Mitochondria were then pelleted, resuspended at 37 °C in pre-warmed DTT-free buffer 1 containing 10 nM $DiOC_6(3)$, and fluorescence was measured by flow cytometry. The shift of the temperature from 4 to 37 °C facilitates the opening of Kv1.3 and therefore open channel block by the toxins (33). A right shift of the fluorescence signal by increased binding of $DiOC_6(3)$ indicates a hyperpolarization, whereas depolarization is indicated by a left shift. Complete

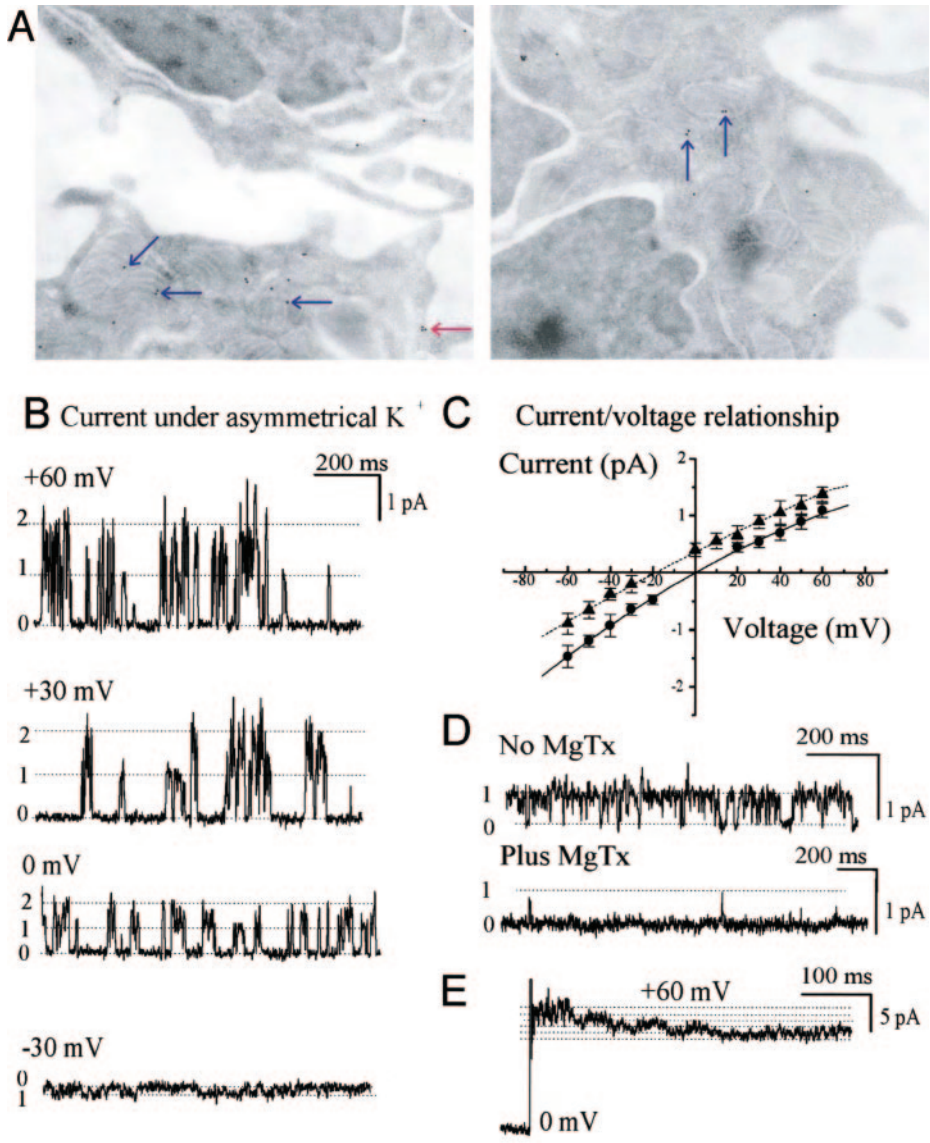

FIG. 1. **Kv1.3 is functionally expressed in the mitochondria of Jurkat lymphocytes.** *A*, transmission electron microscopy on freshly isolated purified human blood lymphocytes reveals the presence of Kv1.3 in mitochondria. *Arrows* indicate gold particles and point to Kv1.3 in mitochondria (*blue arrows*) and the cell membrane (*red arrow*). Kv1.3 in other cell compartments is not indicated. Magnification was 60,000-fold. Control stainings using irrelevant antibodies were negative (not shown). *B–D*, single channel patch clamp recordings on mitoplasts isolated from Jurkat cells show functional expression of Kv1.3 channels in mitoplasts as evidenced by channel conductance (*B–D*), current-voltage relationship (*C*), ion selectivity (*B* and *C*), and sensitivity to MgTx (*D*) in 8 of 13 patches. *B* displays representative current traces under asymmetrical [K^+] (134/50 mM K^+) at +60, +30, 0, and −30 mV. Two channels (indicated by open channel levels 1 and 2) often gated simultaneously. Net outward current at 0 mV indicates potassium selectivity. *C* displays the current voltage relationship of the channel for symmetrical (134 mM [K^+]; *circles*) or asymmetrical (134/50 mM [K^+]; *triangles*) ionic conditions. *D*, treatment of mitochondria from Jurkat cells with the Kv1.3 inhibitor MgTx (2 nM) blocked the channel. Representative traces recorded at +10 mV in asymmetrical [K^+] are shown. *E*, representative trace obtained by averaging nine current records from the same experiment, elicited by stepping voltage from 0 to +60 mV. Ionic conditions were symmetrical. The capacitative current was subtracted.

depolarization of mitochondria was achieved with 1 μM carbonylcyanide *m*-chlorophenylhydrazone for 10 min at room temperature. The experiments were repeated with tetramethylrhodamine ethyl ester showing very similar results (not shown).

RESULTS

To determine the cellular localization of Kv1.3, we first performed transmission electron microscopy studies on freshly isolated peripheral human blood lymphocytes. These experiments surprisingly demonstrated the presence of the channel protein in mitochondria (Fig. 1*A*) and confirmed the previously defined localization of Kv1.3 in the plasma membrane and in intracellular vesicles, most likely of the endoplasmic reticulum. The typical double membrane and the presence of cristae identified mitochondria. The presence of Kv1.3 was confirmed by using two different antibodies, *i.e.* a rabbit polyclonal antibody directed against amino acids 204–220 (Fig. 1*A*, *left panel*) and a rabbit polyclonal antibody directed against the C terminus of Kv1.3 (kindly provided by Dr. O. Pongs) (*right panel*). The specificity of this latter antibody is indicated by the fact that the same antibody, when used in Western blots on whole-cell lysates of lymphocytes, reacted exclusively with a single 65-kDa band only in Kv1.3-containing cells (see Fig. 2*A*). Close inspection of the transmission electron microscopy studies revealed that Kv1.3 localizes to the IMM (Fig. 1*A*).

To show functional expression of Kv1.3 in mitochondria, we performed patch clamp experiments on mitoplasts from Jurkat cells known to endogenously express Kv1.3 (34). In 8 of 13 patches, the mitoplasts were endowed with a channel with biophysical characteristics very similar to those of Kv1.3 in the cell membrane. These characteristics included conductance (Fig. 1, *B–D*), single open channel current-voltage relationship (Fig. 1*C*), and potassium selectivity (Fig. 1, *B* and *C*) and sensitivity to the very specific Kv1.3 inhibitor MgTx (Fig. 1*D*) or the K^+ channel blocker TEACl (not shown). The slope conductance was ~25 pS, and a weak rectification was observed at negative potentials in symmetrical high K^+ solution, as expected for Kv1.3 (34, 35). The observed reversal potential (−20 ± 6 mV) was close to the theoretical E_K (−25 mV) demonstrating potassium selectivity for the channel. The activity was only slightly voltage-dependent (Fig. 1*B* and not shown). The inactivation kinetics of Kv1.3 in mitoplasts could not be systematically studied because application of large voltage steps across the mitoplast membrane resulted in loss of the seal. In a representative case (Fig. 1*E*), application of depolarizing 60-mV voltage steps from 0 mV induced a rapid activation followed by modest inactivation, consistent with the typical behavior of Kv1.3 (see "Discussion"). Channel activity is characterized by flickering behavior, in accordance with the

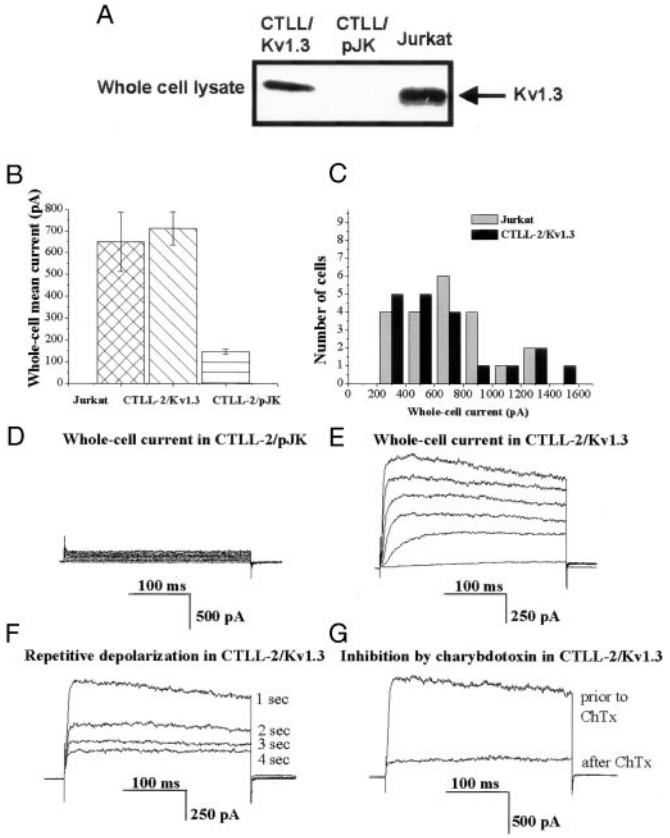

FIG. 2. **Expression of plasma membrane Kv1.3 in CTLL-2 cells.** *A*, Western blots of whole-cell lysates confirm the specific expression of Kv1.3 in CTLL-2/Kv1.3 cells. The 65-kDa Kv1.3 protein was absent in whole-cell lysates of CTLL-2/pJK cells. Proteins were separated, blotted onto nitrocellulose, and developed using polyclonal rabbit anti-Kv1.3 antibodies. Shown are representative blots from three experiments. *B–G*, the biophysical characteristics of Kv1.3 in CTLL-2/Kv1.3 cells resemble those of Kv1.3 in Jurkat cells. *B*, transfection of Kv1.3 into CTLL-2 cells results in a mean whole-cell peak current almost identical to that observed in Jurkat cells endogenously expressing the channel. The mean whole-cell peak currents measured at 50 mV are reported for Jurkat, CTLL-2/Kv1.3, and CTLL-2/pJK cells ($n = 15$). *C*, the distribution of whole-cell peak currents (at 50 mV) confirms a similar expression of Kv1.3 in single Jurkat and CTLL-2/Kv1.3 cells. Representative whole-cell currents of CTLL-2/pJK (*D*) or CTLL-2/Kv1.3 (*E*) cells were elicited by changing the membrane potential in six distinct 20-mV steps (−70 to 50 mV). Voltage pulses were applied every 45 s. The data reveal the absence of Kv1.3 in CTLL-2/pJK cells (*D*) and a current typical for Kv1.3 in CTLL-2/Kv1.3 cells (*E*). *F*, use-dependent inactivation of Kv1.3 in CTLL-2/Kv1.3 cells was detected by stepping up the potential from −70 to 50 mV at 1-s intervals. The inactivation kinetics are identical to those measured in Jurkat T cells. *G*, addition of 20 nM charybdotoxin (*ChTx*), a Kv1.3 inhibitor, inhibited the K^+ current in CTLL-2/Kv1.3 cells. Finally, exchange of the bath solution containing 5 mM KCl with a medium containing 150 mM KCl resulted in a shift of the reversal potential from −70/−80 mV (theoretical reversal potential of −84 mV) to 0 ± 8 mV ($n = 3$), indicating potassium selectivity of Kv1.3 in CTLL-2/Kv1.3 (not shown).

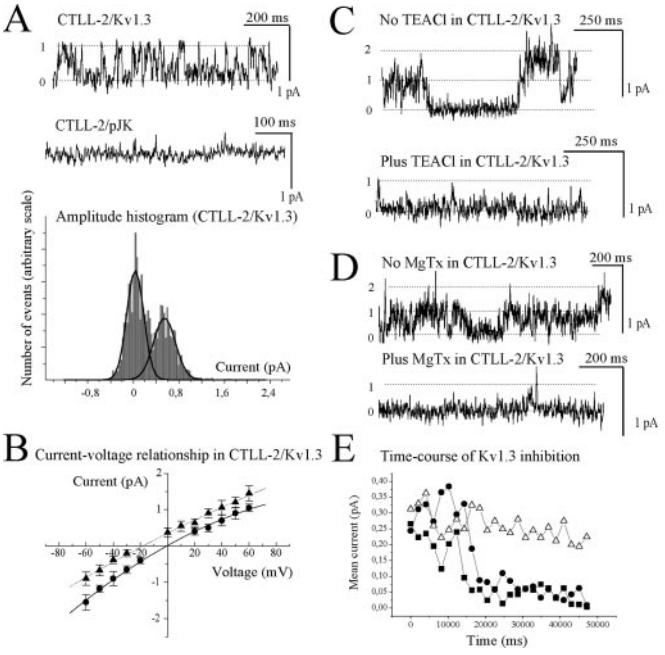

FIG. 3. **Transfection of Kv1.3 into CTLL-2 cells results in functional expression of the potassium channel in mitochondria.** *A–E*, patch clamp experiments on isolated mitoplasts from transfected CTLL-2 cells show functional expression of Kv1.3 channels (detected in 7 of 9 patches) closely resembling those detected in mitochondria from Jurkat cells. *A*, the panel displays representative current traces recorded in CTLL-2/Kv1.3 under symmetrical conditions at 30 mV. Recordings in CTLL-2/pJK mitoplasts under the same conditions indicate the absence of this channel in mitochondria from cells lacking Kv1.3 ($n = 9$). The amplitude histogram shows open and closed current levels. *B*, the panel shows the current voltage relationship of the channel in CTLL-2/Kv1.3 mitoplasts under asymmetrical (134/50 mM $[K^+]$; *triangles*) or symmetrical (134 mM $[K^+]$; *circles*) ionic conditions. *C* and *D*, treatment of mitochondria isolated from CTLL-2/Kv1.3 with 100 mM TEACl (*C*) or 2 nM MgTx (*D*) blocks the channel. Currents were recorded at 10 (*C*) or 0 mV (*D*) in asymmetrical $[K^+]$. MgTx had no effects on mitoplasts from CTLL-2/pJK (not shown), excluding an unspecific effect of the toxin. *E* shows the time course of the mean current with TEACl (*closed squares*) ($n = 3$) or MgTx (*closed circles*) ($n = 5$) present in the pipette and without inhibitor (*open triangles*) ($n = 4$) from experiments performed on mitoplasts from CTLL-2/Kv1.3 and Jurkat cells. None of the activities reported in *B–E* were detected in mitochondria of Kv1.3-deficient cells.

reported single channel activity of Kv1.3 in the plasma membrane (33, 34).

To exclude detection of another mitochondrial channel, displaying similar characteristics, we used a genetic model and transfected Kv1.3-deficient CTLL-2 cells (29) with an expression vector for Kv1.3 (*pJK-Kv1.3*) (cells designated CTLL-2/Kv1.3) or the control vector (*pJK*) (designated CTLL-2/pJK) (36). Control Western blots from whole-cell lysates (Fig. 2*A*) and patch clamp studies in whole-cell configuration (Fig. 2, *B–G*) ($n = 15$) confirmed the expression of Kv1.3 exclusively in transfected cells and indicated a functional expression level of the channel very similar to that in Jurkat cells (Fig. 2, *B* and *C*), making overexpression artifacts very unlikely. The biophysical characteristics of Kv1.3 in the plasma membrane of CTLL-2/Kv1.3 cells were identical to those of endogenous Kv1.3 in Jurkat T lymphocytes (Fig. 2, *E–G*). These data indicate the suitability of this genetic model to analyze Kv1.3 activity.

Next, the expression and function of Kv1.3 in mitochondria were investigated by employing this genetic system. Patch clamp studies in mitoplasts from CTLL-2/Kv1.3 cells revealed in 7 out of 9 patches potassium channel activity similar to endogenous Kv1.3 in Jurkat mitoplasts (Fig. 3, *A–E*). This current was absent in mitoplasts from control CTLL-2/pJK cells ($n = 9$) (Fig. 3*A*). The channel in Kv1.3-transfected CTLL-2 cells was sensitive to TEACl and to the Kv1.3 inhibitor margatoxin (Fig. 3, *B* and *C*). The detection of this current exclusively in mitoplasts of Kv1.3-transfected CTLL-2 (Fig. 3*A*) and Jurkat cells (Fig. 1, *B–D*), but not in mitoplasts of control CTLL-2/pJK cells, indicates functional expression of Kv1.3 in mitochondria and excludes detection of another channel.

To confirm unambiguously that Kv1.3 localizes to the IMM, we tested whether patches with Kv1.3 activity also contained other known mitochondrial ion channels (Fig. 4). The 107-pS channel and the permeability transition pore (PTP) were used in these studies as markers for the IMM (37, 38). Both channels were identified on the basis of their biophysical and pharma-

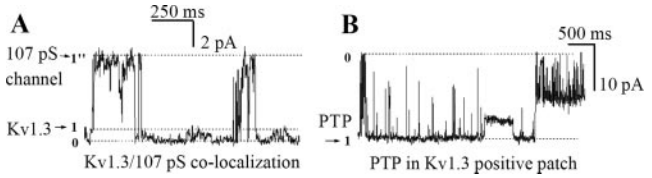

FIG. 4. **Kv1.3 co-localizes with the 107-pS channel and the PTP in the inner mitochondrial membrane.** *A*, Kv1.3 (*level 1*) and the typical IMM 107-pS channel (*level 1″*) were recorded in the same membrane patch. The representative trace was recorded at +60 mV under symmetrical $[K^+]$ (134 mM K^+) conditions. The amplitude of the small channel activity was 0.96 pA with a predicted value of 1 pA for Kv1.3 at this potential. The presence of Kv1.3 in the same patch as the 107-pS channel was detected in 3 of 8 Kv1.3-containing patches from Jurkat cells and 2 of 7 patches from CTLL/Kv1.3 cells. *B*, to detect the mitochondrial megachannel in the same patches as Kv1.3, we first measured Kv1.3 in the patch and then increased the $[Ca^{2+}]$ in the bath to a concentration of 0.1 mM (150 mM KCl, 0.1 mM $CaCl_2$, 20 mM HEPES/K^+, pH 7.3). The trace shows activity of the mitochondrial megachannel from such an experiment at −20 mV. Similar results were obtained in 2 of 7 patches from CTLL-2/Kv1.3 and 5 of 8 patches from Jurkat mitoplasts. Finally, in 2 patches (1 patch from CTLL-2/Kv1.3 and 1 patch from Jurkat), we observed the co-existence of all three channels, *i.e.* Kv1.3, the 107-pS channel, and, after Ca^{2+} increase, the PTP. The PTP and the 107-pS channel were identified by their typical biophysical and pharmacological characteristics.

cological properties. The experiments revealed the simultaneous activity of Kv1.3 and the 107-pS channel in 5 patches of 15 obtained from CTLL-2/Kv1.3 (Fig. 4*A*) and Jurkat mitoplasts, whereas patches from CTLL-2/pJK contained the 107-pS channel but never showed any Kv1.3 activity (not shown). To determine whether PTP and Kv1.3 can be recorded in the same membrane, we increased the Ca^{2+} concentration on the matrix side of patches containing Kv1.3. The $[Ca^{2+}]$ increase results in activation of the PTP. The data revealed that 7/15 of all patches with Kv1.3 activity also contained the PTP (Fig. 4*B*). Co-localization of both channels was only observed in mitoplasts from CTLL-2/Kv1.3 and Jurkat cells (Fig. 4*B*), whereas mitoplasts from CTLL-2/pJK cells displayed PTP activity but lacked any Kv1.3. Moreover, two patches on mitoplasts from CTLL-2/Kv1.3 and Jurkat cells contained all three channels, *i.e.* Kv1.3, the 107-pS channel, and, after a Ca^{2+} increase, the PTP. The simultaneous presence of Kv1.3 and the 107-pS channel and/or the PTP in mitoplasts from CTLL/Kv1.3 or Jurkat cells demonstrated functional expression of Kv1.3 in the IMM.

The mitochondrial localization of Kv1.3 was also confirmed by biochemical analysis, *i.e.* Western blots performed on purified mitochondria from CTLL-2/Kv1.3, CTLL-2/pJK, and Jurkat cells (Fig. 5*A*). However, Percoll-purified mitochondria employed for the Western blots might be contaminated by plasma membrane and/or endoplasmic reticulum containing Kv1.3. In order to exclude this possibility, CTLL-2/Kv1.3 and CTLL-2/pJK cells were transiently transfected with a vector encoding an enhanced yellow fluorescent protein (EYFP) fused either to the mitochondrial targeting sequence of cytochrome *c* oxidase (Mito-EYFP) or a target sequence for the endoplasmic reticulum of calreticulin (ER-EYFP). Transfected cells were sorted by flow cytometry (FACS), and mitochondria were gradient-purified, stained with Cy3-labeled anti-Kv1.3 antibodies, and analyzed by FACS. The outer membrane was permeabilized in order to allow access of the antibody to the inner membrane. The mitochondrial population was identified as Mito-EYFP-positive and by its typical light scattering pattern. Co-staining of Mito-EYFP and Cy3-labeled anti-Kv1.3 was exclusively detected in mitochondria derived from CTLL-2/Kv1.3 (Fig. 5*B*) and Jurkat cells (not shown), but not in mitochondria derived from CTLL-2/pJK cells (Fig. 5*B*). Transfection of the ER-EYFP construct showed the absence of contaminating particles from the endoplasmic reticulum in the mitochondrial fraction (Fig. 5*B*). Mito-EYFP-positive particles did not react with an anti-CD3 antibody, excluding contamination of isolated mitochondria by Kv1.3-containing cell membrane fragments (Fig. 5*B*). To prove further the inner membrane localization of Kv1.3, we stained isolated and purified mitochondria with Cy3-coupled anti-Kv1.3 and FITC-labeled anti-Tim23 antibodies. The latter detected the inner membrane protein Tim23 that is part of the protein translocation machinery. The FACS analysis indicated that Tim23-positive particles, *i.e.* mitochondria, were also positive for Kv1.3, suggesting the presence of Kv1.3 in the inner mitochondrial membrane. None of the antibodies stained mitochondria that were not permeabilized (not shown).

The above data indicate the functional expression of Kv1.3 in the inner mitochondrial membrane of lymphocytes. Therefore, we investigated whether Kv1.3 activity regulates $\Delta\Psi m$. Because the electrochemical gradient of energized mitochondria incubated in a medium containing ~100 mM K^+ leads to a potassium influx, *i.e.* a depolarizing inward potassium current, a blockade of a K^+ channel should hyperpolarize the membrane and vice versa. Specific activators of Kv1.3 do not exist, but MgTx from scorpion (39) and the sea anemone *Stichodactyla helianthus* toxin Shk (40) are known potent blockers of Kv1.3, which do not act on other potassium channels at concentrations used in the low nanomolar range (31, 34). Both toxins have a sufficiently low molecular weight to be able to diffuse through the outer membrane, *e.g.* via porins. To promote rapid diffusion to the inner membrane, and in order to ensure complete inhibition in a high potassium medium (41), we employed relatively high concentrations of these toxins (low nanomolar range) with respect to their K_m. Our results show that incubation of isolated, intact, and energized mitochondria from Jurkat cells with MgTx or Shk rapidly hyperpolarized mitochondria as indicated by the right shift of the fluorescence signal in the FACS analysis. Nontreated mitochondria had a stable membrane potential over more than 20 min (Fig. 6). Incubation of the toxins with recombinant GST-Kv1.3 prior to their addition to the mitochondria completely prevented hyperpolarization (Fig. 6*A*). The specificity of the action of the toxins on Kv1.3 is also illustrated by the result that CTLL-2/Kv1.3 mitochondria hyperpolarized upon application of MgTx or Shk, whereas the $\Delta\Psi m$ of mitochondria of CTLL-2/pJK cells did not change (Fig. 6*B*). The fact that the two toxins did not alter the membrane potential in Kv1.3-deficient mitochondria indicates that the toxins exclusively act on Kv1.3 and do not affect other mitochondrial channels and proteins. Most importantly, the same changes were detected upon addition of both toxins to mitochondria isolated from genetically manipulated (CTLL-2/Kv1.3) and nonmanipulated cells (Jurkat), indicating again that the activity of Kv1.3 in mitochondria is not observable because of an overexpression artifact. Complete depolarization of mitochondria was achieved with 1 μM carbonylcyanide *m*-chlorophenylhydrazone and served as control for integrity of the mitochondria (yellow traces in each experiment).

DISCUSSION

Our data indicate a novel, mitochondrial localization of the potassium channel Kv1.3. This channel thus represents the first mitochondrial potassium conductance identified from a molecular point of view. Several lines of evidence localize Kv1.3 to mitochondria. First, electron microscopy studies on peripheral blood lymphocytes showed the channel in the inner mitochondrial membrane. Second and most important, the observation that Kv1.3, PTP, and the 107-pS channel are present in the same patches of mitochondria from normal Jurkat cells and Kv1.3-transfected CTLL-2 cells proves the localization of Kv1.3 in the inner mitochondrial membrane. Neither the PTP nor the 107- pS channels have been described to be present in other

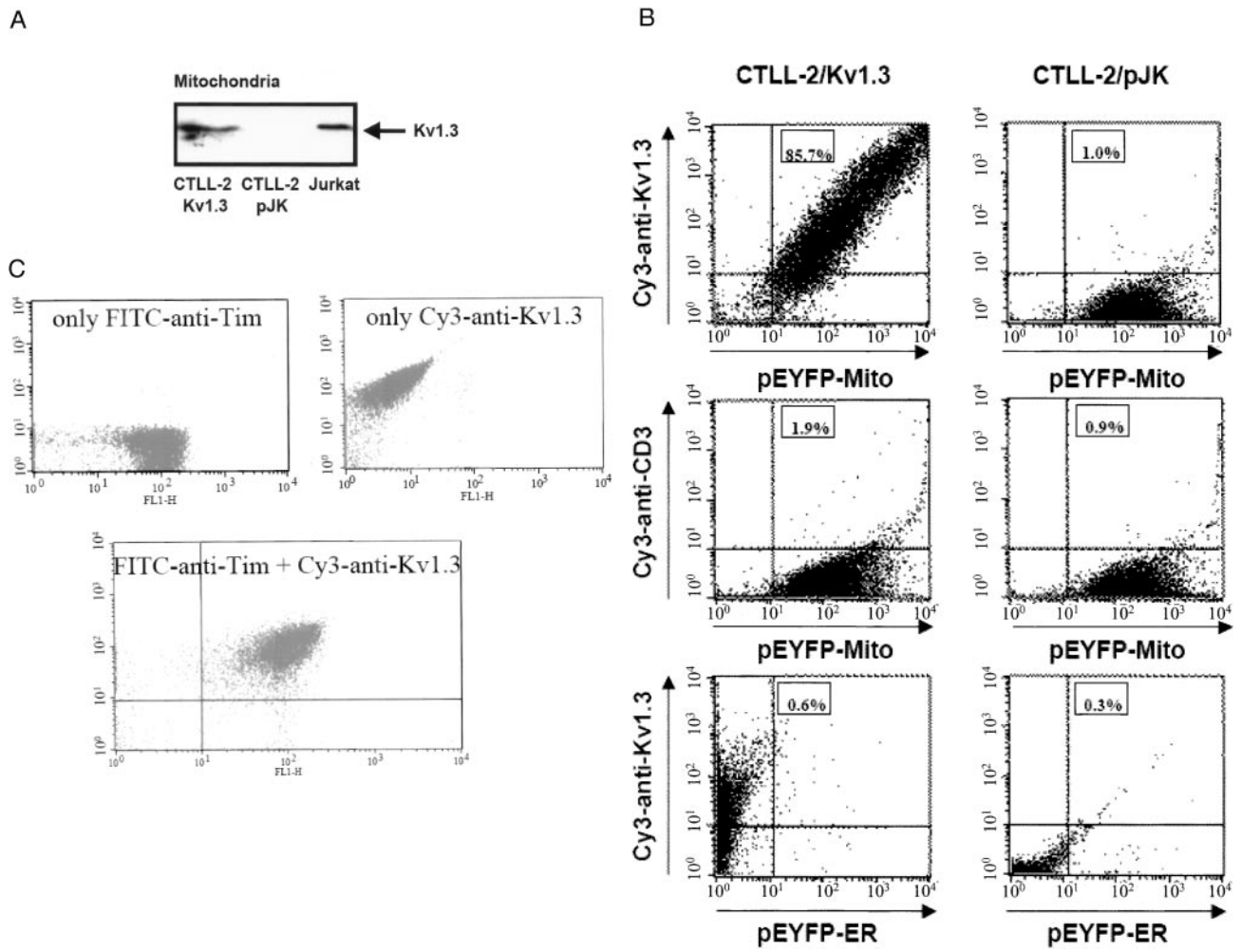

FIG. 5. **Detection of Kv1.3 in mitochondria is not because of contamination.** *A*, Western blot analysis of purified mitochondria from CTLL-2/Kv1.3 and Jurkat cells demonstrates expression of Kv1.3 in CTLL-2/Kv1.3 cells, whereas mitochondria from control-transfected CTLL-2/pJK cells lack the 65-kDa channel protein. *B*, transient transfection of CTLL-2/Kv1.3 or CTLL-2/pJK cells with pEYFP-Mito or control pEYFP-ER reveals expression of Kv1.3 in mitochondria. Staining of purified mitochondria with Cy3-coupled anti-Kv1.3 antibodies showed expression of Kv1.3 in mitochondria obtained from CTLL-2/Kv1.3 cells and the absence of the channel in mitochondria from CTLL-2/pJK cells. Contamination of the mitochondria with Kv1.3-containing cell membrane fragments or endoplasmic reticulum was excluded by staining with Cy3-labeled anti-CD3 antibodies or by transfection of an EYFP protein fused with an endoplasmic reticulum (*pEYFP-ER*) targeting sequence. *C*, mitochondria were isolated from Jurkat lymphocytes, permeabilized, and stained with FITC-labeled anti-Tim23 and Cy3-coupled-anti-Kv1.3 antibodies. Mitochondria were defined as particles positive for Tim23 and analyzed by FACS for the presence of Kv1.3. The *upper panels* show the single staining with FITC-anti-Tim23 (FL-2) or anti-Cy3-anti-Kv1.3-antibodies (FL-2), respectively, and the *lower panel* displays the simultaneous analysis.

locations, in particular the endoplasmic reticulum or the cell membrane. Third, the analysis of mitochondria from Kv1.3-deficient and Kv1.3-reconstituted CTLL-2 cells proves the identity of Kv1.3 and excludes detection of another channel with similar characteristics. We would like to point out that our patch clamp studies revealed a channel with the same characteristics in mitochondria from Jurkat cells that endogenously express Kv1.3 and in Kv1.3-transfected CTLL-2 cells, whereas this channel was absent from mitochondria isolated from Kv1.3-deficient CTLL-2 cells. The fact that transfection of Kv1.3-deficient cells with Kv1.3 restored the conductance of the ion channel in mitochondria strongly suggests that we detect Kv1.3 in mitochondria and do not deal with another similar channel. We would like to stress that the presence of the channel in mitochondria was also revealed in Jurkat cells, which were not manipulated genetically. Because Kv1.3-transfected CTLL-2 cells express lower or normal levels of Kv1.3 compared with those of Jurkat cells endogenously expressing Kv1.3, an overexpression artifact as cause for the mitochondrial expression of Kv1.3 can be also excluded.

The ionic conditions in our patch clamp experiments on mitoplasts were chosen to prevent stimulation of Ca^{2+}-activated, Mg^{2+}-inhibited, or alkaline (>8) pH-activated channels. Under these conditions, the K_{ATP} channel (8), the anion-selective "107-pS" channel (37), porin (42), and perhaps the pores formed by the protein import apparatus, *i.e.* Tom40 (43), Tim23 (44), and Tim22 (45), might be active. Tom40 and porin belong to the outer mitochondrial membrane, and we have not observed either of these channels in the experiments reported here. The mitochondrial Ca^{2+}-activated K^+ channel (15) was suppressed by the low Ca^{2+} concentration used in the present experiments. This and the other channels mentioned above can be easily distinguished from Kv1.3 by their different conductances, selectivity, voltage dependence, and sensitivity to ATP, Ca^{2+}, MgTx, and glibenclamide (not shown). Thus, all other K^+ channel activities known to be present in the IMM are clearly different from the activity described here. In summary, the combination of biophysical, biochemical, pharmacological, and genetic data proves the functional expression of Kv1.3 in lymphocyte mitochondria.

The orientation of the channel seems to be the same in both plasma membrane and mitochondrial inner membranes. This

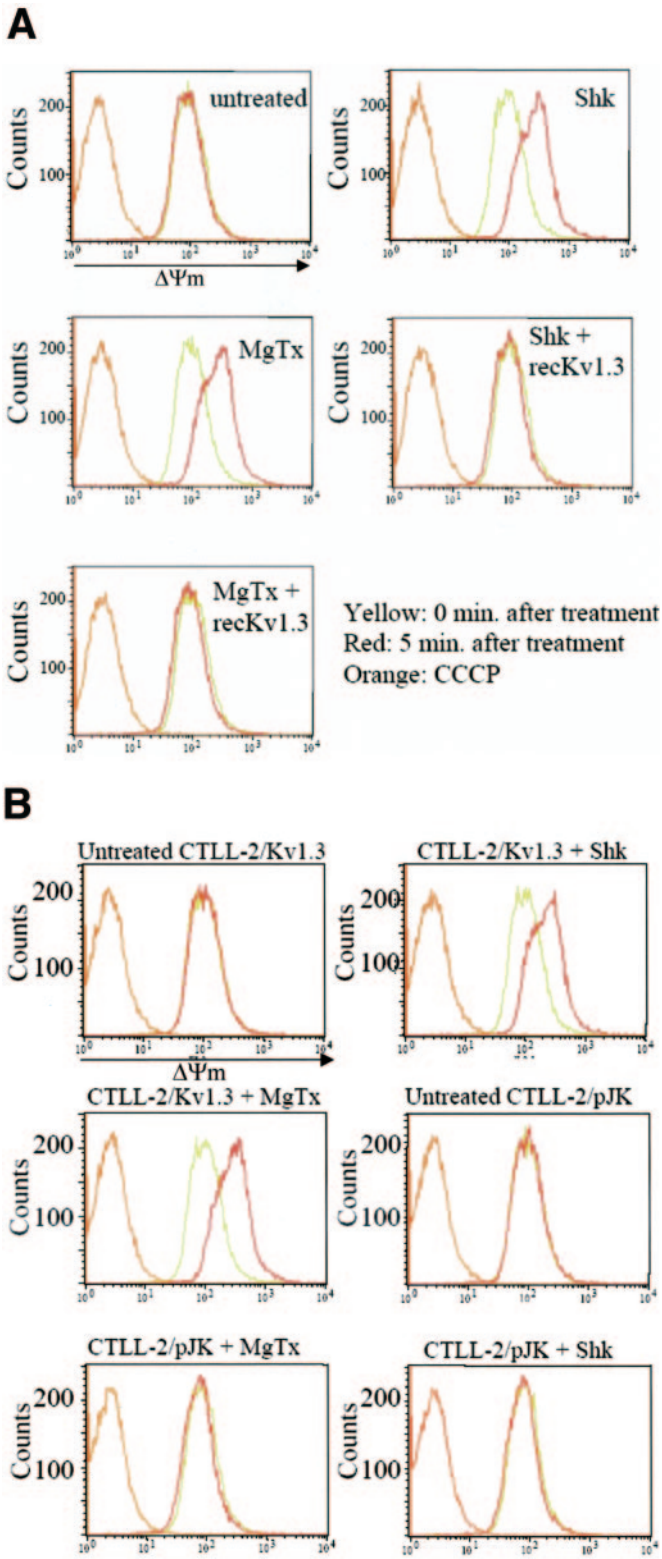

FIG. 6. **Inhibition of mitochondrial Kv1.3 alters mitochondrial membrane potential.** Purified Kv1.3-expressing mitochondria respond to incubation with Shk or MgTx with hyperpolarization of the mitochondrial membrane potential $\Delta\Psi m$ (*A*). Addition of recombinant GST-Kv1.3 during the 30-min incubation blocked the effects of Shk or MgTx on Kv1.3-positive mitochondria isolated from Jurkat cells (*A*). Kv1.3-deficient mitochondria from CTLL-2/pJK cells failed to respond to Shk or MgTx (*B*). All experiments were repeated at least three times.

is indicated by the sensitivity of the channel to MgTx, which acts by binding in the outer-facing vestibule, the characteristics of the open channel I/V curve in symmetrical high K^+ solution, and the labeling of permeabilized mitochondria in the FACS analysis by anti-Kv1.3 antibodies, raised against an extracellular portion of the protein. The sensitivity to margatoxin from the outer side also indicates that endoplasmic reticulum vesicles containing Kv1.3 do not contribute to the observed phenomena, because the orientation of the channel in the vesicles is expected to be the opposite.

The data presented show that inhibition of Kv.1.3 by pharmacological inhibitors, *i.e.* Shk and MgTx, results in hyperpolarization of mitochondria suspended in K^+ medium within 5 min. The time scale of the response is compatible with a hyperpolarization because of changes in ion fluxes. Kv1.3-positive mitochondria isolated from Jurkat and CTLL/Kv1.3 were sensitive to these toxins, whereas deficiency of Kv1.3 in CTLL/pJK or neutralization of the toxins by recombinant Kv1.3 proteins prevented mitochondrial effects of the toxins (Fig. 6*B*). This indicates the specificity of the observed interaction between Kv1.3 and Shk or MgTx.

Electrophysiological experiments on the plasma membrane of the T lymphocyte reveal that only a small fraction of Kv1.3 is open at resting potential (V_r, approximately −50 mV) thus mediating the efflux of potassium ions from the cytoplasm. Still, these open channels significantly contribute to the maintenance of V_r, given that their inhibition by margatoxin induces a depolarization by 30 mV (30). The fact that specific inhibitors of Kv1.3 alter the $\Delta\Psi m$ of isolated mitochondria points to the presence of a potassium channel that is active at negative resting mitochondrial potentials and mediates the influx of potassium into mitochondria according to the electrochemical gradient, as stated above. Unfortunately, it is impossible to investigate by patch clamp whether Kv1.3 is in fact active at physiological potentials, because mitoplast membrane patches do not resist voltages higher than 120 mV in our hands. In any case, the fluorescence data mentioned above together with the lack of strong voltage dependence of Kv1.3 in mitoplasts indicate that the regulation of Kv1.3 is different in the plasma membrane and in the mitochondria, at least under our experimental conditions. This might be mediated by the presence of unknown factors, which attenuate voltage dependence and/or a marked hyperpolarizing shift in the voltage dependence of activation ($V_{½}$). In this respect it is interesting to note that the plant Shaker channel AKT2 switches between two gating modes, giving rise to either leak-like or to time-dependent, hyperpolarization-activated currents. This switch is controlled by an unidentified post-translational factor (46). Similarly, the animal KCNK2 channel behaves as a leak channel when dephosphorylated and as a depolarization-activated outward rectifier upon phosphorylation (47). In general, several factors may be important for the regulation of mitochondrial Kv1.3. For instance, the mitochondrial inner membrane contains little or no cholesterol. Cholesterol depletion or inhibition of sphingolipid synthesis has been shown to alter Kv1.5 function by inducing a slight hyperpolarizing shift in $V_{½}$, (48). Accordingly, a right shift in $V_{½}$ occurred in the case of Kv1.3 upon cholesterol loading (49) or the addition of sphingosyl-phosphorylcholine (50). Another factor that may originate the differences between the properties of plasma membrane and the mitochondrial Kv channel might be glycosylation of the channel protein, because *N*-glycolysation of Kv1.1 has been reported to affect the gating of the channel (51). Gating properties of Kv channels may be regulated also by the presence or absence of β-subunits (52–54). For instance, RCK1, a fast inactivating Shaker Kv channel, became slowly inactivating in the absence of β-subunits (52). A potassium channel β-subunit has been identified recently by proteomics in plant mitochondria (55), but whether β-subunits are present in mammalian mitochondria is not

known. In the important planar bilayer study of Spencer *et al.* (41) with purified α-subunits, the loss of the β-subunit and/or of a regulatory factor may be the reason for the actual lack of inactivation and of a strong voltage dependence of Kv1.3. Thus, the properties of Kv1.3 observed in our studies in mitochondria are within the range of Kv1.3 behavior reported in the literature.

Our studies show the presence of Kv1.3 in the plasma and inner mitochondrial membranes and provide further evidence that the same protein can have multiple localizations within a cell. Kv1.3 does not exhibit a classical basic N-terminal mitochondrial targeting presequence. Moreover, the TargetP algorithm (56) predicts no mitochondrial localization for this protein (probability of 0.049). Several mitochondrial carriers of the inner membrane that are characterized by numerous transmembrane segments, similarly to Kv1.3, also lack an N-terminal sequence but become imported into the mitochondria in a Tim22 complex-dependent pathway. The targeting information seems to comprise several distant amino acids spread throughout the entire protein (57). With regard to mitochondria, a dual distribution has also been reported for other proteins, namely for the voltage-dependent anion channel (58, 59), the ryanodine receptor (60), and the Ca^{2+}-activated BK-potassium channel (15), whose activities are present both in mitochondrial membranes and in the plasma membrane. The sodium/potassium ATPase α-subunit (61) and the calcium-transporting ATPase (62) of plasma membrane have also been found in purified mitochondria by proteomic approach. Mitochondrial chloride intracellular channels have been localized to the cytoplasm and mitochondria (63) and recently even to the nucleus (64). Although specific post-translational protein modifications and differential splicing have been hypothesized to account for organellar protein targeting, the mechanisms responsible for the simultaneous targeting of a protein to different cellular compartments are not known and are beyond the focus of the present work.

The present work identifies a previously unknown mitochondrial K^+ channel, which is most likely to be partly responsible for the much studied "basal" K^+ conductance in these organelles (1, 4). Most interestingly, examination of Kv1.3-deficient mice (65) led to the discovery that Kv1.3, which seems to be abundantly expressed in brown and white fat, regulates energy homeostasis and body weight (22). Kv1.3 might therefore exert its regulatory effect on the basal metabolic rate directly in the mitochondria. Our finding that mitochondria contain Kv1.3 may also provide a plausible explanation for the fact that in actinomycin D-induced cell death mitochondria-associated events required expression of Kv1.3 (28). In addition, mitochondrial potassium influx, independently of the nature of the involved channel, is emerging as a crucial factor in mitigating hypoxia injury (17, 66). Thus, mitochondrial Kv1.3, at least in certain tissues, may represent an important player in the apoptotic signal transduction, a hypothesis that will be investigated by exploiting the genetic model used in this study.

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