

# Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes

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The potassium channel Kv1.3 has recently been located to the inner mitochondrial membrane of lymphocytes. Here, we show that mouse and human cells that are genetically deficient in either Kv1.3 or transfected with siRNA to suppress Kv1.3-expression resisted apoptosis induced by several stimuli, including Bax over-expression. Retransfection of either Kv1.3 or a mitochondrial-targeted Kv1.3 restored cell death. Bax interacted with and functionally inhibited mitochondrial Kv1.3. Incubation of isolated Kv1.3-positive mitochondria with recombinant Bax, t-Bid, or toxins that bind to and inhibit Kv1.3 successively triggered hyperpolarization, formation of reactive oxygen species, release of cytochrome *c*, and marked depolarization. Kv1.3-deficient mitochondria were resistant to Bax, t-Bid, and the toxins. Mutation of Bax at K128, which corresponds to a conserved lysine in Kv1.3-inhibiting toxins, abrogated its effects on both Kv1.3 and mitochondria. These findings suggest that Bax mediates cytochrome *c* release and mitochondrial depolarization in lymphocytes, at least in part, via its interaction with mitochondrial Kv1.3.

ion channels | mitochondria

Mitochondria are key organelles of the intrinsic apoptotic pathway, mediating cell death in pathological and stress conditions via the release of proapoptotic factors (1). This release is often a direct consequence of the activation of proapoptotic Bcl-2 family proteins, in particular Bax and Bak (2), although release can also be induced in cells lacking these proteins (3). However, at present, it is unknown how Bax and Bak mediate these apoptotic events.

We investigated the role of mitochondrial ion channels in apoptosis mediated by Bax. Recent pharmacological data demonstrated an important role of calcium-dependent (4) and ATP-regulated (5) mitochondrial potassium channels in the control of apoptotic death. Although apparently conflicting data exist on the function of these channels in apoptosis, their activation appears to protect against cell death induced by massive ischemia (4, 5). Furthermore, LETM1 (leucine zipper-, EF-hand-containing transmembrane protein 1), a putative component of the K<sup>+</sup>/H<sup>+</sup> antiporter of the inner mitochondrial membrane (IMM), has recently been shown to control cell viability (6). Thus, potassium fluxes across the IMM are emerging as regulators of cell death in various systems.

Here, we define the molecular role of the mitochondrial potassium channel Kv1.3 in apoptosis. Activation of plasma membrane Kv1.3 is intimately involved in T cell proliferation and maturation (7), but studies employing actinomycin D to induce cell death also suggest the importance of Kv1.3 in apoptosis (8). We have recently reported that, like other channels expressed in multiple subcellular locations (9–12), the potassium channel Kv1.3 is present in a functionally active form not only in the plasma membrane, but also in the IMM of lymphocytes (13). The present study demonstrates that IMM Kv1.3 interacts with Bax in a toxin-like mode to trigger cell death.

## Results

**Mitochondrial Kv1.3 is Critically Involved in Apoptosis.** To address the role of Kv1.3 in apoptosis, we used a genetic model in which

Kv1.3-deficient CTLL-2 cells (14) were transfected with either an expression vector for Kv1.3 (*pJK-Kv1.3*) (cells designated CTLL-2/Kv1.3) or control vector (*pJK*) (designated CTLL-2/pJK) (13, 15).

Incubation of Kv1.3-reconstituted CTLL-2/Kv1.3 cells, Jurkat cells, or activated human peripheral blood lymphocytes (PBL) with TNF $\alpha$ , staurosporine, sphingomyelinase, or C<sub>6</sub>-ceramide resulted in DNA fragmentation, cytochrome *c* release, mitochondrial depolarization, and morphological alterations typical of apoptosis, whereas Kv1.3-deficient CTLL-2/pJK cells were resistant (Fig. 1*A*, and data not shown). Prolonged (24–36 h) incubation of CTLL-2/pJK cells with these stimuli finally resulted in apoptosis, suggesting that Kv1.3 is involved in an amplification loop, such as the mitochondrial pathway that mediates apoptosis.

To confirm the role of Kv1.3 in apoptosis, we transfected either activated human PBL or Jurkat cells with either siRNA, which almost completely suppressed both the expression and activity of Kv1.3 in the transfected population [supporting information (SI) Fig. S1], or control siRNA, respectively, which was without effect on Kv1.3. Suppression of Kv1.3 inhibited the proapoptotic effect of staurosporine that was used as a paradigmatic drug to stimulate the mitochondrial pathway of apoptosis (Fig. S1*A* and *B*). Almost identical results were obtained for the induction of apoptosis in Jurkat cells transfected with siRNA to target Kv1.3 (data not shown).

To specifically address the role of intracellular Kv1.3 in apoptosis, we transfected CTLL-2/Kv1.3 and control CTLL-2/pJK cells with an expression vector for Bax and determined whether apoptosis was triggered by over-expression of Bax (Fig. 1*C*). Bax over-expression triggered massive apoptosis of Kv1.3-positive cells (CTLL-2/Kv1.3 and Jurkat), whereas Kv1.3-deficient CTLL-2 cells were resistant to Bax. These data establish a critical role of Kv1.3 in the induction of apoptosis and suggest an important role of intracellular Kv1.3 in Bax-induced apoptosis.

To directly test the role of mitochondrial Kv1.3 in apoptosis, we transfected Kv1.3-deficient CTLL-2 cells with an expression vector for an EYFP-Kv1.3 construct that specifically targets the expression of Kv1.3 in mitochondria (*pJK/mito-EYFP-Kv1.3*). Transfection with this mitochondrial Kv1.3 construct restored apoptosis in CTLL-2 cells when treated with either TNF $\alpha$  or staurosporine (Fig. 1*B*), whereas cells lacking Kv1.3 did not show significant apoptosis 12 h after stimulation. Mitochondrial expression (Fig. S2*A*) and orientation of Kv1.3 (Fig. S2*B*), mito-EYFP-Kv1.3 (Fig. S2*C*), or

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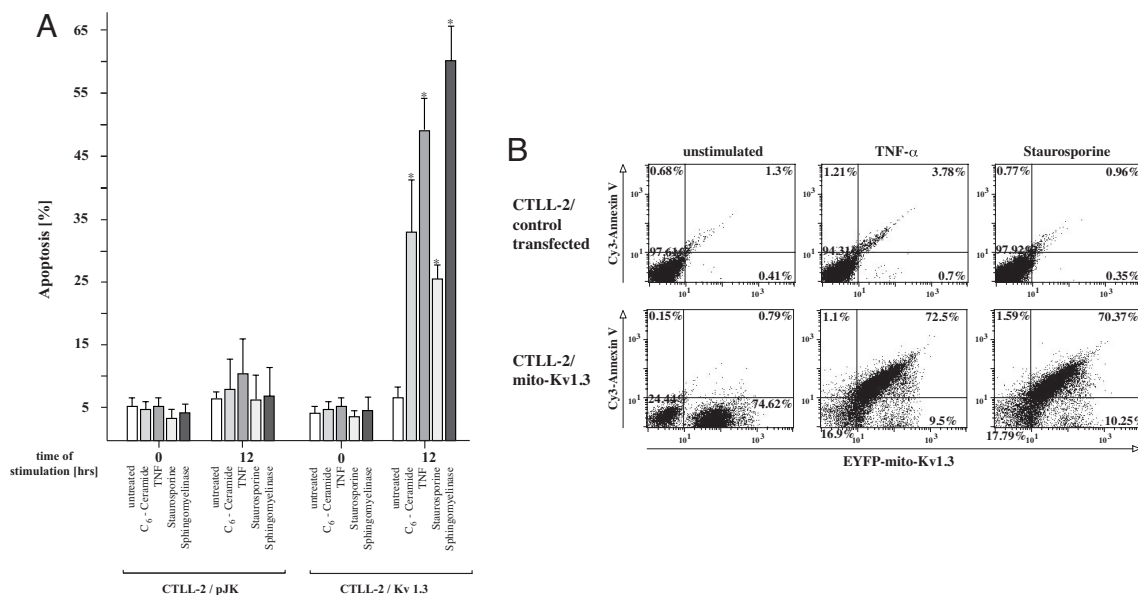
The authors declare no conflict of interest.

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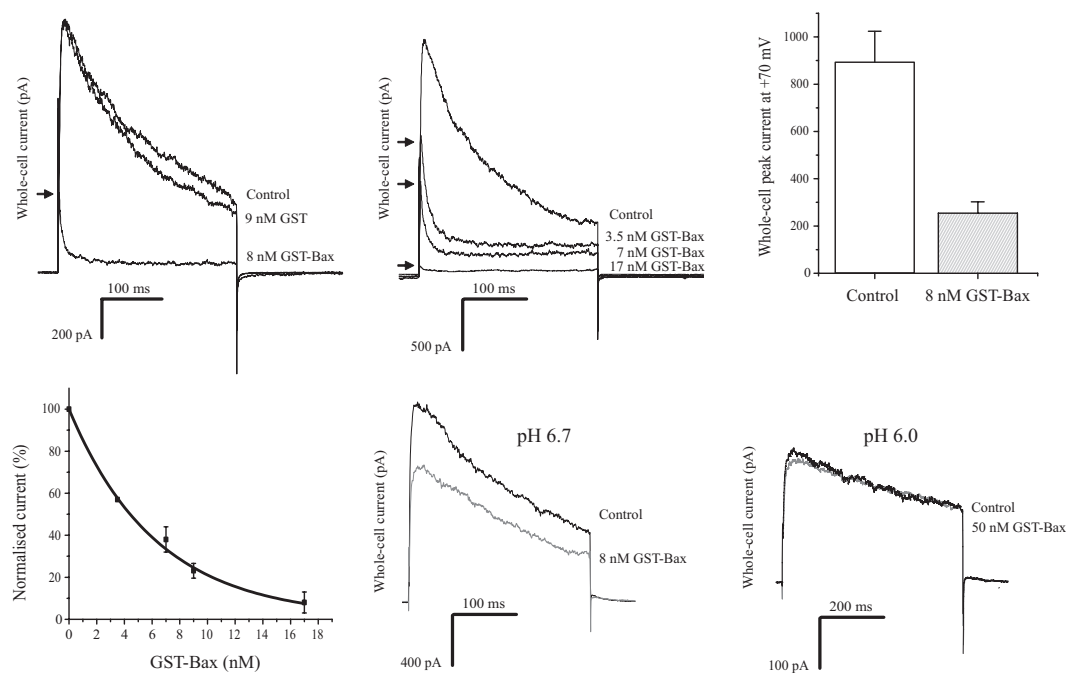


**Fig. 1.** Kv1.3 is required for Bax-, TNF $\alpha$ -, staurosporine-, sphingomyelinase-, and C<sub>6</sub>-ceramide- induced apoptosis. (A) Treatment of CTLL-2/Kv1.3 cells with 20  $\mu$ M C<sub>6</sub>-ceramide, 1  $\mu$ M staurosporine, 100 ng/ml TNF $\alpha$ , or 1 u/ml sphingomyelinase results in DNA fragmentation indicative of apoptosis. CTLL-2/pJK cells lacking Kv1.3 were resistant. Means  $\pm$  SD are shown ( $n = 3$ ; \*,  $P \leq 0.05$ ,  $t$  test). (B) Transfection of Kv1.3-deficient CTLL-2 cells with a construct that specifically targets Kv1.3 expression to mitochondria (pJK/EYFP-mito-Kv1.3) restores apoptosis in these cells when stimulated with 100 ng/ml TNF $\alpha$  or 1  $\mu$ M staurosporine. Cells were stained with fluorescent Annexin to determine apoptosis. Shown are representative data of each three independent experiments.

translocation of Bax (Fig. S2 D and E) were confirmed in control experiments.

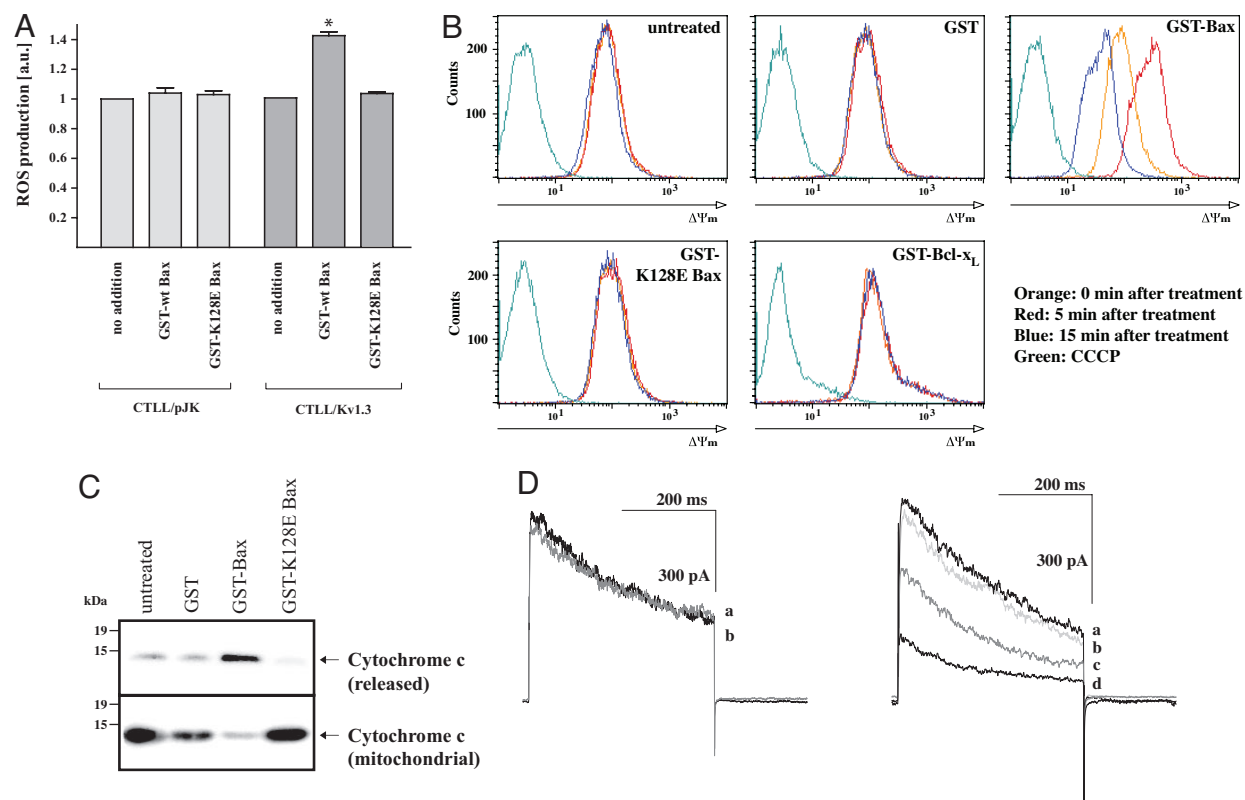
To test the role of an additional Kv channel for apoptosis triggered by staurosporine, we transfected CTLL-2 cells that lack

expression of all known Kv channels with an expression vector of either Kv1.1 or control vector. The results (Fig. S3) demonstrate that Kv1.1 is localized in mitochondria after transfection of CTLL-2 cells and, most importantly, that expression of Kv1.1 in CTLL-2



**Fig. 2.** Bax physically interacts with and inhibits Kv1.3. Patch-clamp studies reveal that recombinant GST-Bax inhibits Kv1.3. (*Upper*) (*Left*) Whole-cell currents (voltage from  $-70$  to  $+70$  mV at 45-sec intervals) in Jurkat lymphocytes. GST-Bax (8 nM) added to the same patch inhibited current within 135 sec; control GST-protein (9 nM) did not decrease the current within 450 sec. (*Center*) Currents elicited at  $+70$  mV at various GST-Bax concentrations. Arrows indicate peak currents after addition of GST-Bax. (*Right*) whole-cell mean peak currents (at  $+70$  mV) for controls ( $n = 12$ ) and GST-Bax-treated cells ( $n = 8$ ). (*Lower*) (*Left*) Dose-response curve ( $n = 3$ ) yielding an  $IC_{50}$  of 4 nM. (*Center and Right*) A change of pH to 6.7 strongly reduces the inhibitory effects of GST-Bax on Kv1.3; at pH 6.0 of the bath solution, inhibition of Kv1.3 is abolished even with 50 nM GST-Bax. Currents are recorded (at  $+70$  mV) in the absence (black line) and presence (gray line) of Bax.  $IC_{50}$  for GST-Bax at pH 6.7: 12 nM ( $n = 3$ ) and at pH 6.0:  $\gg 50$  nM ( $n = 4$ ). In another set of experiments, currents at  $+70$  mV were measured in control conditions at pH 6.7 ( $1,076 \pm 127$  pA,  $n = 14$ ) and with 20 nM GST-Bax at pH 6.7 ( $320 \pm 76$  pA,  $n = 4$ ).





**Fig. 4.** K128 of Bax is critical to block Kv1.3. (A–C) Mutation of Bax at K128 to glutamate (5 nM GST-Bax K128E) abrogated proapoptotic effects of Bax and prevented the release of ROS (A), changes in  $\Delta\Psi_m$  (B), and release of cytochrome c (C), which occurred after incubation with 5 nM GST-Bax. (\*,  $P \leq 0.05$  compared with control,  $n = 3$ ,  $t$  test). (D) (Left) K128E Bax (10 nM) does not decrease Jurkat whole-cell Kv1.3 currents (at +70 mV). Black: control; gray: 405 sec after addition of K128E Bax to bath. In five similar experiments, addition of K128E Bax did not significantly alter the current ( $-12 \pm 7\%$ ,  $P = 0.663$ ). Preincubation with K128E Bax did not induce a significant change in peak current at 70 mV [ $693 \pm 68$  pA for control ( $n = 15$ ) and  $583 \pm 64$  pA for K128E Bax-treated cells ( $n = 11$ ),  $P = 0.274$ ]. (Right) Current traces obtained from the same patch in trace a: control conditions; trace b: after addition of 3 nM K128E Bax. Traces c and d were obtained after washing and subsequent addition of wild-type Bax to a final concentration of 3 nM in trace c and 12 nM in trace d.

because the orientation of the channel in mitochondria is the same as in the plasma membrane as suggested by the sensitivity of the mitochondrial channel to MgTx (13). Previous studies indicated that the binding of the highly specific Kv1.3 inhibitor toxin (e.g., MgTx), which docks in the outer-facing vestibule of Kv1.3, is pH dependent, and that protonation of Histidine 404 of Kv1.3 weakens the pore-toxin interaction (18). Accordingly, the  $IC_{50}$  of GST-Bax increased from 4 to 12 nM when the bath solution pH was lowered to pH 6.7 and to a value  $\gg 50$  nM at pH 6.0, indicating a toxin-like interaction between Bax and the external vestibule of Kv1.3 (Fig. 2). The Bax-Kv1.3 interaction involves parts of the channel that face the extracellular (or IMM) space (Fig. S5).

Coimmunoprecipitation experiments confirmed that Kv1.3 and Bax physically interacted in both mouse and human cells only upon induction of apoptosis (Fig. S6).

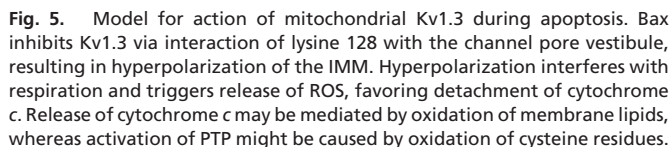
Finally, we isolated mitochondria from either untreated or staurosporine-treated PBL or CTLL-2/Kv1.3 cells. Bax associated with mitochondrial Kv1.3 only in those mitochondria that were isolated from apoptotic cells (Fig. S6). In the unstimulated sample, a small fraction of Bax was present in mitochondria as the protein has been previously shown to be loosely associated with the outer mitochondrial membrane, but was not inserted into the membrane (19) and does not interact with IMM-located Kv1.3. In summary, the data indicate a direct interaction of Kv1.3 with Bax.

**Bax Plus t-Bid Triggers Kv1.3-Mediated Hyperpolarization, Depolarization, and Cytochrome c Release in Isolated Mitochondria.** To determine whether the interaction of Kv1.3 with Bax is critically involved in apoptosis, we incubated isolated, purified mitochondria with

recombinant full-length Bax, recombinant GST-Bax, or control GST. Whereas GST-Bax is active without further modification, full-length Bax requires t-Bid-mediated activation. The latter was added together with a very low concentration of t-Bid (0.1 nM), which was previously shown to be without effect if added alone (18, 20). We also treated the mitochondria with 5 nM t-Bid which was previously shown to trigger activation of Bak and Bax, which are either constitutively present in mitochondria or, to some degree, loosely bound to the outer mitochondrial membrane, respectively (20, 21). Recombinant full-length Bax, GST-Bax, and t-Bid (5 nM) triggered hyperpolarization, followed by depolarization of the mitochondrial membrane (Fig. 3A) only in Kv1.3-positive mitochondria. Hyperpolarization is compatible with the inhibition of Kv1.3 that carries a depolarizing  $K^+$ -influx and was prevented by substitution of external potassium with sodium (Fig. S7). Incubation of isolated Kv1.3-positive mitochondria with either Bax or t-Bid also resulted in a rapid release of cytochrome c from isolated Kv1.3-positive mitochondria, indicating apoptotic changes in these organelles (Fig. 3B). GST was without effect on mitochondria (Fig. 3A and B). Importantly, similar changes, both qualitatively and quantitatively, were detected when either full-length Bax or GST-Bax were added to mitochondria that were isolated from genetically manipulated (CTLL-2/Kv1.3) and nonmanipulated (Jurkat) cells.

Next, we measured whether GST-Bax induces a Kv1.3-dependent increase in ROS release (see also *SI Text*). The data show that GST-Bax induced a 1.5- to 2-fold increase in the rate of ROS production in Kv1.3-expressing mitochondria, whereas mitochondria from CTLL-2/pJK cells did not respond (Fig. 3C). ROS release induced by GST-Bax was similar to that observed in the





presence of the respiratory chain inhibitor Antimycin A, previously shown to trigger ROS production and apoptosis (22) (Fig. 3C). The antioxidants DTT, Tiron, and BHT and the PTP inhibitor CSA did not affect hyperpolarization but prevented mitochondrial depolarization (Fig. S8 A and B). These antioxidants also inhibited the release of cytochrome *c* (Fig. 3D and data not shown).

**Kv1.3 Toxins Trigger Apoptotic Changes in Isolated Mitochondria.** To confirm that inhibition of Kv1.3 by Bax mediates apoptotic changes in mitochondria, we tested whether pharmacological inhibitors of Kv1.3 (i.e., ShK, MgTx, and Psora-4) induce the same alterations as Bax. ShK and MgTx are positively charged peptide toxins that interact with negatively charged residues in the Kv1.3 protein vestibule to block the pore. Psora-4 [5-(4-phenylbutoxy-psoralen)] (23) is the most potent synthetic inhibitor of Kv1.3 available. All three compounds triggered the same changes as Bax in Kv1.3-positive isolated mitochondria (Fig. S9). Kv1.3-deficient mitochondria from CTLL/pJK cells did not respond to the toxins (Fig. S9), indicating the specificity of the effects of the inhibitors. Depolarization induced by toxins was also prevented by DTT and cyclosporin A (data not shown).

**Bax Interacts with Kv1.3 Via a Toxin-Like Mechanism.** All toxins that block Kv1.3 contain a lysine residue, which is critical for the interaction with Kv1.3 (7, 24) (Fig. S10). A model of the structure of the membrane-integrated Bax monomer indicates that at least amino acids 127 and 128, located between the 5th and 6th helices of Bax, protrude from the outer mitochondrial membrane into the intermembrane space (25). The amino acid in position 128 is a highly conserved, positively charged lysine, which may mimic the action of the critical lysine in Kv1.3-blocking toxins (Fig. S10) by binding to the ring of four aspartate residues (24) of the channel vestibule, which faces the intermembrane space in mitochondria (13). Furthermore, antiapoptotic proteins contain a negative charge in the corresponding position (amino acid 158 for Bcl-x<sub>L</sub>) (Fig. S10). Mutation of lysine 128 in GST-Bax to a negatively charged glutamate (GST-K128E Bax) prevented ROS release (Fig. 4A), hyperpolarization/depolarization of the mitochondrial membrane potential (Fig. 4B), and release of cytochrome *c* (Fig. 5C); this mutation also prevented the inhibition of Kv1.3 in whole-cell patch clamp experiments (Fig. 4D). The mutant GST-K128E Bax still integrated into the mitochondrial membrane and formed ion channels when reconstituted into black lipid bilayers (M.Z., unpublished results), indicating that the protein conformation is not significantly altered by the mutation. GST, Bcl-x<sub>L</sub>, and Bcl-2 were without effect in all of these experiments.

Our results identify mitochondrial Kv1.3 as a target for Bax and indicate that Kv1.3 is required for induction of apoptosis by Bax, at least in lymphocytes. The amino acid residue K128 in Bax seems to be particularly important for the interaction of Bax with Kv1.3 because mutation of this lysine to a glutamic acid abrogated inhibition of Kv1.3 by Bax and the proapoptotic activity of Bax. The finding that mutation of a single, critical amino acid controls the interaction of a protein/peptide with a channel finds a precedent in the interaction of agitoxin with KcsA (26). Our data suggest that Bax and the toxins ShK and MgTx act on Kv1.3 in a functionally similar way.

Bax-mediated inhibition of Kv1.3 results in hyperpolarization and ROS release, which are upstream of both cytochrome *c* release (27) and PTP activation as indicated by the experiments with radical scavengers. ROS may also play multiple roles in apoptosis, i.e., to induce the dissociation of cytochrome *c* from the IMM, PTP activation, and the release of cytochrome *c* from mitochondria upon formation of pores by oligomerized Bax (28).

Because the inactivation of Kv1.3 by Bax, ShK, MgTx, and Psora-4 provides a positive signal, i.e., hyperpolarization of the mitochondrial membrane and ROS release, which are absent in Kv1.3-deficient cells, deficiency of Kv1.3 is clearly not equivalent to inhibition of the channel. A similar situation was found in rodent beta islet cells, in which either glucose- or drug-induced  $K_{ATP}$  channel closure led to insulin secretion, whereas the lack of a functional channel resulted in greatly reduced rather than increased glucose-induced insulin release (29).

The interaction of Bax with ion channels may not be restricted to Kv1.3; other mitochondrial Kv channels, such as Kv1.5 (16), may have similar functions. Furthermore, because up-regulation of Kv1.1 (and of a chloride channel) occurs in genetically modified Kv1.3 knockout mice (30), Kv1.1 up-regulation may reconstitute apoptosis in these animals as suggested in the present studies. CTLL-2 cells do not express Kv1.1 (13, 14) enabling us to study the role of Kv1.3 in apoptosis. Conversely, mitochondrial  $K_{ATP}$  channels (31) are not central to Bax-induced apoptosis in CTLL-2/Kv1.3 cells, although this does not exclude a function of  $K_{ATP}$  channels in other cells and contexts.

In summary, our studies establish a direct link in lymphocytes between mitochondrial potassium conductance and the proapoptotic machinery in the cytoplasm, indicating a channel-blocking action of Bax (Fig. 5).

**Cell Culture, Recombinant Proteins, Transient Transfections, and Cellular Apoptosis Assays.** All cells were cultured as previously described and detailed in the *SI Text*. Bax (amino acids 1–170) was cloned into pGEX-3X, expressed in BL21A1, and purified from bacterial lysates using glutathione-Sepharose (for details see *SI Text*). The K128E mutant of Bax was obtained by a site-directed mutagenesis PCR technique (Stratagene), cloned into pGEX-3X, and expressed and purified as a GST-fusion protein as above. Controls confirm that K128E Bax still integrates into the mitochondrial membrane, indicating that the protein conformation is not significantly altered by the mutation.

Full-length recombinant hBax (32) was kindly provided by J. C. Martinou (University of Geneva, Geneva). Purified t-Bid was purchased from Axxora.

For the siRNA experiments, cells were transiently transfected with 20 nM Alexa Fluor 488- or Cy3-coupled siRNA molecules by electroporation at 400 V with 5 pulses, 3 msec each, using a BTX electroporator. Dead cells were removed via Ficoll purification after 24 h. The cells were used for apoptosis or patch-clamp assays 36–48 h after transfection.

CTLL-2/pJK or CTLL-2/Kv1.3 cells ( $2 \times 10^7$  cells per sample) were cotransfected with 10  $\mu$ g of an expression vector of GFP-tagged actin (pcDNA-EGFP-actin) and 50  $\mu$ g of pcDNA-Bax (kindly provided by M. Weller, University of Tuebingen, Germany). Cells were electroporated as above and cultured for an additional 24 h with IL-2. IL-2 was then removed as above, cells were incubated for 8 h, stained with Cy3-Annexin, and analyzed by fluorescence microscopy. Transfected cells were identified by the expression of GFP-tagged actin and at least 400 GFP-positive cells per sample were scored for apoptosis.

Transient transfection of mito-EYFP-Kv1.3. Expression of Kv1.3 that was specifically targeted to mitochondria was achieved by electroporation of  $10^7$  CTL-2 cells with 25  $\mu$ g of an EYFP-tagged Kv1.3 construct that was fused to the mitochondrial targeting sequence of subunit VIII of human cytochrome c oxidase (Clontech) in the expression vector pJK (pJK-mito-EYFP-Kv1.3).

Apoptosis was determined by DNA fragmentation, cytochrome c release, and morphological analysis as described in *SI Text*.

**Patch-Clamp Experiments.** Whole-cell currents were recorded with an EPC-7 amplifier (List) (filter: 1 kHz, sampling rate: 5 kHz), as described in ref. 13 and *SI Text*.

**Coimmunoprecipitations, Anti-Kv1.3 Antibodies.** In the coimmunoprecipitation studies, either cells or mitochondria were lysed in 4% CHAPS, 5 mM  $\text{MgCl}_2$ , 137 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl (pH 8.0), and 10  $\mu$ g/ml both aprotinin and leupeptin for 5 min at 4°C, insoluble material was cleared by centrifugation for 5 min, and the supernatants were subjected to coimmunoprecipitation experiments with either rabbit anti-Kv1.3 or anti-Bax antibodies. West-

ern blots were developed with the corresponding antibodies using the ECL system as detailed in *SI Text*.

**Cytochrome c and ROS Release From, and Membrane Potential in, Isolated Mitochondria.** Cells were homogenized by using a Dounce homogenizer and the soluble fraction isolated. Cytochrome c was analyzed by Western blotting as described in *SI Text*. Amplex Red fluorimetric assays and the change of cytochrome c absorption were used to determine ROS production by isolated mitochondria. The membrane potential of isolated mitochondria was determined by FACS analysis of DiOC<sub>6</sub> (3)-stained mitochondria treated as indicated.

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