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Role of Kv1.3 mitochondrial potassium channel in apoptotic signalling in lymphocytes

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ABSTRACT

Mitochondria have been shown to play a pivotal role in apoptotic signalling in various cell types. We have recently reported that in lymphocytes the voltage-gated potassium channel Kv1.3, known to reside in the plasma membrane, is active also in the inner mitochondrial membrane. Upon induction of apoptosis, outer-membrane inserted Bax binds to and inhibits Kv1.3 resulting in hyperpolarization, an increase in reactive oxygen species production and cytochrome *c* release. In cells lacking Kv1.3 these events do not take place. Here, we present new data which further corroborates an important role of this channel in the sequence of events leading to Bax-induced cytochrome *c* release. Recombinant Kv1.3, when pre-incubated with Bax, prevents the actions of Bax at the level of mitochondria. Furthermore, we report the presence of Kv1.3 protein in mitochondria from PC3 and MCF-7 cancer cells, suggesting that this channel might play a role in the apoptotic signalling not only in lymphocytes but also in other cells.

1. Introduction

Several plasma membrane ion channels play an essential role for cell proliferation. A cell cycle-dependent function has been demonstrated for some voltage-gated potassium channels (e.g. ether à go-go [1]), Ca^{2+}-dependent potassium channels as well as calcium and chloride channels (for reviews see e.g. [2–5]). Along with other membrane conductances, these channels control the membrane voltage and Ca^{2+} signalling as well as intracellular ion concentration, cytosolic pH and cell volume in proliferating cells and thus participate in the regulation of the cell cycle, known to be altered in cancer cells. Ion channels have an impact also on programmed cell death (apoptosis), a process shown to be defective in many cancer types. Various plasma membrane channels have been shown to be regulated during apoptosis. Voltage-gated potassium channels [6–8], IP3R [9], the ATP-gated ion channel P(2X1) [10], an outwardly rectifying swelling-activated chloride channel [11,12] and calcium release-induced calcium channel (I_{CRAC}) [13] were among the first ion channels shown to be regulated upon induction of apoptosis by various stimuli in different cell types. In general, the plasma membrane-located channels can be easily targeted by specific drugs, therefore ion channels are emerging targets for anti-tumor therapy. Several different channel inhibitors have been shown to impair tumor growth both *in vitro* and *in vivo* (for recent review see [14]).

In addition to plasma membrane-located ion channels, several mitochondrial ion channels, including the permeability transition pore (PTP) and the voltage-dependent anion channel (VDAC) have been implicated in regulation of apoptosis, especially of events taking place at mitochondria (for review see e.g. [15–17]). Mitochondrial potassium fluxes are important for controlling the proton motive force in energized mitochondria [18–20]. Several agents are being developed for possible tumor therapy that act on mitochondrial potassium channels (for review see e.g. [21]). For example, the potassium channel openers diazoxide and cromakalim, known to affect the mitochondrial as well as the plasma membrane K_{ATP} channels [22], have anti-tumor potential in human neuroblastoma and human astrocytoma [23]. Benzothiazine diazoxide have been shown to decrease the division of leukemic cells by causing mitochondrial membrane depolarization [24]. However certain potassium channel openers such as minoxidil have been shown to

Abbreviations: CHO, Chinese hamster ovary cell line; CTLL-2, interleukin-2 dependent murine cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; IMM, inner mitochondrial membrane; IP3R, inositol triphosphate receptor; MCF-7, human breast adenocarcinoma cell line; MPT, mitochondrial permeability transition; MgTx, Margatoxin; OMM, outer mitochondrial membrane; PC3, human prostate cancer cell line; PMCA, plasma membrane calcium ATP-ase; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATP-ase; ShK, *Stichodactyla* toxin; TNF, tumor necrosis factor α; VDAC, voltage-dependent anion channel

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stimulate the growth of breast cancer cells, while potassium channel blockers like amioradone and dequalinium inhibit it [25]. In accordance, glibenclamide, a K_{ATP} channel blocker acts as an anti-tumor agent for a human gastric cell line [26]. The lack of specificity of most drugs for mitochondrial versus plasma membrane potassium channels as well as contradictory observations (see above) make it difficult to assign a specific role of mitochondrial potassium channels in the regulation of tumor cell growth and/or apoptosis by using only pharmacological strategies.

We have recently set up a genetic model in order to clarify the role of Kv1.3 in the regulation of apoptosis in lymphocytes [27]. Kv1.3 is the predominant type of voltage-gated Kv channel expressed in the plasma membrane in human lymphocytes. Its activation is a key event in T cell proliferation [28]. In accordance, specific inhibitors of Kv1.3 have a strong immunosuppressive effect [29]. As a genetic model, we used interleukin-2 dependent murine cytotoxic T lymphocytes (CTLL-2), known to be deficient for Kv1.3 [30] (CTLL-2/pJK), and stably transfected these cells with Kv1.3 (CTLL-2/Kv1.3) [27]. Either absence (in CTLL-2/pJK cells), or downregulation of Kv1.3 by siRNA in human peripheral T lymphocytes blunted death induced by various apoptotic stimuli [31]. Multiple evidence was obtained in favour of previously not described mitochondrial inner membrane (IMM) localization of the Kv1.3 (mitoKv1.3) in CTLL-2/Kv1.3 as well as in Jurkat lymphocytes [32,33]. In T cells, mitochondria provide a powerful, generally decisive potentiation of the apoptotic process induced by death receptor engagement (for reviews see e.g. [34,35]). The molecular identification of the potassium conductance in T lymphocyte mitochondria (mitoKv1.3) allowed us to determine a critical role of this channel in the regulation of apoptosis. In a previous work [31] we provided evidence that the pro-apoptotic Bcl-2 family member Bax interacts with and inhibits mitoKv1.3 via a lysine residue in position 128. Events known to take place at mitochondria in various apoptotic models, like reactive oxygen species (ROS) production, membrane potential changes and cytochrome *c* release were all dependent on the presence of mitoKv1.3.

In the present work we provide further experimental evidence in favour of our model according to which, at least in lymphocytes, Bax interaction with mitoKv1.3 is a crucial step, which precedes cytochrome *c* release during apoptosis.

2. Materials and methods

Mitochondria were isolated, mitochondrial membrane potential and cytochrome *c* release were determined as described previously [31].

2.1. Confocal microscopy

Cells were washed in phosphate saline buffer (PBS), fixed in PBS-buffered 2% paraformaldehyde (pH 7.3) for 10 min, washed again and permeabilized for 5 min with 0.1% Triton X-100. Cells were washed again and blocked with PBS/1% FCS for 10 min, washed and stained with FITC-labeled rabbit anti-Bax antibodies (UBI) for 45 min at room temperature. Samples were washed 3-times in PBS and incubated for 45 min with Cy3-labeled murine anti-cytochrome *c* antibodies (clone 7H8.2C12, BD-Biosciences). After extensive washing, confocal microscopy was performed and analysed on a Leica confocal microscope DMIRE 2. Sequential scanning was performed in order to exclude cross-detection of FITC signal in the Cy3 channel and vice versa.

2.2. Reactive oxygen species (ROS) formation in intact cells

To determine the formation of reactive oxygen species cells were stimulated as indicated or left untreated. Cells were lysed in 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 10 mM EDTA, 25 mM HEPES pH 7.3, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 1 mg/ml cytochrome *c* (Sigma) as previously described in [36,37]. Samples were immediately transferred to a cuvette, covered with mineral oil and absorbance at 550 nm was determined.

2.3. Expression, purification and polyacrylamide gel (PAGE) analysis of recombinant Bax and Kv1.3

Bax (aminoacid aa 1–170) and Kv1.3 (either aa 319-523 or full length (accession number NM_002232) or full length) were cloned into pGEX-3X, expressed in *E. coli* BL21A1 and purified from bacterial lysates using glutathione-sepharose. Bacteria were lysed in 50 ml of 25 mM HEPES, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF, Na_3VO_4, sodium pyrophosphate, 10 µM each aprotinin and leupeptin (A/L), and 1 mg/ml lysozyme. Samples were incubated on ice for 15 min, brought to 30 mM $MgCl_2$, 5 µg/ml DNAaseI was added, and samples were incubated for an additional 30 min. Insoluble material was clarified by a 50-min centrifugation at 11,000 *g* at 4 °C. The supernatant was collected and 300 µl of glutathione Sepharose (GE Healthcare) were added to immobilize GST-fusion proteins for 1 h at 4 °C. The beads were pelleted by centrifugation at 500 *g* for 2 min, and the supernatant was discarded. The pellet was washed twice with 50 ml of the lysis buffer (minus lysozyme). After the last wash, 49.5 ml of the supernatant was removed, the pellet was resuspended in 5 ml of HEPES/saline (H/S) supplemented with 20 mM glutathione (pH 7.4) and incubated for 30 min at 4 °C to detach the GST-fusion proteins. Samples were centrifuged at 500 *g* for 2 min, the supernatant was collected, diluted with 15 ml of H/S, and concentrated by a 60-min centrifugation through size-exclusion columns (cut-off, 10,000 Da; Viva Science, Sartorius) for Bax and Kv1.3 to 1 ml. The samples were again diluted with 20 ml of H/S and purified via size-exclusion columns. This procedure was performed for a total of five times to ensure elimination of the detergents. Finally, Bax was resuspended in H/S, Kv1.3 in H/S supplemented with micellular 0.1% NP40.

GST-Kv1.3 or GST (each 20 µg) were separated on a 5% polyacrylamide gel (PAGE) containing 0.1% SDS. The sample buffer contained 41 mM Tris/HCl pH 6.8, 10% glycerol and Bromophenol Blue, without dithiothreitol (DTT) and sodium dodecyl sulphate (SDS) and the sample was not boiled before loading for PAGE (Fig. 4). The gel was soaked for one hour before transblotting to polyvinylidene-fluoride (PVDF) membrane in a sampling buffer containing 9% SDS. The blots were developed with rabbit anti-Kv1.3 antibodies (provided by O. Pongs) and ECL. A similar method has previously been shown to allow detection of multimeric forms of a potassium channel from *Streptomyces lividans*, according to Cortes and Perozo [38].

2.4. Binding of Margatoxin to GST-Kv1.3

We immobilized approximately 2 nmol of CHAPS-solubilized, partly tetrameric GST-Kv1.3 on glutathione-agarose, washed the beads, incubated with 0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 4 nmol Biotin-labeled MgTx (volume: 1 ml), washed again and incubated the samples with 1 µg/ml alkaline phosphatase-coupled anti-Biotin antibodies. The samples were washed again and then incubated with AP (alkaline phosphatase) substrate (diaminobenzidine DAB tablets) to convert the colourless substrate into a red dye. The absorption of the samples was determined to measure binding of MgTx-Biotin to GST-Kv1.3.

2.5. Immunoprecipitation

Fusion proteins in the soluble form were subjected to co-immunoprecipitation experiments with a rabbit anti-mouse Kv1.3 antibody, kindly gifted by O. Pongs, directed against the external epitope 409–525 of human Kv1.3 (cross-reactive with rat and mouse) or a commercial rabbit anti-Kv1.3 antibody (from Alamone Lab.)

directed against the epitope aa 471–523 of human Kv1.3 (cross-reactive with mouse Kv1.3). Both antibodies gave the same results, revealing co-immuno-precipitation of Kv1.3 with Bax. Western blots were done either with the rabbit anti-Kv1.3 antibody from O. Pongs (all data on murine cells) or antibody from Alamone, both antibodies gave the same results.

3. Results and discussion

Recombinant Bax as well as full length Bax has previously been shown to inhibit Kv1.3 channel activity and to physically interact with the mitoKv1.3 protein, only in mitochondria isolated from apoptotic cells [31]. The electrochemical gradient for K^+ in energized mitochondria predicts that K^+ flow through an IMM-located potassium channel should be inward. If an influx of positive charge through mitoKv1.3 is inhibited, a hyperpolarization is expected. Hyperpolarization in turn results in the reduction of respiratory chain components such as Fe/S centers, cytochromes and the ubiquinone pool, and in enhanced production of reactive oxygen species (ROS) (e.g.: [39,40]). In agreement, addition of recombinant Bax or of toxins, known to inhibit Kv1.3 and mitoKv1.3 with high specificity (like MgTx or ShK), to isolated mitochondria in suspension resulted in hyperpolarization and ROS production [31]. ROS production would therefore be expected to be associated with Kv1.3-dependent apoptosis. Fig. 1 illustrates that treatment of CTLL-2/Kv1.3 intact cells with the antioxidants Tiron and N-acetylcysteine prevents the release of ROS as

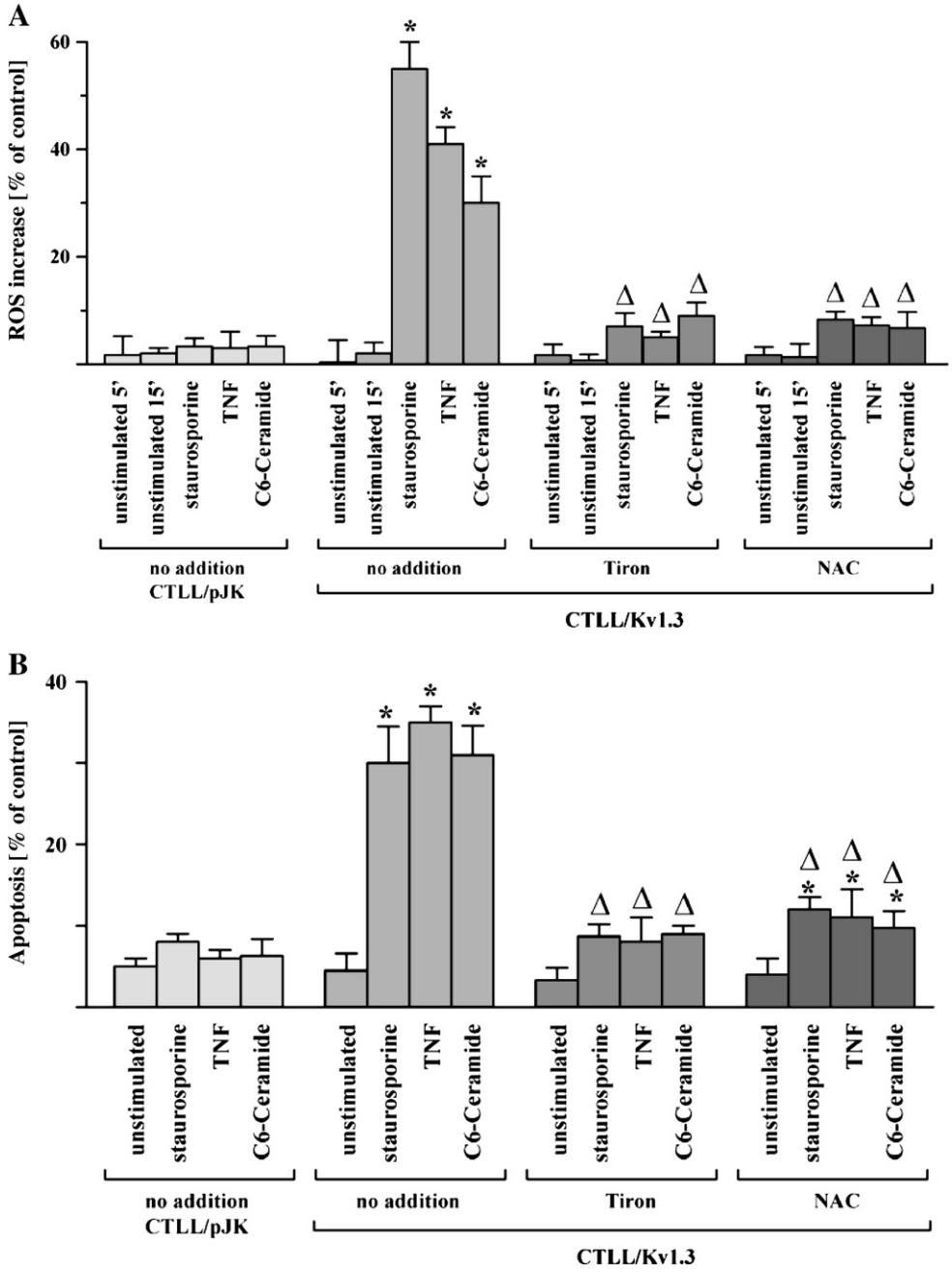


Fig. 1. ROS release and apoptosis are correlated in intact cells. (A) CTLL-2/Kv1.3 cells were pre-treated for 30 min with Tiron (1 mM) or N-acetylcysteine (1 mM, NAC) and then stimulated with 20 μM C_6-ceramide, 1 μM staurosporine or 100 ng/ml TNFα, respectively. ROS was measured as described in the Materials and methods section. (B) Apoptosis was measured by FACS analysis after staining with FITC-Annexin (Roche) for 15 min at 22 °C. Given are the mean ± SD of 3 independent experiments each (*$p < 0.05$, *t*-test to controls, $p > 0.05$ to untreated samples).

well as induction of apoptosis after treatment with staurosporine, C_6-ceramide and tumor necrosis factor (TNF) indicating that indeed apoptosis induced by various factors is dependent on ROS in our system.

ROS are able to oxidize thiol groups and thus to elicit mitochondrial depolarization by activation of the PTP [41–43]. PTP opening and consequent $\Delta\psi m$ decrease downstream of transient mitochondrial hyperpolarization and/or increase in ROS production has been reported in several studies employing drugs or Ca^{2+}-overload to induce apoptosis (e.g.: [44–46]). In our case, Bax-induced hyperpolarization was indeed followed by CSA-sensitive depolarization, indicating that PTP opening was induced [31]. Our data are consistent with previous studies showing a transient hyperpolarization and/or increase of ROS followed by a MPT-mediated decrease of mitochondrial membrane potential upon treatment with, e.g., the cytostatic drug BMD188, ceramide, oxygen–glucose-deprivation or staurosporine to name a few [45,47–49].

ROS have also been shown to oxidize cardiolipin resulting in the release of cytochrome *c* from the inner mitochondrial membrane [50,51]. In accordance, the presence of the channel has been shown to be crucial for both ROS release and Bax-induced cytochrome *c* release as previously assayed by Western blots [31]. Fig. 2 shows confocal microscopy studies on CTLL/pJK and CTLL/Kv1.3 that illustrate translocation of Bax into mitochondria in both cell types upon treatment with staurosporine but release of cytochrome *c* only in Kv1.3-positive cells. These latter cell types express also mitoKv1.3 in their mitochondria, as shown previously [31,32]. The cells were stained with anti-cytochrome *c* and anti-Bax antibodies. Confocal microscopy clearly proves that Bax migrated to mitochondria after staurosporine treatment and was not only present in a layer directly above or below the mitochondria. The signal for cytochrome *c* released in the cytoplasm is relatively weak, since confocal microscopy visualizes thin sections. Please note that T cells are characterized by a U-shaped nucleus, causing the observed distribution of fluorescent signal within the cell. Data of Fig. 2 together with previously published data indicate a translocation of Bax into mitochondria of CTLL-2/pJK cells without release of cytochrome *c*, while the CTLL/Kv1.3 cells do release cytochrome *c* into the cytoplasm upon translocation of Bax.

Our data suggest that inhibition of mitoKv1.3 triggers the release of ROS, which finally mediate release of cytochrome *c*. If hyperpolarization, triggered by the inhibition of mitoKv1.3 by Bax, was necessary for cytochrome *c* release to occur, replacing KCl by NaCl in the suspension medium of isolated mitochondria should prevent these events, since K^+-selective mitoKv1.3 does not carry an inward current under these conditions. Our data confirmed this hypothesis, and showed that Bax-induced hyperpolarization and cytochrome *c* release were prevented by substitution of external potassium with sodium [31]. This result however might seem to be in contradiction with results published by Uren et al. [52] who have shown that NaCl and LiCl (at least 50 mM) worked as well as KCl in permitting the release of cytochrome *c* upon treatment of mitochondria with t-Bid, a BH3-only pro-apoptotic Bcl-2 family member, which activates endogenous Bax that is attached to mitochondria. In the absence of external salt, cytochrome *c* is not released because of its strong electrostatic interactions with negative lipids in the inner mitochondrial membrane. In the studies by Uren et al. [52] 10 mM potassium was however present in all experiments. In mitochondria from Jurkat cells (Fig. 3), 10 mM KCl in the medium is sufficient to allow hyperpolarization of the mitochondrial membrane and ROS production upon addition of Bax. However, cytochrome *c* release does not take place with 10 mM KCl (and no other salt), while it does occur at high salt, in agreement with the findings by Uren et al [52]. In our experiments on KCl substitution with NaCl the salt concentration was the same (100 mM), therefore the interaction of cytochrome *c* with negatively charged phosphatidylserine is fully expected to be equally weakened in the two cases. However, no KCl was present when the NaCl-based medium was used. Thus, the ion substitution experiment indicates that indeed cytochrome *c* release requires potassium as well as a high ionic strength of the external medium for complete detachment.

If our model is correct, addition of recombinant Kv1.3 channel protein which competes with endogenous mitoKv1.3 for Bax binding should be able to prevent the effects of Bax on mitochondria. A fusion protein consisting of a GST-tag and aa 319–523 of Kv1.3 was chosen for these experiments. A full length GST-Kv1.3 fusion protein was also produced, although with low expression efficiency. Full length Kv1.3 has previously been shown to be purified with similarly low efficiency

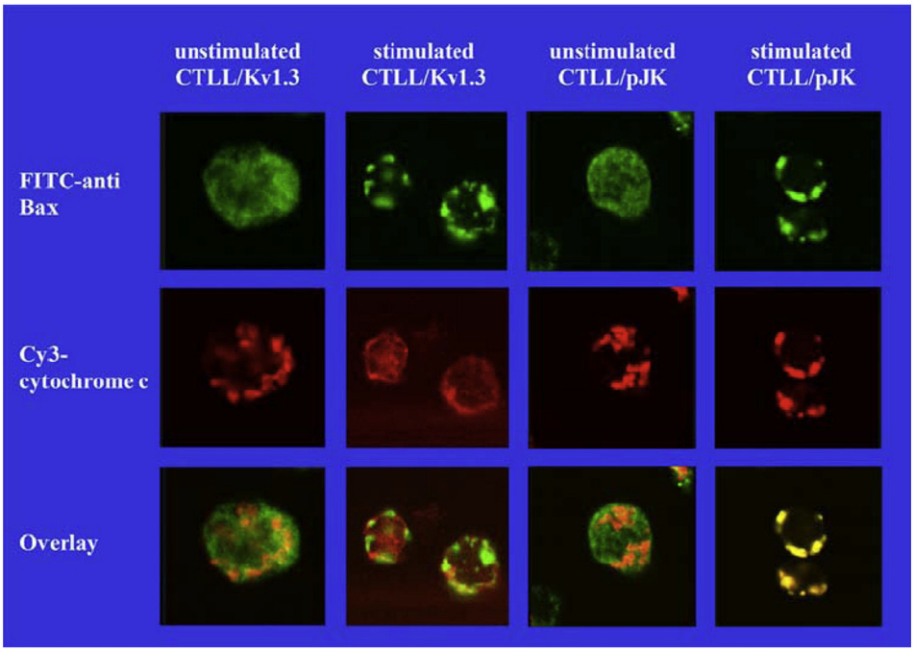


Fig. 2. Bax migrates to mitochondria but does not induce the release of cytochrome *c* in Kv1.3-deficient CTLL-2/pJK cells. CTLL/Kv1.3 or CTLL/pJK cells were stimulated for 1 h with 1 μM staurosporine, and stained with anti-Bax antibodies and with anti-cytochrome *c* antibodies. Confocal microscopy images are shown.

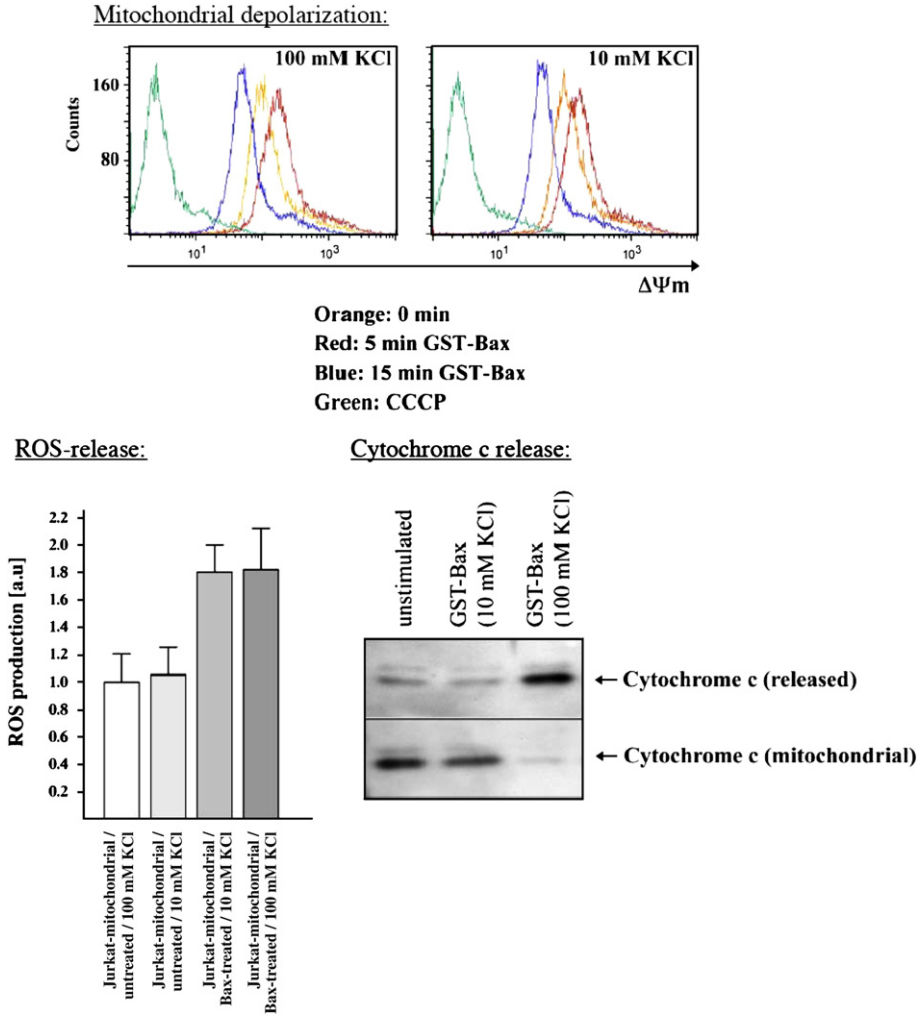


Fig. 3. Cytochrome *c* release requires K^+ and a high concentration of salt. The data show hyperpolarization and ROS formation after treatment of isolated mitochondria with 5 nM GST-Bax in the presence of about 10 mM KCl (70 mM sucrose, 210 mM mannitol, 1 mM EDTA, and 10 mM HEPES/KOH, pH 7.5), while no release of cytochrome *c* from isolated mitochondria was observed under these conditions. Increasing the concentration of potassium to 100 mM in the experimental medium allowed cytochrome *c* release. Mitochondrial membrane potential, cytochrome *c* release and ROS-generation in isolated mitochondria was measured as described in Material and methods of [31].

from Kv1.3-expressing CHO cells in the presence of deoxycholate as detergent [53]. In our experiments, the protein was purified from bacterial extracts with glutathione-agarose in the presence of detergents SDS and deoxycholate. In most experiments we used GST-Kv1.3 (aa 319–523), however, critical experiments were confirmed with a GST-fusion protein of full length Kv1.3. Native Kv1.3 is a homotetrameric channel. To address the issue of whether tetrameric protein is present in our preparation, we performed non-denaturing gels (containing 0.1% SDS, according to [38]) that were blotted with polyclonal rabbit anti-Kv1.3 antibodies. The results of 3 independent studies show bands for monomeric, dimeric and tetrameric constructs indicating the presence of tetrameric GST-Kv1.3 (Fig. 4A).

The tetrameric, functionally active nature of the recombinant GST-Kv1.3 fusion protein was indicated also by the observation that it was able to bind Margatoxin, its specific inhibitor, with high affinity. To determine MgTx binding, we immobilized soluble GST-Kv1.3 on glutathione and tested the binding of Biotin-labeled MgTx. The data obtained show a half maximal binding upon addition of approximately 1.1 nmol MgTx-Biotin for 2 nmol GST-Kv1.3, and near-saturation at about 2 nmol of added MgTx-Biotin. While precision is not sufficient to determine an accurate KD, the experiments show that the affinity of GST-Kv1.3 for MgTx-Biotin is very high, in the same range as that of membrane Kv1.3 for MgTx (110 pM, [54]). The data also demonstrate that MgTx competes with MgTx-Biotin for binding to immobilized GST-Kv1.3 with very similar affinity, since half-saturation of MgTx-Biotin binding is observed when equal amounts of MgTx and MgTx-Biotin are added.

Previous studies indicated that binding of the highly specific Kv1.3 inhibitor toxins, which dock in the outer-facing vestibule of Kv1.3 (involving D386 residues), is pH dependent, and that protonation of Histidine 404 of Kv1.3 weakens pore–toxin interaction [55]. H404 and D386 are both present in the GST-Kv1.3 fusion protein used in our experiments. We have previously shown that the IC_{50} of GST-Bax for Kv1.3 binding increased from 4 to 12 nM when in patch clamp experiments on T lymphocytes the bath solution pH was lowered to pH 6.7 and to a value ≫50 nM at pH 6.0. These data indicated a toxin-like interaction of Bax with the external vestibule of Kv1.3 and mitoKv1.3 [31]. Here we show that co-incubation of GST-Bax with GST-Kv1.3 resulted in strong association of the two proteins (Fig. 4B) as revealed by immunoprecipitation experiments. The strength of this interaction significantly decreased when pH was lowered to 6.0, confirming again that Bax binds to Kv1.3 in a manner similar to Margatoxin. To further prove the specificity of the interaction of Bax and Kv1.3 we added MgTx (5 μM) to GST-Kv1.3 (~2 nM) prior to addition of GST-Bax. As MgTx specifically binds to the pore of Kv1.3 with very high affinity (IC_{50}: 110 pM), an excess of MgTx (5 μM) will prevent binding of Bax to Kv1.3, but only if Bax binds in the pore region of Kv1.3. Our results show that Margatoxin indeed prevented

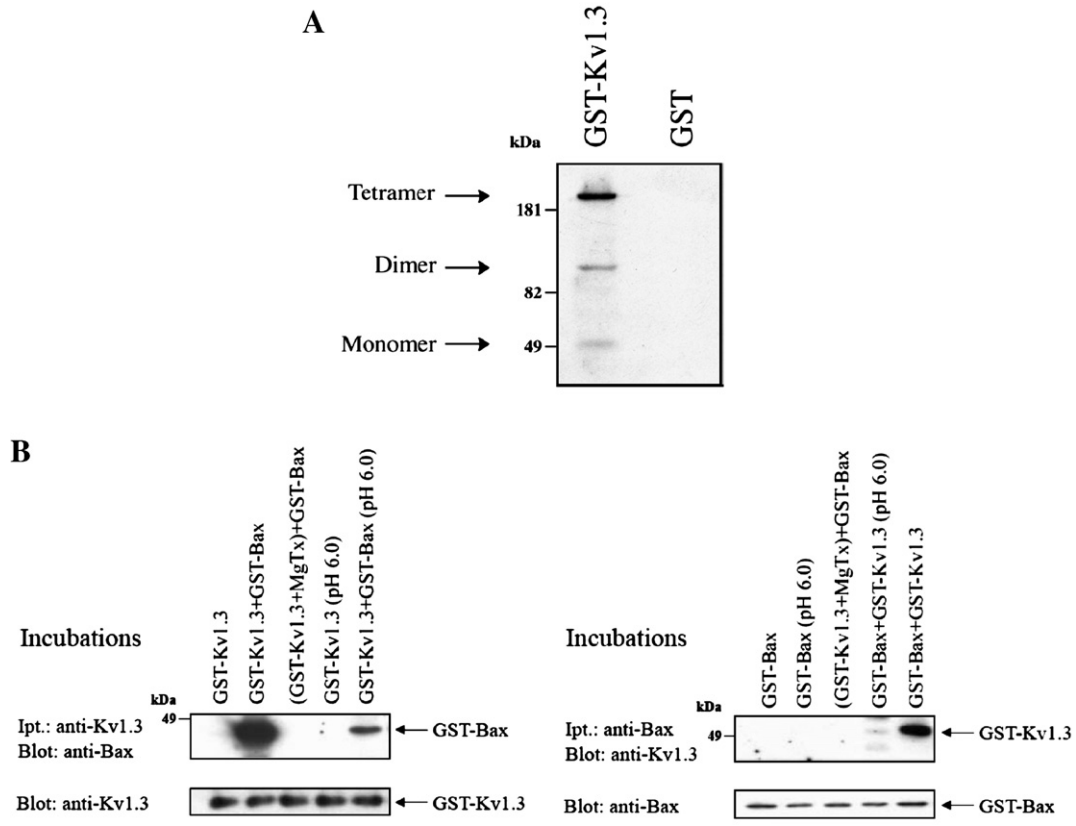


Fig. 4. Recombinant GST-Kv1.3 interacts with GST-Bax *in vitro*. (A) Monomeric, dimeric and tetrameric forms of GST-Kv1.3 are visible on non-denaturing PAGE. (B) Co-incubation of GST-Kv1.3 with GST-Bax (~200 ng/ml each) reveals an association of the two proteins, which is blocked by pre-incubation and neutralization of GST-Kv1.3 with Margatoxin (5 μM). The association of the two proteins is strongly decreased by altering the pH from 7.4 to 6.0. Shown are Western blot experiments performed with anti-Bax and anti-Kv1.3 antibodies, respectively, which are representative of 3 very similar results. The lower blots demonstrate that similar amounts of protein were loaded in all lanes.

the interaction of GST-Kv1.3 with GST-Bax excluding a non-specific interaction of the two proteins (Fig. 4B).

Finally, we tested the effect of the recombinant GST-Kv1.3 on membrane potential changes of isolated mitochondria induced by toxins or Bax, as well as on Bax-induced cytochrome *c* release. Pre-incubation of Margatoxin or ShK (Fig. 5A) as well as of Bax (Fig. 5B) with recombinant GST-Kv1.3 prevented their effects on changes in mitochondrial membrane potential. Neither early hyperpolarization, nor later depolarization occurred, indicating that the recombinant protein competed with endogenous mitoKv1.3 for Bax and the toxins. The same kind of experiment was performed when assessing cytochrome *c* release (Fig. 5C), further demonstrating that Bax interacts with mitoKv1.3 and the importance of this interaction for cytochrome *c* release.

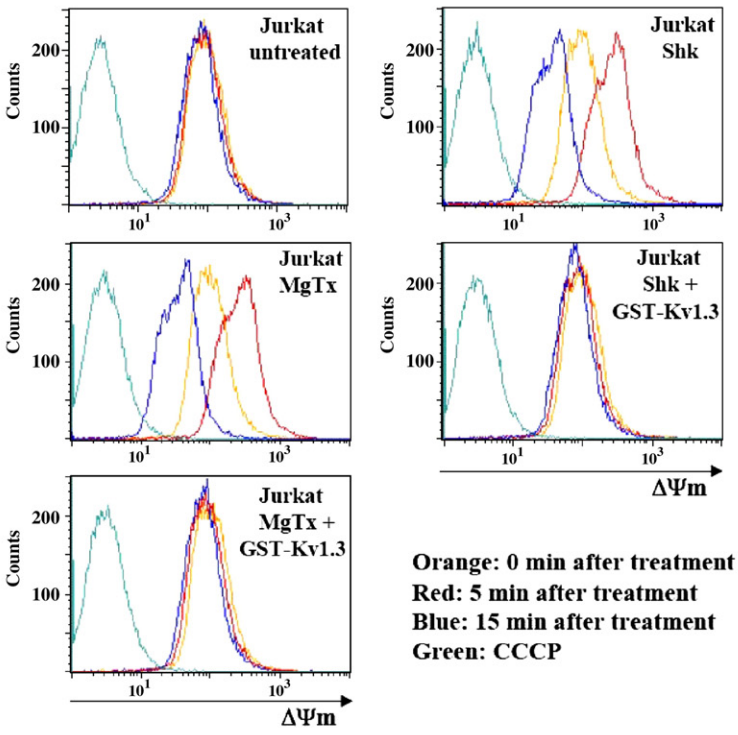
Kv1.3, as plasma membrane channel, is known to be expressed in different tissues and cell types, including brain, lung, thymus, spleen, lymph node, fibroblasts, B lymphocytes, T lymphocytes, tonsils, macrophages, microglia, oligodendrocytes, osteoclasts, platelets, and testis [56]. An altered expression level of Kv1.3 has been found in several types of cancer [14], including prostate and breast cancer [25,57,58]. Therefore, we checked whether Kv1.3 might be present in the mitochondria not only of lymphocytes but also of other types of cells. Fig. 6 shows that a mitochondrial location of Kv1.3 can be identified in prostate cancer PC3 and in breast cancer MCF-7 cell lines. At equal protein quantities loaded, intensity of mitochondrial markers Bak and prohibitin, as well as of Kv1.3 increases in purified mitochondria with respect to whole-cell lysate, whereas the intensity of the plasma membrane marker Ca^{2+}-ATP-ase PMCA and of the ER marker Ca^{2+}-ATP-ase SERCA decreases. These data indicate that the presence of mitoKv1.3 in mitochondria is not restricted to lymphocytes. Furthermore, a functional mitoKv1.3 seems to be present also in hippocampal mitochondria [59]. Whether mitoKv1.3 in these cells may represent a possible target for modulating apoptosis remains to be clarified.

4. Conclusion

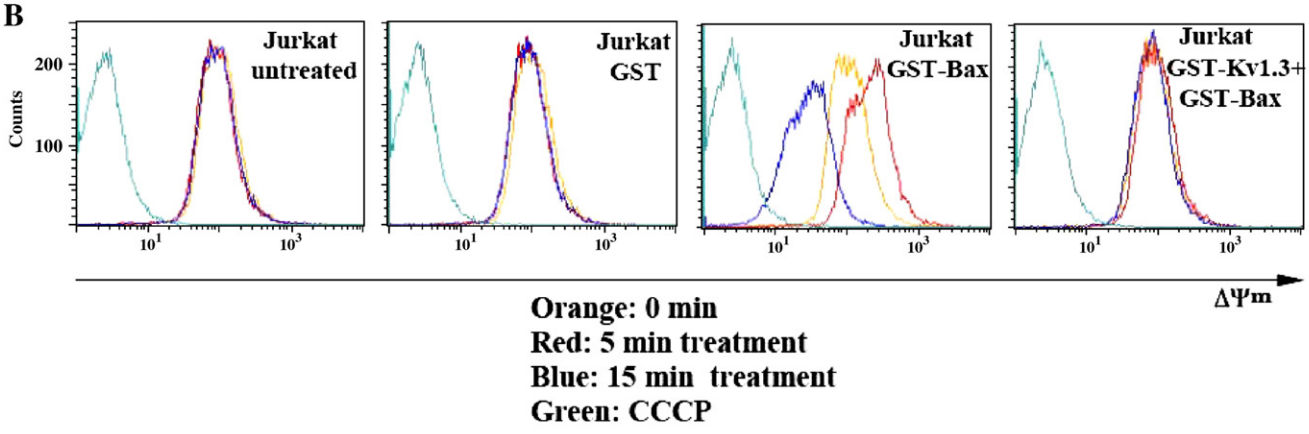
The data presented above further confirm the importance of mitoKv1.3 for apoptosis in lymphocytes and point to a direct interaction of mitoKv1.3 with Bax. Inhibition of mitoKv1.3 by outer-membrane inserted Bax leads to early hyperpolarization (within 5 min), ROS release, a later depolarization (15 min after treatment) and cytochrome *c* release. We used low nanomolar concentrations of Bax in our experiments. They do not exclude that Bax may also form oligomeric pores in the membranes or that it interacts with other mitochondrial proteins. Bax-induced cytochrome *c* release may well

Fig. 5. Pre-incubation of Bax with GST-Kv1.3 abolishes its effects on membrane potential changes in mitochondria and on cytochrome *c* release from isolated mitochondria. Incubation of purified, Kv1.3-positive mitochondria from Jurkat with 20 nM MgTx or 10 nM ShK (A) or 5 nM GST-Bax (B) resulted in hyperpolarization, followed by depolarization. The presence of 100 nM recombinant GST-Kv1.3 during the 30-min incubation neutralized the toxins and Bax and blocked their effects. Control GST was without effect. The flow cytometry panels show plots representative of 3 independent experiments reporting $\Delta\psi$m in isolated Jurkat mitochondria stained with 10 nM $DioC_6(3)$. Complete depolarization upon application of CCCP served as control for mitochondria integrity. (C) Purified Kv1.3-expressing mitochondria responded to incubation with GST-Bax with release of cytochrome *c*, whereas pre-incubation of Bax with GST-Kv1.3 abolished the effect of Bax. Cytochrome *c* release was determined by Western blotting. Blots are representative of 5 similar experiments.

A


Jurkat untreated
Jurkat Shk
Jurkat MgTx
Jurkat Shk + GST-Kv1.3
Jurkat MgTx + GST-Kv1.3
Counts
200
100
10^1
10^3
ΔΨm
Orange: 0 min after treatment
Red: 5 min after treatment
Blue: 15 min after treatment
Green: CCCP

B


Jurkat untreated
Jurkat GST
Jurkat GST-Bax
Jurkat GST-Kv1.3+ GST-Bax
Counts
200
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Orange: 0 min
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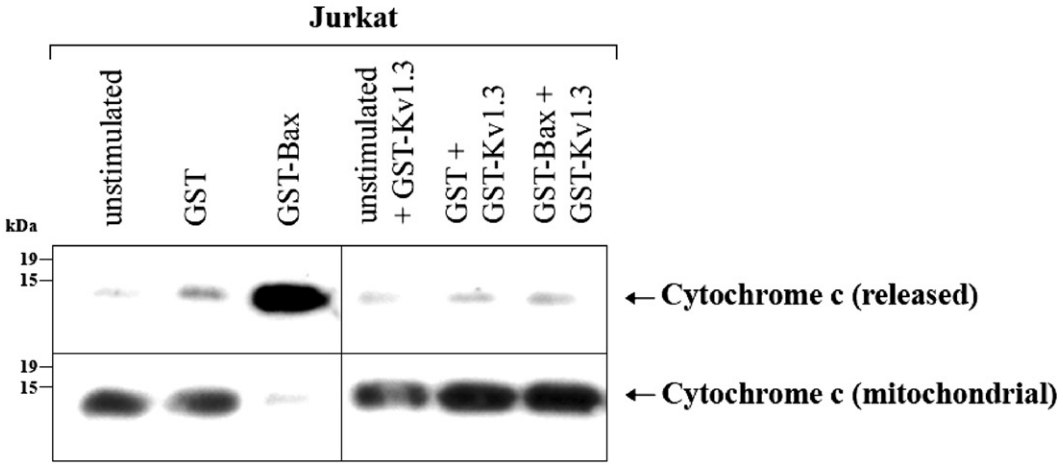
Blue: 15 min treatment
Green: CCCP

C


Jurkat
unstimulated
GST
GST-Bax
unstimulated + GST-Kv1.3
GST + GST-Kv1.3
GST-Bax + GST-Kv1.3
kDa
19
15
← Cytochrome c (released)
← Cytochrome c (mitochondrial)

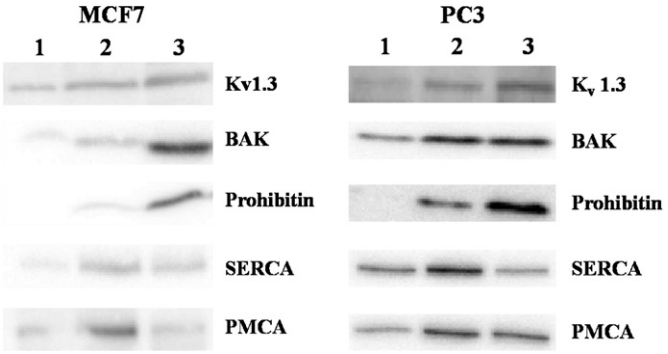


Fig. 6. Kv1.3 is located to mitochondria also in PC3 and MCF-7 cancer cells. 50 μg of total protein per lane was loaded of whole-cell lysate (1), a membrane-enriched fraction (2) and Percoll-purified mitochondria (3). See description in the text. Kv1.3 was detected with an apparent MW of 64 kDa. Blots with marker proteins were obtained by stripping and re-blotting. Differences in background are due to different exposure time and/or development method.

be a multi-stage process, inhibition of mitoKv1.3 by presumably monomeric Bax representing only an early step, to be followed by other processes, such as ROS release, detachment of cytochrome *c* from the surface of the IMM, and PTP activation. Bax has recently been shown to be inserted into the OMM first as monomer and then undergoing oligomerization [60]. Formation of Bax oligomers and pore formation by Bax in the OMM may well occur independently of the presence of Kv1.3. Although our data indicate PTP activation downstream of Bax–Kv1.3 interaction, we should stress that they do not discriminate whether cytochrome *c* efflux is directly linked to PTP opening – which may induce distension of cristae and increased availability of cytochrome *c* for efflux – or is triggered independently of PTP — for instance, by oxidation of cardiolipin.

In summary, previous data as well as those described in the present manuscript indicate an important function of mitoKv1.3 in lymphocyte apoptosis. The presence of mitoKv1.3 in other cell types as well suggests that the action of mitoKv1.3 might not be restricted to lymphocytes only.

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