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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.)