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Activation of the Permeability Transition Pore by Bax via Inhibition of the Mitochondrial BK Channel

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Key Words

Mitochondrial ion channels • Permeability transition pore • Mitochondrial BK channel • Bax • Bcl-xL • Apoptosis

Abstract

Mitochondria are crucially involved in the intrinsic pathway of apoptosis. Upon induction of apoptosis, proapoptotic proteins of the Bcl-2 family, in particular Bax and Bak, transfer the death signal to the organelle. The outcome is release of proapoptotic factors, such as cytochrome c, and mitochondrial changes, such as depolarization. Details of the mechanism by which Bax mediates mitochondrial alterations, however, are unknown. Using the single-channel patch-clamp method, we studied mitoplasts (vesicles of inner membrane) from rat astrocyte and liver mitochondria and intact murine glioma mitochondria to determine the action of proapoptotic Bax and antiapoptotic Bcl-xL on the mitochondrial Ca^{2+}-activated channel (mtBK) and the permeability transition pore (mtPTP). Bax (1 nM) inhibited the open probability of the mtBK, whereas Bcl-xL or control proteins had no effect. Incubating mitochondria with iberiotoxin, an inhibitor of mtBK, induced the release of cytochrome c. Bcl-xL inhibited the effects of Bax on mtBK. Furthermore, in patch-clamp studies Bcl-xL inhibited the mtPTP itself, whereas Bax had no direct effect on the mtPTP. We conclude that Bax exerts its proapototic effect by inhibiting mitochondrial K^+ channels, whereas Bcl-xL exerts its antiapoptotic effect by inhibiting the effects of Bax on mitochondrial potassium channels and by direct inhibition of the mtPTP.

Introduction

Apoptosis is a key process during the physiological turnover of tissues and development. However, it is also an important process in many diseases, such as neurodegenerative diseases or malignancies that are characterized by an imbalance between proliferation, cell differentiation, survival, and apoptosis. [1, 2]. Several pathways have been found to mediate apoptosis. For example, mitochondria are key organelles in apoptosis and are necessary for many stimuli that trigger apoptosis. The integrity or dysfunction of mitochondria during

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apoptosis is controlled by proteins of the Bcl-2 (B cell lymphoma 2) family. Bax (Bcl-2 associated protein X), Bak (Bcl-2 homologous antagonist/killer), Bad (Bcl-2-associated death promoter), Bid (BH 3 interacting domain death agonist), and other proteins exert a proapoptotic effect on cells, Bcl-2 itself and Bcl-xL (B cell lymphoma extra large) are antiapoptotic [3].

Many apoptotic stimuli induce the activation (i.e., a conformational change) of Bax and Bak. Bax may be associated with the mitochondrial outer membrane (OM) or, upon activation, may translocate from the cytoplasm to the OM. Bak is located to the OM. It has been demonstrated that amino acids 127 (threonine) and 128 (lysine) of Bax protrude into the intermitochondrial space [4]. Although it has been clearly established that Bax and Bak are necessary for the induction of mitochondrial changes during apoptosis, the mechanisms by which Bax and Bak mediate these alterations require definition [5].

The release of cytochrome c from cardiolipin in the inner mitochondrial membrane constitutes a key step in the proapoptotic changes of mitochondria. Cytocrome c and other proapoptotic factors, such as apoptosis-inducing factor (AIF) or Omi/HtrA1, are finally released from the intermitochondrial space into the cytoplasm by a sudden increase in the permeability of the OM of larger solutes [6]. The opening of a special pore, the rupture of the OM, or the channel properties of Bcl-2-family members themselves have been proposed as release mechanisms [7, 8]. Furthermore, many stimuli of apoptosis induce a depolarization of the mitochondrial membrane potential ($\Delta\Psi$) and dramatically reduce the organelle's ability to retain mitochondrial Ca^{2+}. These findings suggest an increase in the permeability of the inner membrane [9]. This increase has long been known to be a response to an increase in the concentration of cytosolic Ca^{2+} and has been dubbed the permeability transition [10]. Consequently, the structure assumed to be responsible for this increase has been called the permeability transition pore (mtPTP).

Pharmacological studies have identified a large number of agonists and inhibitors of the mtPTP [11]. Patch-clamp techniques have demonstrated that channel events with characteristic features, such as sensitivity to Ca^{2+} and cyclosporin A (CsA), and extraordinarily high single-channel conductance (γ) of more than 1 nS, combined with several substates of lower conductance, could be detected in mitoplasts (i.e., tiny vesicles of the inner membrane [IM] devoid of OM) [12-14]. Unfortunately, however, the molecular nature of the mtPTP has remained enigmatic.

Recent studies have shown that voltage-dependent potassium channels (Kv channels) are expressed in the inner mitochondrial membrane [15, 16] and that Bax interacts with mitochondrial Kv1.3 in a toxin-like manner and inhibits the channel [15]. This inhibition finally results in the release of cytochrome c and in mitochondrial depolarization [4]. It is unknown whether Bax also interacts with other potassium channels and whether the inhibition of mitochondrial potassium channels may be a universal motif that triggers apoptosis. Thus, we investigated whether Bax and Bcl-xL interact with mitochondrial BK channels (mtBK), which are found most frequently in mitoplasts of human glioblastoma cells, rat astrocytes, and guinea pig ventricular cells [17-19]. In hepatocyte mitochondria we find them at much lower density. mtBK is similar to its plasma membrane counterpart in γ, voltage dependence, and inhibition by toxins, such as iberiotoxin and charybdotoxin. Studies using a combined approach of Western blot analysis, high-resolution immunofluorescence, and immunoelectron microscopy identified the BK beta4 subunit (KCNMB4) in the inner mitochondrial membrane of neuronal mitochondria in rat brain and cultured neurons [20]. Accumulating evidence suggests that mtBK interacts with the mtPTP, thereby regulating the opening of the mtPTP [11].

In the present study we demonstrate that Bax inhibits mtBK and that this inhibition finally results in the activation of the mtPTP and the release of cytochrome c. Bcl-xL prevents the inhibition of mtBK by Bax and, in addition, blocks mtPTP activity. These findings suggest that mitochondrial potassium channels play a central role in the induction of apoptosis by Bax.

Materials and Methods

Preparation of hepatocytes and their mitochondria

Wistar rats (age, ~7 weeks; weight, 200 g) were sacrificed by decapitation as approved by the Animal Health and Care Committee of the State of Sachsen-Anhalt, Germany. The liver was removed and washed twice in ice-cold medium containing 250 mM sucrose (pH=7.4). After the ligaments and blood clumps had been removed, the liver was minced, homogenized in a solution containing 250 mM sucrose and 1 mM EDTA (pH=7.4), and centrifuged for 5 min at 800 x g. The supernatant was filtered through sterile gauze and centrifuged for 4 min at 5,100 x g. The pellet was resuspended and homogenized again in a solution of 250 mM sucrose (pH=7.4), and then centrifuged for 2 min at 12,300 x g. Resuspension and centrifugation were repeated for 10 min at 12,300 x g. The pellet consisting to major extent out of mitochondria was finally resuspended in 1 to 2 mL of a solution

containing 250 mM sucrose and 0.5 mM EDTA (pH=7.4). All procedures were performed at 4 °C.

Cell culture and preparation of mitochondria from astrocytes

Isolation of astrocytes from the embryonal rat brain has been described by Chamaon et al. in detail [21]. Astrocytes were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 µg/mL tylosin at 37 ºC in a humidified atmosphere with 5% CO_2 (media from PAA Laboratories, Pasching, Austria). The cells were fed and reseeded every third day. The culture medium was removed, and the cells were washed twice with Hank's BSS medium. Astrocytes from 4 to 6 culture flasks were then collected in 25 mL Hank's BSS medium and centrifuged for 10 min at 800 x g. The pellet was resuspended and homogenized in a solution of 250 mM sucrose and 5 mM HEPES (pH=7.2). Mitochondria were separated from diluted proteases and from the other cellular organelles by fast (9,200 x g, 10 min) and slow (800 x g, 10 min) centrifugation. They were then transferred into a storage solution by centrifuging them twice for 10 min at 9,200 x g. The pellet was resuspended in 1 mL of a solution containing 150 mM KCl and 10 mM HEPES (pH=7.2). All procedures were performed at 4 °C.

Preparation of mitoplasts and patch-clamp experiments

We treated mitochondria from rat liver (RLM) and from astrocytes with a hypotonic solution (5 mM HEPES, 0.2 mM $CaCl_2$, pH=7.2) for 45 seconds to swell mitochondria by the osmotic gradient and to obtain mitoplasts, i.e., round and fragile vesicles of the inner mitochondrial membrane. The isotonicity was restored by the addition of a hypertonic solution (750 mM KCl, 30 mM HEPES, 0.2 mM $CaCl_2$, pH=7.2). A pipette of borosilicate glass (Harvard Apparatus, Kent, UK) with a resistance of 15 to 20 MΩ was filled with isotonic solution (150 mM KCl, 10 mM HEPES, 0.2 mM $CaCl_2$, pH=7.2). The high Ca^{2+} concentration of the solutions was found to ease formation and to improve stability of Giga seals between the glas and the mitoplast membrane. Patches from which it was recorded were thus either in the mitoplast-attached or (less likely) in the inside-out configuration. We used a flow system driven by a peristaltic pump to apply the test solution from the bath, and we used the back-filling technique to apply substances through the patch pipette. The tip of the pipette was filled with isotonic solution by negative pressure, and the rest of the pipette was filled from the back with solution containing test substance. The test substance reached the ion channel by diffusion. Single-channel recording was performed by an EPC-7 amplifier (HEKA Electronics, Lambrecht, Germany). The records were low-pass filtered at a corner frequency of 0.5 kHz (Compumess Elektronik, Unterschleißheim, Germany) and sampled at a frequency of 2.5 kHz by the Clampex 9.2 software (Axon Instrument, Foster City, CA, USA). Negative potentials are referring to the inner side of the mitoplast and inward currents are deflecting downward. The holding potential (E_H) is the potential to which the membrane is clamped and the reversal potential (E_{rev}) is the potential at which the single-channel currents reverse sign.

Data analysis

Data were analyzed with the Clampfit 9.2 software. The open probability (P_o) of the mtBK was calculated by using the single-channel search mode of Clampfit 9.2. The P_o of the mtPTP was determined by all-points analysis [14]. Data are presented as mean ± SEM and were analyzed with t-test by using the software program OriginPro 7.5 ($p<0.05$ marked by *; $p<0.01$ by **; and $p<0.001$ by ***). Numbers of independent patches used for statistics (n) are given either in the results section or in the figure legends.

Recombinant proteins

Bax (amino acids 1-170) was cloned into pGEX-3X as a GST (gluthathione-S-transferase) fusion protein, expressed in BL21A1, and purified from bacterial lysates as previously described [4]. Briefly, 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 of NaF, Na_3VO_4, and sodium pyrophosphate; 10 µM each of aprotinin and leupeptin (A/L); and 1 mg/mL lysozyme. DNA was degraded with 5 µg/mL DNaseI in 30 mM $MgCl_2$ for 30 min. Insoluble material was removed by centrifugation. GST-Bax was then purified by the addition of 300 µL glutathione sepharose (GE Healthcare, Chalfont St. Giles, UK) for 1 hour at 4 °C. The beads were pelleted by centrifugation and were extensively washed. GST-Bax was eluted by the addition of 20 mM glutathione, which was finally removed by dialysis. In this study we used a GST-ΔC-Bax, which lacks the C-terminal transmembrane domain and does not require activation by truncated Bid (tBid) for membrane insertion [22].

Release of cytochrome c *from isolated mitochondria*

Mitochondria (obtained from $1x10^6$ murine glioma cells, GL261) were isolated as above and incubated for 30 min on ice in 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 µg/mL A/L, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, and 50 µg/mL creatine kinase in the presence or absence of 300 nM iberiotoxin so that the toxin could bind to mitochondria. The mitochondria were then centrifuged, and the supernatants were discarded. The mitochondria were resuspended in prewarmed buffer (37 °C), incubated for 5 minutes at 37 °C, and centrifuged at 16,300 x g at 4 °C. The supernatants were added to 5x SDS sample buffer. The samples were analyzed for the release of cytochrome c by 12.5% SDS/PAGE, the proteins were blotted on nitrocellulose membranes, and the blots were developed with an anti-cytochrome c antibody (AP-coupled anti-mouse antibody) and were then incubated with an AP-coupled secondary antibody and electrochemoluminescence.

Results

Mitoplasts from cultured non-neoplastic rat astrocytes exhibited the previously described iberiotoxin-sensitive mtBK with a single-channel conductance (γ) of

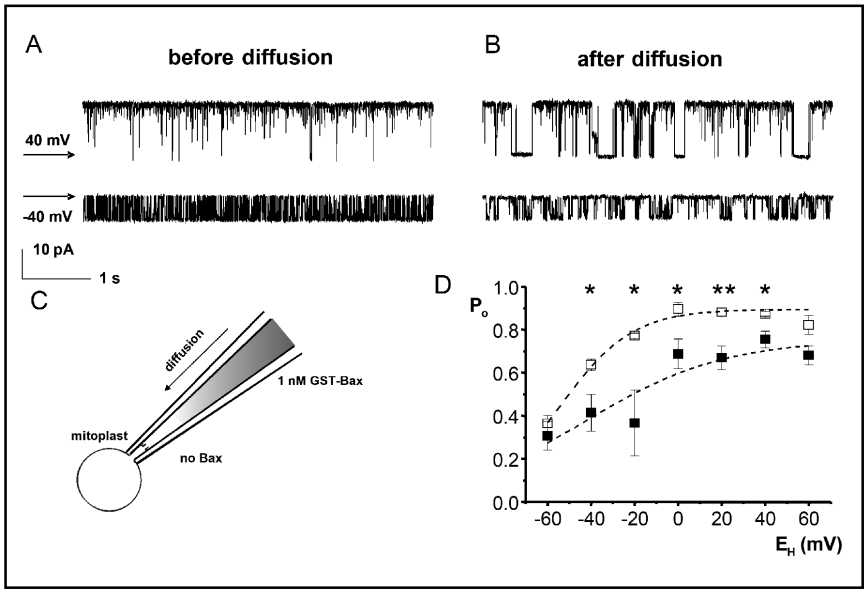


Fig. 1. Applying 1 nM GST-Bax by backfilling the measuring pipette (i.e., the membrane outside) reduces the open probability (P_o) of the mtBK in mitoplasts of astrocytes. A: Single-channel current traces at +40 mV and -40 mV immediately after formation of the patch. B: Diffusion of GST-Bax to the patch results in longer closed times (90 min later), as shown in the same patch as in panel A. Arrows indicate the closed state of the channel. E_{rev} = -17 mV in this mitoplast. C: Schematic drawing of the measuring situation. D: P_o versus E_H demonstrates voltage dependence of the mtBK (reduced P_o at negative E_H) and a reduction of the P_o by GST-Bax (n=6); the reduction is statistically significant between -40 mV and +40 mV ($p<0.05$) and at +20 mV ($p<0.01$). □: immediate recording, ■: recording after ~90 min.

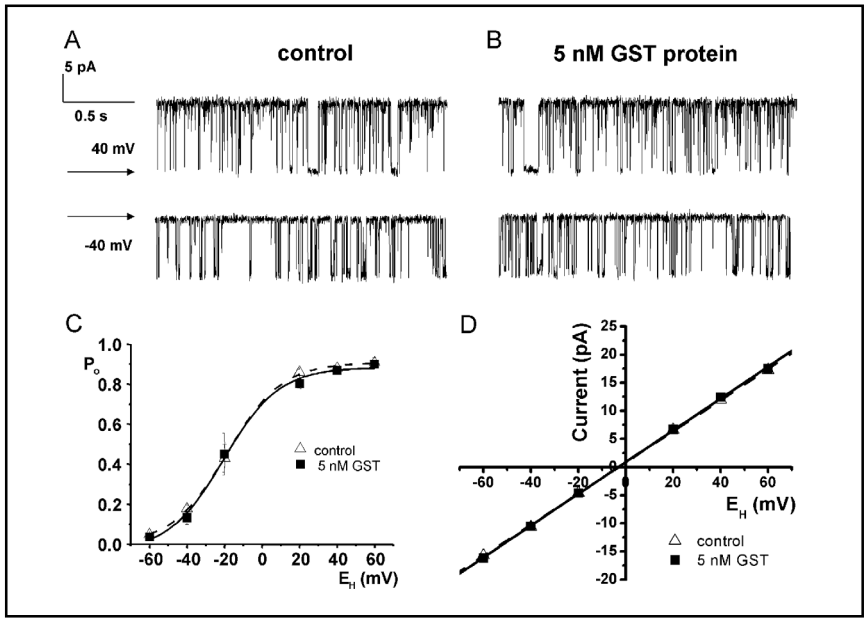


Fig. 2. Applying 5 nM GST does not reduce the open probability (P_o) of the mtBK in astrocyte mitoplasts. The experimental conditions were identical to those described in Fig. 1. A: Single-channel current recorded at +40 mV and -40 mV immediately after formation of the patch. B: Recording from the same patch 99 min later. C: The P_o versus E_H plot shows no significant difference between the two conditions (n=3). Δ: early control; ■: 5 nM GST. D: GST had no effect, as indicated by an unchanged relationship between i and E_H. The continuous straight line indicates a single-channel conductance of 295 pS. Symbols as in C.

295 pS (Fig. 1, A and B) [17, 19]. We used back-filling of the measuring pipette to apply 1 nM GST-Bax to the outer side of the mitoplast membrane (Fig. 1C). Immediately after the seal was formed, the P_o was significantly larger than it was after 90-min incubation when GST-Bax had diffused to the membrane of the patch (Fig. 1D). To determine whether the observed effect could be caused simply by a run-down of P_o, we performed another experiment without back-filling the measuring pipette. Even after 2 h we observed no decrease in P_o throughout the potential range of ±60 mV (not shown). Thus, it seems reasonable to assume that the major part of the delay is due to diffusion of GST-Bax through the tip of the pipette to the patch. We also confirmed that GST itself had no effect on mtBK (Fig. 2, A-D).

Because it is impossible to exchange the test solution under these conditions, we determined whether 1 nM GST-Bax has the same irreversible inhibiting effect on mtBK when applied through the flow system from the bath side to the mitoplast. Bax reduced P_o primarily in the depolarizing potential range (Fig. 3). Mean values from 3 experiments demonstrate irreversible reductions in P_o (Fig. 4 A). Again, GST-Bax had no effect on the amplitude of the single-channel current (Fig. 4B). Higher concentrations of GST-Bax (3 nM and 5 nM) did not exert an additional effect on the decline of P_o. The onset

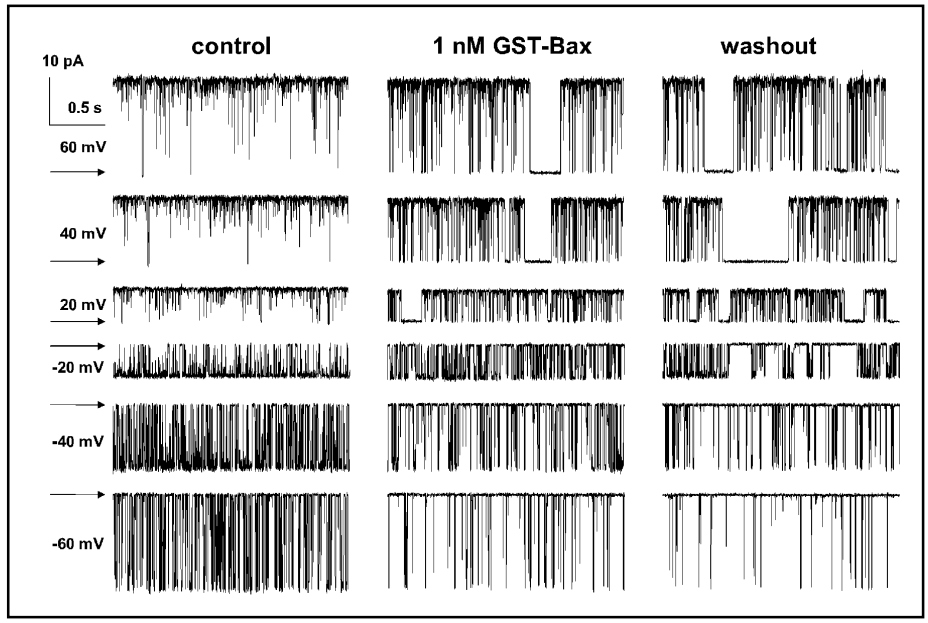


Fig. 3. Applying 1 nM GST-Bax through the pipe of the flow system irreversibly reduces the open probability (P_o) of the mtBK in astrocyte mitoplasts. Demonstrated are original current traces at the indicated holding potentials. Arrows indicate closed states. The panel is representative of 4 experiments with similar results.

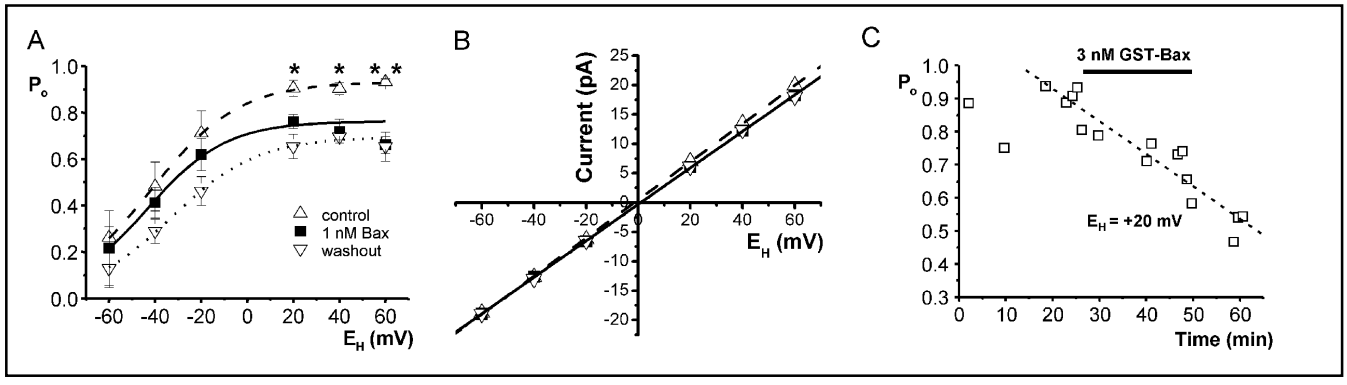
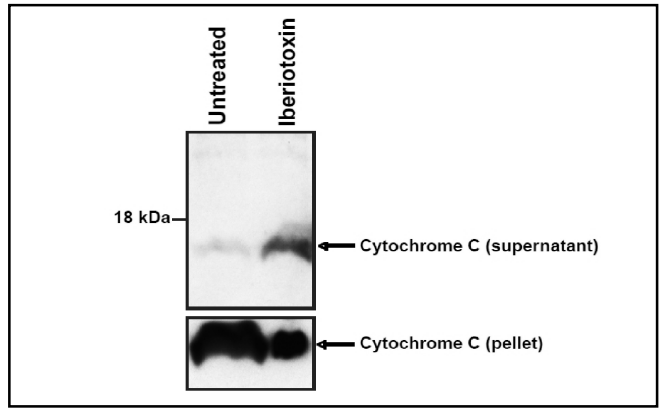


Fig. 4. A: Applying 1 nM GST-Bax by the flow system irreversibly reduces the open probability (P_o) of the mtBK in astrocyte mitoplasts, preferentially at positive E_H (depolarizing direction). Upon negative E_H the effect was not statistically significant. Error bars indicate the standard error of the mean (SEM) (n=4). Δ: control before; ■: 9 min 24 s after switching to 1 nM GST-Bax (mean time of 4 experiments); ∇: 9 min 47 s after switching to washout. Curves were calculated by using the Botzmann equation. B: GST-Bax did not change the relationship between i and E_H. Symbols as in A. C: Development of P_o during an experiment with 3 nM Bax (application indicated by bar). Onset of the Bax-induced decline is rapid (1% per min), and the decline in P_o can be described by a linear regression line (dashed); the decline continues even after the switch back to control solution. The dotted line indicates the mean value of the P_o before the application of Bax.

Fig. 5. Iberiotoxin induces the release of cytochrome c from isolated mitochondria. Glioma mitochondria were isolated, separated into aliquots, and treated with 300 nM iberiotoxin. The release of cytochrome c into the supernatant was determined by Western blotting. The results are representative of 3 studies with almost identical results.



of the effects of GST-Bax was observed within less than 1 min after the addition of GST-Bax (Fig. 4C). In this experiment, 3 nM GST-Bax already produced a decrease in P_o with a 1% reduction per minute; the decrease

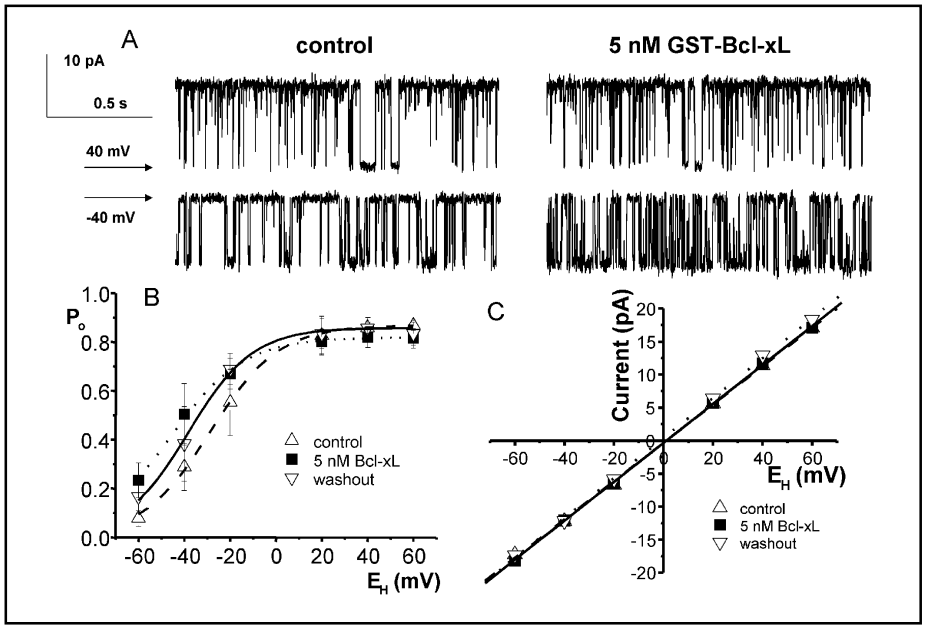


Fig. 6. Applying GST-Bcl-xL through the flow system does not directly affect the mtBK in astrocyte mitoplasts. A: Single-channel current traces in control solution and in 5 nM GST-Bcl-xL show comparable activity. B: The open probability (P_o) vs. E_H plot shows no statistically significant change upon application of Bcl-xL (n=4). C: The relationship between i and E_H is unchanged by the application of 5 nM GST-Bcl-xL. Δ: control before; ■ : 3 nM GST-Bax + 5 nM GST-Bcl-xL; ∇: washout.

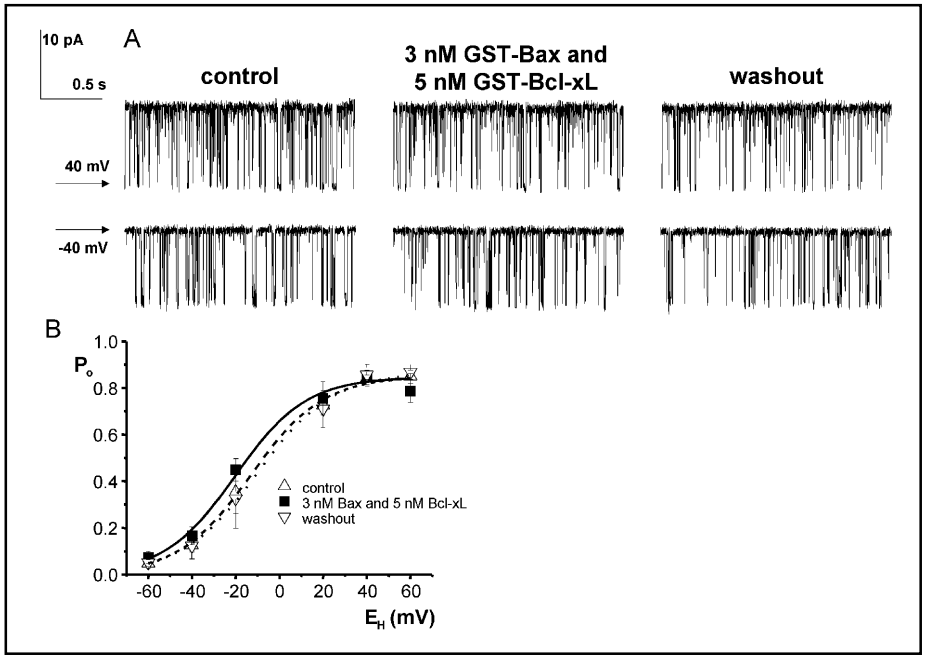


Fig. 7. GST-Bcl-xL antagonizes the effects of GST-Bax on the mtBK in astrocyte mitoplasts. A: Shown are current traces at the indicated potentials before, during, and after simultaneous application of 3 nM Bax and 5 nM GST-Bcl-xL. B: The open probability (P_o) versus E_H plot shows an increase (not statistically significant) rather than a decline in P_o throughout the tested voltage range (n=3). Δ: control before; ■ : 3 nM GST-Bax + 5 nM GST-Bcl-xL; ∇: washout.

continued with the same kinetics after washout (dashed line).

To further demonstrate the role of mtBK channels in the induction of apoptosis by Bax, we incubated isolated mitochondria with iberiotoxin, an inhibitor of BK channels, and used Western blot analyses to determine whether the inhibition of mtBK channels is sufficient to release cytochrome c and to alter the potential of the mitochondrial membrane. The results indicate that iberiotoxin triggers the release of cytochrome c from isolated mitochondria (Fig. 5). These findings demonstrate that the inhibition of mtBK channels is sufficient to trigger the typical apoptosis-associated changes in mitochondria.

The effects of Bax have been shown to be inhibited by Bcl-xL. To determine the level at which Bcl-xL blocks Bax, we investigated whether Bcl-xL also affects mtBK channels. Our patch-clamp studies did not show a significant modification of mtBK by GST-Bcl-xL, thereby excluding a direct effect of Bcl-xL on mtBK (Fig. 6A-C). To further investigate the mechanisms how Bcl-xL antagonizes Bax, we added 3 nM GST-Bax and 5 nM GST-Bcl-xL to the test solution inside the flow system (Fig. 7A). The plots of P_o and E_H demonstrate that Bcl-xL inhibits the effects of GST-Bax on mtBK (Fig. 7B). As expected, it exerted no effect on single-channel conductance. Thus, Bcl-xL antagonized the effects of

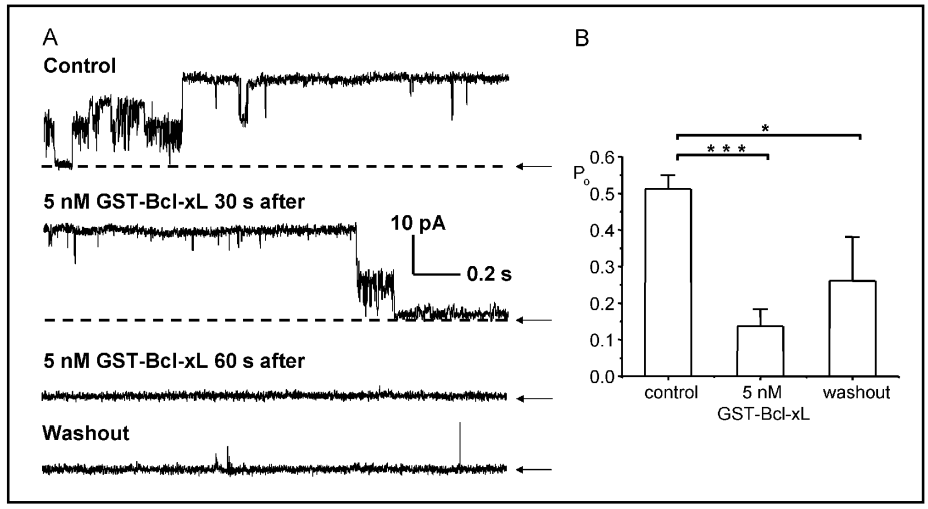


Fig. 8. GST-Bcl-xL inhibits the mtPTP in liver mitoplasts. A: The panel displays representative single-channel current traces from RLM mitoplasts in control solution, 30 s and 60 s after switching to 5 nM GST-Bcl-xL, and after washout of GST-Bcl-xL. Arrows and dashed lines mark the closed state. B: Shown is the mean open probability (P_o) ($\bar{x}$ ± SEM, n=6) in controls, after 5 nM Bcl-xL, and during washout. *, $p<0.05$; ***, $p<0.001$.

Bax either by binding to Bax itself or by shielding a putative binding site for Bax at the pore of the mtBK.

To further determine how Bcl-xL *functionally* antagonizes proapoptotic Bax, we also studied the effects of GST-Bcl-xL on the mtPTP; this large pore is detected most frequently in RLM [14]. GST-Bcl-xL completely blocked the mtPTP as quickly as 1 min after addition and at a very low concentration (Fig. 8A). As little as 5 nM Bcl-xL caused a significant inhibition of the mtPTP ($p<0.001$); this inhibition was only partially reversible (Fig. 8B). Furthermore, we determined whether 1 nM GST-Bax directly activates mtPTP. To abolish the activity of the mtPTP we lowered the Ca^{2+} concentration to 1 μM for either back-filling of the pipette (n~35) or application by the flow system (n~35). However, under this condition we did not observe a stimulatory effect of GST-Bax on the mtPTP (P_o=0).

Discussion

Mitochondria respond to proapoptotic stimuli of the intrinsic signaling cascade by a number of reactions, all of which disturb the ion and voltage homeostasis at the inner membrane. The findings of a recent study demonstrated that the Kv1.3 channel is a target of Bax to trigger death [4]. In this study we found that Bax also inhibits another mitochondrial channel; this finding suggests that the mitochondrial effects of Bax might be mediated by a general inhibition of at least several potassium channels. Bax seems to fold in the conserved pore of potassium channels and to close the pore as a plug [4]. This finding suggests a comprehensive model for the action of Bax: Bax binds to and inhibits mitochondrial potassium channels. The inhibitory effect seems to be specific to potassium channels but is not restricted to a certain potassium channel. Rather it seems likely that Bax inhibits several types of mitochondrial potassium channels. Inhibiting the potassium flux results in mitochondrial hyperpolarization and release of reactive oxygen species that oxidize cardiolipin to release cytochrome c into the intermitochondrial space and to open the mtPTP. This finding provides additional evidence for our hypothesis that open K^+ channels tend to keep the mtPTP closed, and it seems irrelevant which of the K^+ channels is involved [4, 11]. The observation that the effects of Bax do not increase at concentrations higher than 1 nM may indicated saturation even at this very low concentration of Bax.

The Bax effect was irreversible: even after the solution was switched to a control solution, the P_o continued to decline. Thus, inhibition of the mtBK by Bax is progressive and differs from inhibition by other substances, such as Chtx, which produce a reversible effect [23-25]. It is unknown whether the continuing Bax-induced decline in P_o is due to strong binding, whether it reflects slow diffusion through the mitoplast to the membrane patch from which it is recorded, or whether it reflects the slow formation of Bax dimers [26]. The progressive effect could also be due to continued equilibration of Bax out of the mitoplasts matrix space to the channel through the narrow neck of the Ω-shaped patch even after washout has eliminated Bax from the bath side. We found no indication that Bax itself could have channel properties [27-29]. No additional ion channels with a different fingerprint (i.e., γ, P_o, kinetics), either in RLM or in astrocyte mitoplasts, were found to open after application of proteins from the Bcl-2 family. Thus, it seems that the channel properties which have been predicted by the structure of e.g. Bax [28] are

irrelevant in the system described here, although the proteins may behave differently when incorporated into the OM or into artificial bilayers.

Our results indicate that the well-known proapoptotic Bax-induced activation of the mtPTP is not achieved directly but rather by inhibition of mtBK and Kv1.3. The data confirm previous studies that also observed no direct effect of Bax on the mtPTP [30]. The missing Bax effect in swelling and other experiments on intact mitochondria might be owed to the low K^+-concentrations used for the media (300 mM sucrose; [31, 32]). The antiapoptotic Bcl-xL protein antagonizes Bax inhibition of the mtBK. In this study, the P_o did not change significantly when both proteins were added. This finding indicates that Bcl-xL can abolish the inhibitory effect of Bax. The way in which Bcl-xL relieves the effect of Bax on mtPTP and on the release of cytochrome c has been debated [33, 34]. Some researchers have suggested that Bcl-xL forms a heterodimer with Bax through the interaction of their BH 3 domains [35-37]. This theory is supported by our results. In our solutions, if 5 nM GST-Bcl-xL and 3 nM GST-Bax heterodimerized in a 1:1 relationship, almost no free GST-Bax remained to inhibit the mtBK. Alternatively, the direct effects of Bax on mtBK could be also inhibited by shielding the channel with Bcl-xL, although this shielding does not alter the potassium flux.

Several of the reactions typical of apoptosis can be related to the opening of the mtPTP, such as loss of $\Delta\Psi$, loss of mitochondrial Ca^{2+} retention capability, and swelling due to the negative colloidal osmotic pressure of the matrix proteins [38]. In this study we used single-channel measurements to determine the effect of two Bcl-2–like proteins directly at the mtPTP.

Our findings indicate that Bcl-xL directly inhibits mtPTP and neutralizes the effects of Bax on mtBK. Bcl-xL is an antiapoptotic factor that is believed to stabilize the mitochondrial membrane and to prevent the release of cytochrome c into the cytosol [39]. Inhibition of the mtPTP by Bcl-xL has been previously demonstrated by experiments with intact mitochondria [40]. The patch-clamp experiments described here have for the first time demonstrated this inhibition on the single-channel level. Taken together, these results and those of earlier studies indicate that Bcl-xL exerts its antiapoptotic activity by inactivating Bax, very likely by directly interacting with Bax and thereby preventing the binding of Bax to potassium channels. In addition, Bcl-xL stabilizes the mitochondrial membrane by inhibiting mtPTP, as also shown here.

The use of mitoplasts for research on mitochondrial ionic currents has been repeatedly proved reliable and is now a widely accepted method [4, 18, 41, 42]. Many results of experiments with mitoplasts agree with the results of experiments with intact mitochondria. Though, depending on the protocol used for purification, plasma membrane particles may contaminate the mitoplast preparation, we do not have difficulties to distinguish mitoplasts by phase contrast optics [8]. We used mitoplasts from a non-neoplastic astrocytic cell line to study the mtBK, and we used mitoplasts from freshly dissected rat liver to study the mtPTP. Because these preparations had previously been used extensively, we could rely on earlier results to determine the identity and the mitochondrial location of the observed channels [14, 19, 20, 43-45].

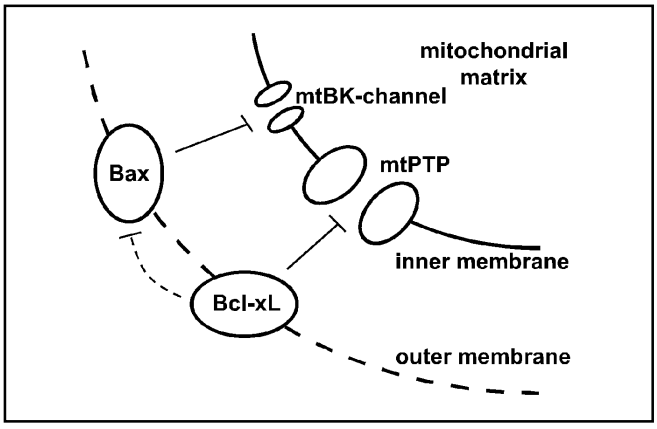


Fig. 9. Schematic drawing of the results.

In conclusion, the findings we have obtained from single-channel experiments explain the effects of Bax and Bcl-xL on mtBK and mtPTP at the single-channel level. Our results are in agreement with a scenario in which Bax inhibits mtBK and thereby opens the mtPTP and releases cytochrome c into the cytoplasm. We further show that Bcl-xL prevents the interaction of Bax with mtBK and, in addition, directly blocks the mtPTP, whereas Bax has no effect on the mtPTP. Our findings indicate that proapoptotic and antiapoptotic proteins may be able to act either as channel inhibitors or as pore inhibitors but that each of these actions requires the targeting of a different channel. The results are summarized in Fig. 9.

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