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Bax affects intracellular Ca^{2+} stores and induces Ca^{2+} wave propagation

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Abstract

In the present study, we evaluated proapoptotic protein Bax on mitochondria and Ca^{2+} homeostasis in primary cultured astrocytes. We found that recombinant Bax (rBax, 10 and 100 ng/ml) induces a loss in mitochondrial membrane potential ($\Delta\Psi_m$). This effect might be related to the inhibition of respiratory rates and a partial release of cytochrome *c*, which may change mitochondrial morphology. The loss of $\Delta\Psi_m$ and a selective permeabilization of mitochondrial membranes contribute to the release of Ca^{2+} from the mitochondria. This was inhibited by cyclosporin A (5 μM) and Ruthenium Red (1 μg/ml), indicating the involvement of mitochondrial Ca^{2+} transport mechanisms. Bax-induced mitochondrial Ca^{2+} release evokes Ca^{2+} waves and wave propagation between cells. Our results show that Bax induces mitochondrial alteration that affects Ca^{2+} homeostasis and signaling. These changes show that Ca^{2+} signals might be correlated with the proapoptotic activities of Bax.

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Abbreviations: ER, endoplasmic reticulum; CSA, cyclosporin A; rBax, recombinant Bax; RRed, Ruthenium Red; $\Delta\Psi_m$, mitochondrial membrane potential

Introduction

Bax is a proapoptotic member of the Bcl-2 family located predominantly in the cytosolic compartment and in the monomeric form in healthy cells. After apoptotic stimuli, a significant fraction of Bax protein forms multimers and translocates to the outer mitochondrial membrane.[1] The signals involved in Bax translocation are not fully understood, but may involve conformational changes in the structure of the protein, exposing its C-terminal hydrophobic domain.[2,3] Translocation of Bax to the mitochondria is associated with the release of cytochrome *c* from the mitochondrial intermembrane space and the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$).[4] This release of cytochrome *c* and other apoptogenic factors from the mitochondria, in addition to $\Delta\Psi_m$ decrease, is probably caused by the pore-forming activity of Bax when inserted in mitochondrial membranes.[5] Once in the cytosol, cytochrome *c* activates caspases, which trigger the apoptotic response. On the other hand, the fall in $\Delta\Psi_m$ can lead to a limitation of high-energy phosphates in the cytosol, and may be a sign of mitochondrial dysfunction, possibly leading to necrotic cell death.[6]

Studies have shown that Bax and other proapoptotic members of the Bcl-2 family could modulate Ca^{2+} stores in the endoplasmic reticulum (ER) and change mitochondrial matrix Ca^{2+} (Ca^{2+}_m) contents.[7,8] In addition, mitochondrial membrane integrity is altered by excessive Ca^{2+} uptake.[9] Bax and another multidomain protein, Bak, may translocate or localize in the ER, deplete Ca^{2+} from the ER and activate caspase-12.[10] It is certainly a matter of investigation to understand the role of Bax and other proapoptotic proteins in the ER and mitochondrial interaction and in Ca^{2+} redistribution between these organelles during apoptosis.

Under physiological conditions, transient increases in cytosolic Ca^{2+} (Ca^{2+}_c) levels activate Ca^{2+}_m uptake and promote the accumulation of this ion in the mitochondria.[11,12] In some circumstances, Ca^{2+}_m uptake may increase sufficiently to induce Ca^{2+}_m release through the permeability transition pore (PTP), which promotes a Ca^{2+}-triggered, nonselective inner mitochondrial membrane permeabilization.[13,14] Thus, Ca^{2+} homeostasis is important for cell physiology and its regulation depends on a complex machinery, which maintains Ca^{2+}_c at nanomolar levels.[15]

Alterations of Ca^{2+}_c may trigger apoptotic pathways. Some of these alterations include changes in Ca^{2+}_m uptake promoted by apoptotic stimuli, which increase the probability of PTP activation.[16] Depletion of intracellular Ca^{2+} stores has also been related to the induction of apoptosis,[17] and a decrease in Ca^{2+} concentration within the ER, leading to Ca^{2+}_m accumulation, determines the mode and amount of cytochrome *c* release[18] and apoptosis.[19] On the other hand, recent studies have demonstrated that Bax and Bak deficiency causes a decrease in Ca^{2+} at the ER level and a decrease in mitochondrial Ca^{2+} uptake. In this case, absence of Bax and decrease of Ca^{2+} stores reduced apoptotic cell death induction.[20] Changes in Ca^{2+} levels within intracellular compartments may activate endogenous endonucleases when in the presence of an apoptotic stimulus,[21] amplifying the toxicity of these stimuli and accelerating cell death. Counteracting these proapoptotic effects are the antiapoptotic proteins such as Bcl-2, which can regulate Ca^{2+} homeostasis and prevent Ca^{2+}-induced cytochrome *c* release.[22–24]

Since mitochondria are the main Bax insertion site, are important in Ca^{2+} homeostasis and are intimately associated with the regulation of apoptosis, we decided to investigate the effects of Bax on mitochondrial function, including $\Delta\Psi_m$, respiration, morphology, cytochrome *c* release and Ca^{2+} transport. In order to conduct these studies, we used primary cultures of rat astroglia instead of previously used immortalized cell culture lines.[4] Our data show that Bax leads to respiratory inhibition, which may cause partial decrease of $\Delta\Psi_m$ and release of cytochrome *c*. Bax also causes Ca^{2+}_m release, in a manner dependent on Ca^{2+}_m uptake. This release of Ca^{2+} may cause increase in Ca^{2+}_c, which evokes Ca^{2+} waves and wave propagation. This report shows the effect of recombinant Bax (rBax) on Ca^{2+} homeostasis and respiration *in situ*, and provides original data that support the importance of Bax-induced Ca^{2+}_m regulation, which might be important for the apoptotic process.

Results

Bax decreases mitochondrial membrane potential ($\Delta\Psi_m$) in permeabilized astrocytes

We have previously shown that translocation of Bax protein from the cytosol to cellular membranes,[1,25] especially mitochondrial membranes, after treatment with staurosporine (STS), causes loss of $\Delta\Psi_m$.[4] Since in Bax-overexpressing systems, a small percentage of the protein may be preinserted in membranes and this can alter Bax effect on $\Delta\Psi_m$, we investigated the changes in $\Delta\Psi_m$ after the addition of the recombinant protein to native cells.

In Figure 1, we show astrocytes loaded with the mitochondrial potentiometric dye TMRE (20 nM, 5–10 min). We observed that within 5–10 min the mitochondria were completely loaded with the dye and further incubation was not necessary. However, the experiments were performed in the presence of the same TMRE concentration to replenish dye loss through photobleaching.[13] Thereafter, cells were washed several times in intracellular buffer (IB, see Material and Methods) and digitonin (7 μg/ml) was added to permeabilize selectively the plasma membrane. This permeabilization procedure was complete after 2–3 min of digitonin addition and was monitored by real-time microscopy. After permeabilization, the cells were incubated with IB free of digitonin and supplemented with mitochondrial substrates, protease inhibitors, an ATP regenerative system, TMRE and thapsigargin (Tap, 2 μM), to avoid ER Ca^{2+}-ATP_{ase} influence during the measurements of $\Delta\Psi_m$. Under these conditions, pure rBax, obtained as described below, was tested at different concentrations (10, 100 and 10 followed by 100 ng/ml).

Figure 1 shows, in two different magnifications, the partial loss of $\Delta\Psi_m$ when Bax was added. Rat astrocytes show mitochondria with a typical elongated structure, and in close relation with other organelles that may represent functional interconnection. Fluorescence traces extracted from mitochondrial regions of interest (ROI) show that the decrease of $\Delta\Psi_m$ is rapid but not complete when compared with FCCP added at the end of the experiments (Figure 1b, Supplementary Figure 1 video 1). Most of the analyzed mitochondria

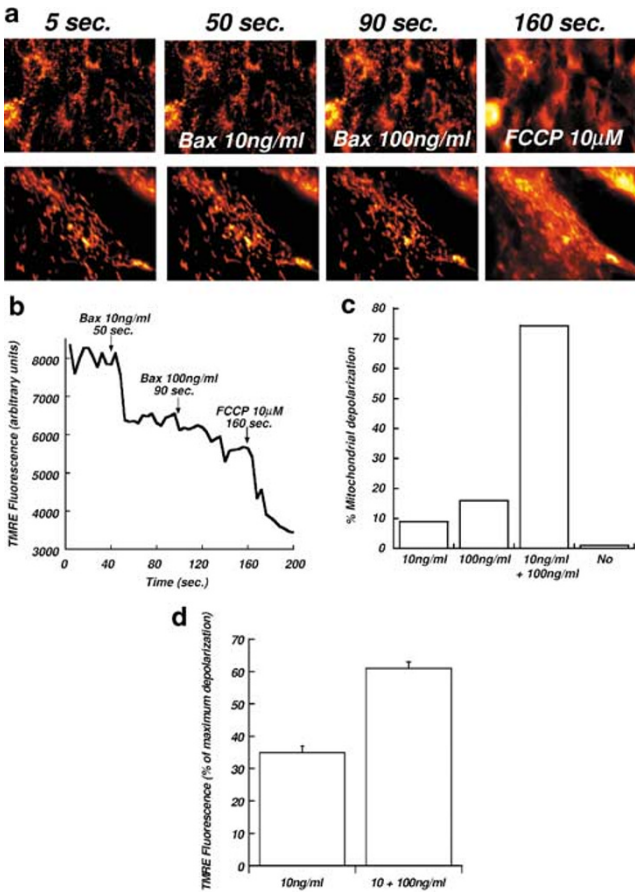


Figure 1 rBax decrease $\Delta\Psi_m$ in permeabilized cells. Rat astrocytes were loaded with the mitochondrial potentiometric dye TMRE (20 nM) and permeabilized with digitonin (7 μg/ml) as described in Material and methods. Images were acquired with $\times$ 43 or with $\times$ 100 with a CCD camera and 10-s delay. (**a**) First row shows astrocytes image acquired with $\times$ 43 objective. Bax was added at 50 (10 ng/ml) and 90 (100 ng/ml) seconds and FCCP (10 μM plus oligomycin 1 ng/ml) at time 160 s. Second row shows astrocyte image acquired with $\times$ 100 objective and FCCP addition at time 160 s (Supplementary Figure 1 video 1). (**b**) Representative graph of the fluorescence traces extracted using ROI tool showing the depolarization induced by each addition. (**c**) Histogram shows the number of mitochondria (in percentage) that depolarized with 10, 100 or 10 + 100 ng/ml of Bax or that showed no response. (**d**) Percentage of $\Delta\Psi_m$ loss (decrease in TMRE fluorescence in relation to maximum depolarization) induced by 10 (34%) and 100 ng/ml (34 + addition 27%) in relation to the maximum depolarization obtained after FCCP addition. Graphs represent means obtained from more than 400 mitochondria extracted from at least six experiments

(over 400 from different cells) were sensitive to the lower concentration of Bax used (10 ng/ml) and, irregularly, sensitive to subnanogram levels (not shown). Figure 1c shows that approximately 10% of the mitochondria responded to 10 ng/ml of Bax. A similar percentage was less sensitive and did not answer to 10 ng/ml but responded with some level of loss of $\Delta\Psi_m$ to 100 ng/ml. However, the majority of the mitochondria responded to 10 and 100 ng/ml in a dose-dependent manner, when added in the same experiment. Only 1% of the mitochondria did not show any change in $\Delta\Psi_m$ after Bax addition. The concentration of 10 ng/ml induced, on average, a 34% of decrease in TMRE fluorescence in the ROI in relation to the maximum decrease induced by FCCP. The 100 ng/ml

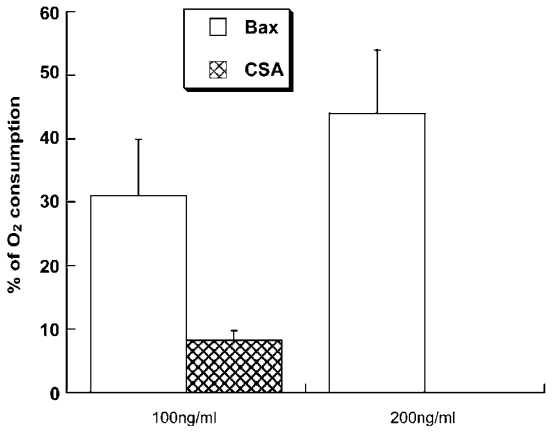


Figure 2 Bax inhibits mitochondrial respiration. Respiration was measured in permeabilized cells. Histogram shows the averaged effects with different concentrations of Bax (100 and 200 ng/ml) that inhibited respiration in a concentration-dependent manner. This effect was strongly suppressed by CSA (5 μM). Data are represented as the percentages of the inhibition of respiratory rates of at least three experiments

induced a further 25–30% (about 60% of the total) decrease in fluorescence in relation to the maximum decrease (Figure 1d). Since most mitochondria responded to these concentrations of Bax, we decided to use them in the following experiments.

Bax inhibits respiration

The next step was to investigate if the addition of Bax would change mitochondrial respiration. We found that Bax (100 and 200 ng/ml) inhibits respiration in permeabilized astrocytes, and that this effect is inhibited by pre-incubation with cyclosproine A (CSA) (Figure 2). Thus, the decrease in respiratory rates induced by Bax could be related to cytochrome *c* release and structural mitochondrial alterations associated with the opening of the PTP. Therefore, we investigated the effect of Bax on cytochrome *c* release under the same conditions.

Cytochrome *c* release following Bax treatment

To associate our findings with the induction of cell death, we investigated mitochondrial cytochrome *c* release after the addition of Bax through immunofluorescence. Mitochondrial location and morphology was simultaneously assessed using MitoTraker Red (MTR, 25 nM). Figure 3 shows confocal images obtained with rat astrocytes that were incubated with MTR and anticytochrome *c* antibody as described in Material and Methods. The first row shows control cells not treated with Bax and the second row shows images obtained from cells that were incubated with 10 ng/ml Bax (Figure 3a). After the addition of Bax, mitochondria changed shape and appeared rounded when compared to the elongated and well-defined organelles present in the control cells (Figure 3b). Cytochrome *c* distribution patterns also changed and appeared more diffused into the cytosol, which might be correlated with a partial, but not complete, release of cytochrome *c* from the mitochondria to the cytosol.

Bax releases Ca^{2+}_m

Next, we studied the effect of rBax on intracellular Ca^{2+} stores such as the ER and mitochondria, using a similar protocol in which the cellular membrane was rendered permeable to Bax by use of digitonin. For these experiments, astrocytes were used in suspension, as described previously.[26] Ca^{2+} levels were measured in the extramitochondrial space of the cell suspension, using Fura-2FF in the presence of the Ca^{2+}-ATP_{ase} (SERCA) inhibitor Tap. Results were normalized in relation to the basal fluorescence and data were expressed as percentage in relation to the total Ca^{2+} (obtained with ionomycin) in the system. To avoid misinterpretation of the effect induced by Bax and other compounds due to the variation in the Ca^{2+} pool sizes, the calculated percentages expressed the total Ca^{2+}. This allowed us to compare results from different cells that exhibited different Ca^{2+} content and pool sizes. Our results show that Bax (10 ng/ml) induced an increase in the fluorescence ratio of Fura-2FF, which indicates Ca^{2+}_m release (Figure 4a). As a negative control to Bax effect, we used the mutant Bax W107 that presents point mutations on Bax sequence specially at the α7, α8 and α9 helices of its structure.[3] The α9 helix of Bax is located at the hydrophobic pocket of the C-terminal tail and in Bax, the C-terminal region is the putative transmembrane domain. Replacement of Trp^{188} (as in the mutant W107) abolished Bax translocation from the cytosol to mitochondria and decrease its toxicity.[2] The experiments with Bax mutant W107 showed that Bax-induced Ca^{2+} release is dependent on Bax structure and localization on mitochondria, since Bax mutant W107 does induce the effect observed with Bax wild type (Figure 4b).

After Bax-induced Ca^{2+} release, ionomycin still induces a further increase in extramitochondrial Ca^{2+}, which indicates that Bax does not completely deplete the stores. Figure 4c shows that addition of Tap induces an increase in Ca^{2+}, such as those promoted by the inhibition of ER Ca^{2+} uptake,[27] and that Bax still induces a Ca^{2+} increase after this treatment. However, this effect was lower than the effect observed in Figure 4a, indicating that Bax may also act on Tap-sensitive stores. Bax was tested after the addition of FCCP (10 μM), which eliminates the driving force for Ca^{2+} uptake (Figure 4d). Thus, FCCP itself can also induce a Ca^{2+}_m release,[13] and under these conditions, Bax-induced Ca^{2+}_m release was inhibited. In these experiments, FCCP was used with oligomycin (1 μg/ml) to avoid rapid ATP consumption.[28] We investigated if Bax effect on Ca^{2+}_m release was sensitive to other inhibitors of mitochondrial Ca^{2+} transport. Histogram in Figure 4e shows the effect of Bax after FCCP, Ruthenium Red (RRed, 1 μg/ml) and CSA (5 μM). Results show that Bax effect was significantly inhibited by the PTP inhibitor CSA and by FCCP. This corroborates the hypothesis that the release of Ca^{2+}_m may involve PTP opening in a transient mode since a full depolarization of the $\Delta\Psi_m$ was not observed. This result may be correlated with findings that, in different conditions, Bax-induced cytochrome *c* release was prevented by inhibitors of the PTP.[29] We also investigated if this process

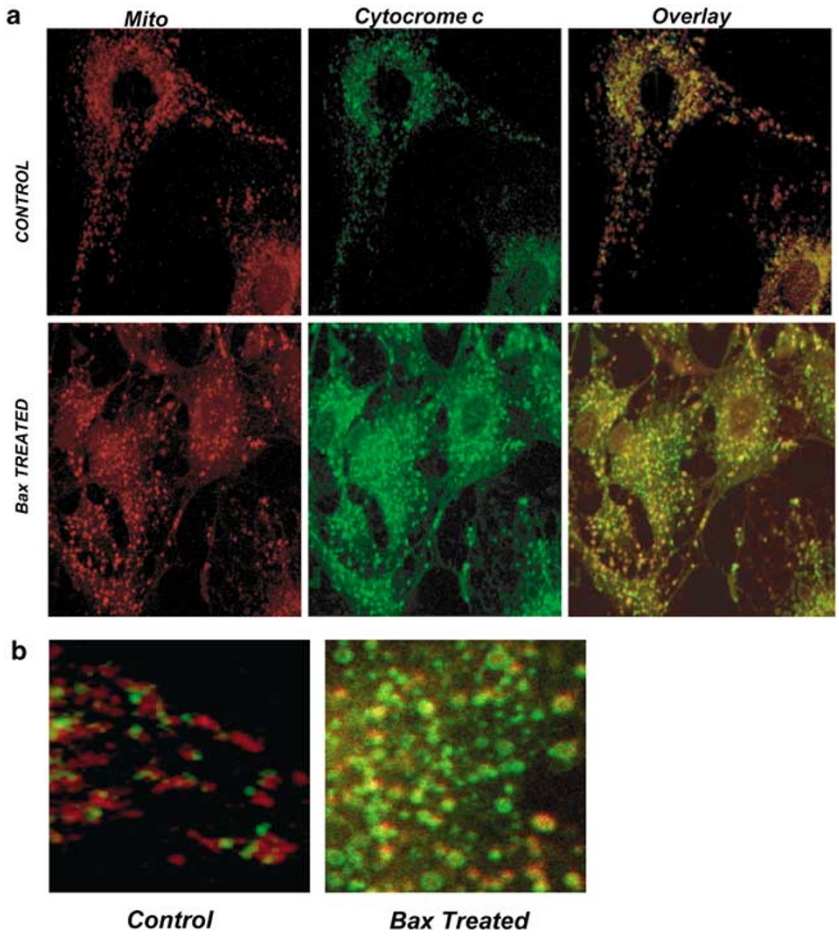


Figure 3 Bax induces mitochondrial swelling and partial release of cytochrome *c*. (**a**) Astrocytes were loaded with MTR (25 nM) for 10 min, permeabilized with saponin (0.01%) and fixed with formaldehyde (2%). Cytochrome *c* staining was obtained with secondary antibody against cytochrome *c* and revealed with FITC-conjugated secondary antibody. In some experiments, after permeabilization cells were treated with 10 ng/ml rBax (second row) for 2 min. Treated cells were compared with control cells that were not treated with Bax (first row). (**b**) shows mitochondria with regular shape and fluorescence in control cells (first panel) and others with rounded aspect and irregular distribution of cytochrome *c* (second panel). Images were acquired using a confocal microscope (LSM 5120)

occurred through the mitochondrial Ca^{2+} uniporter. This Ca_m^{2+} uptake is fueled by $\Delta\Psi_m$, which forms a highly negative charge in the mitochondrial matrix. In the absence of a more negative $\Delta\Psi_m$, the uniporter may work in a reverse mode allowing Ca_m^{2+} to be redistributed to the extramitochondrial space. In the presence of RRed, the effect of Bax (10 ng/ml) was again strongly inhibited (Figure 4e). These results indicate that the decrease in $\Delta\Psi_m$ induced by Bax could induce the uniporter to work in a reverse mode, allowing efflux of Ca_m^{2+} that is inhibited by RRed.

To verify if Bax-induced Ca_m^{2+} release could affect ER Ca^{2+} content, experiments were carried out in similar conditions as described and shown in Figure 4. Thus, cells were first treated with Tap (2 μM), which increased the external Ca^{2+} due to the release of Ca^{2+} from Tap-sensitive stores. In other experiments, Bax (10 ng/ml) was added first (in the absence of Tap) and the effect of Tap was then observed in its presence. In these circumstances, the effect of Tap was significantly lower than its effect before Bax addition. This indicates that previous addition of Bax caused release of Ca^{2+} from the mitochondria and/or Tap-sensitive stores, thus when Tap was added, its effect was decreased. Similar effects were obtained when Tap was added after FCCP, which also causes Ca_m^{2+} release and FCCP + Bax (Figure 5). As noted before, each effect was expressed as percentage in relation to the total Ca^{2+} released calibrated with ionomycin.

Bax induces Ca^{2+} waves that are propagated through the cells

We have also investigated the effect of Bax in an intact nonpermeabilized system. For this study, astrocytes plated in Petri dishes were loaded with Fura-2AM in a regular microscopy buffer.[13] Before the experiment, cells were washed in Ca^{2+}-free buffer to avoid influx of external Ca^{2+} through plasma membrane Ca^{2+} channels. Control experiments were performed in the presence of external Ca^{2+} as well. Ca_c^{2+} levels in isolated cells were investigated using high-resolution digital microscopy controlled by computer software. After placing the coverslips, one cell in the field was chosen for microinjection. When the injector was in the right position and the cell attached, image acquisition started at 3-s intervals between images. After about 40 s of acquisition, rBax (10 ng/ml) was microinjected.

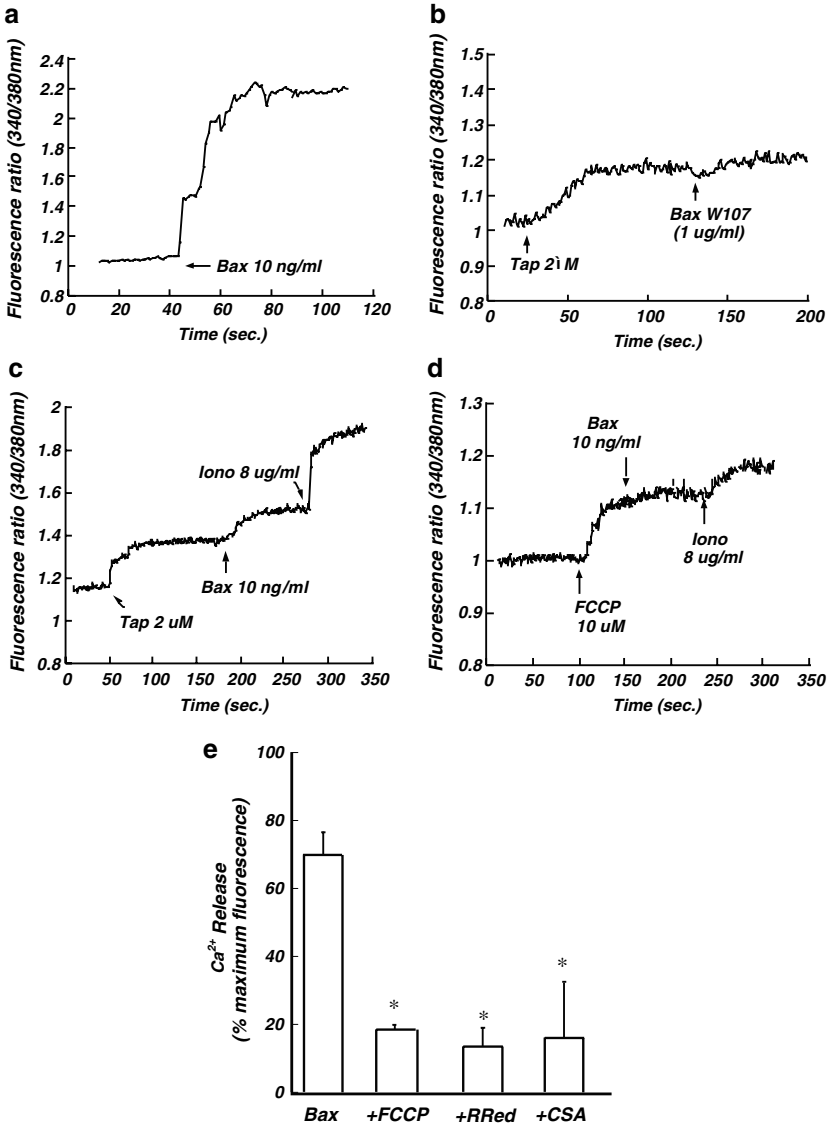


Figure 4 Bax-induced Ca_m^{2+} release is dependent on its structure**.** Astrocytes in suspension were permeabilized with digitonin as described in Material and methods. After digitonin permeabilization, Fura-2FF-free acid (5 μM) was added followed by Tap (2 μM). (**a**) Effect of rBax (10 ng/ml) and the increase in fluorescence ratio, which corresponds to the release of Ca_m^{2+} to the extramitochondrial space. (**b**) Effect of Tap followed by the addition of the Bax mutant (W107 1 μg/ml), which did not change the trace pattern. (**c**) Effect of rBax (10 ng/ml) after Tap and then calibrated with ionomycin (Iono, 8 μg/ml) that was added at the end of each experiment to induce maximum release of Ca^{2+} from intracellular stores. (**d**) Bax was tested in the absence of Tap but in the presence of FCCP (5 μM) plus oligomycin (1 μg/ml). Graphs are represented as fluorescence ratio normalized to the baseline fluorescence. (**e**) Histogram shows the average ratio values that represent the Ca^{2+} released from intracellular stores by each condition tested. It also shows the effect of Bax in the absence (first bar) and after the addition of FCCP (+ FCCP), Ruthenium Red (+ RRed) and CSA (+ CSA). Data are expressed as the percentage of maximum fluorescence obtained with ionomycin added at the end of each experiment. *Significantly different from control $P<0.05$ (Bax effect represented in the first bar). Data represent at least four experiments

Bax induced a Ca_c^{2+} wave immediately after injection (Figure 6, Supplementary Figure 6 video 1). This effect was massive, rapid and transient and reached the peak after 2–4 s. Different wave patterns were induced and in certain cells the transient peak was followed by a more sustained response that persisted for several seconds (Figure 6b–e). The injection of the same buffer in which Bax was diluted did not induce changes in fluorescence (Supplementary Figure 6 video 2). Cells were numbered according to the location of the microjected cell (#1) to show that the increase in Ca_c^{2+} started more rapidly in the cells that were closer to the microjected one cell, which presented a smaller time lag for the beginning of the response. Data show the time when Bax response started and the time to peak in relation to its location (Figure 6f). In some cells, Bax induced not only one wave but also oscillations with several peaks with smaller amplitude and low frequency when compared to the first wave (Figure 7).

Discussion

In this study, we evaluated the effects of Bax on calcium transport in primary cultured astrocytes. The use of primary astrocyte cultures is of interest since these cells do not

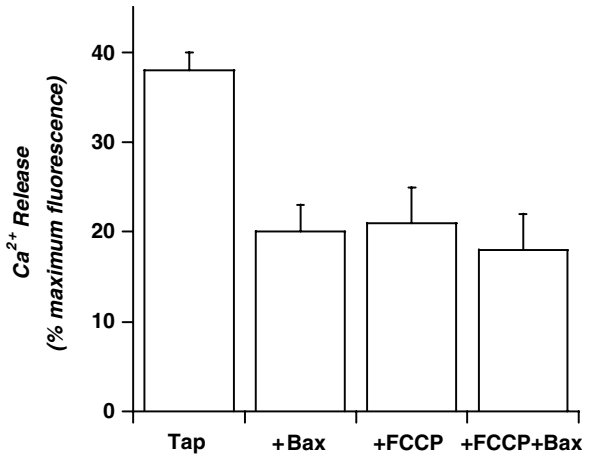


Figure 5 Bax decreases Tap-sensitive stores Ca^{2+} content. Astrocytes in suspension were permeabilized as described before. Histogram compares the effect of Tap (2 μM) before and after the treatment with 10 ng/ml rBax (+ Bax), 5 μM FCCP (+FCCP) and FCCP + Bax (+FCCP + Bax). These results reflect the level of refilling of Tap-sensitive stores after Bax or FCCP treatment. In all experiments FCCP (5 μM) was used with oligomycin (1 μg/ml). Data shown are means of the ratio values that represent the Ca^{2+} released from intracellular compartments. Results are expressed as the percentage of maximum fluorescence obtained with ionomycin added at the end of each experiment. Data represent at least four experiments

present the biochemical and morphological alterations typical of immortalized cell lines usually adopted in these studies.[1,30,31] We found that Bax induces an incomplete loss of $\Delta\Psi_m$ (Figure 1), which can be related to the inhibition of maximal respiratory rates (Figure 2) and cytochrome *c* release (Figure 3). The partial loss of $\Delta\Psi_m$ may also contribute to the release of Ca_m^{2+} through the uniporter in a reverse mode and the PTP opening (Figure 4). This Ca_m^{2+} released may induce further release of the ER Ca^{2+} (Figure 5), which may induce Ca^{2+} wave and wave propagation between cells (Figures 6 and 7).

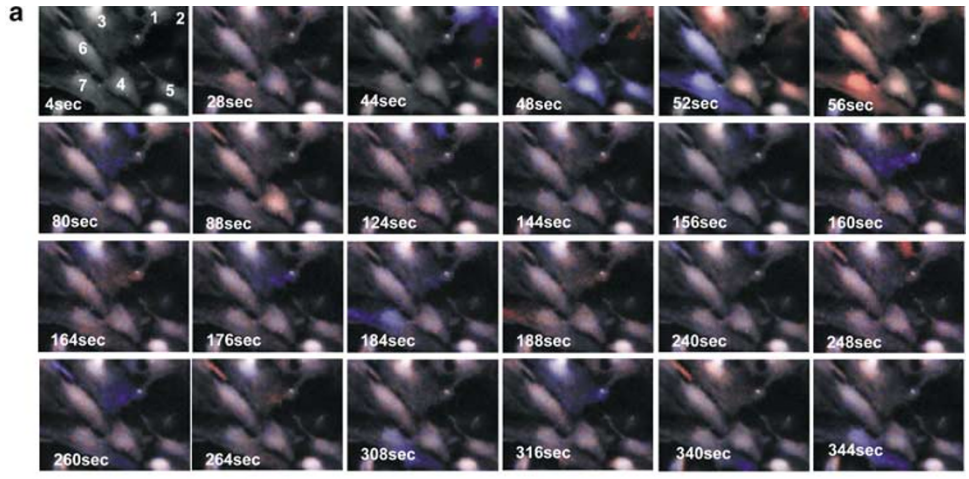
Among the proapoptotic proteins of the Bcl-2 family, Bax has been reported to translocate from the cytosol to mitochondria upon an apoptotic stimulus and release of cytochrome *c* and other apoptotic factors.[1,32] According to several lines of evidence, different hypotheses were drawn to discuss the permeabilization induced by apoptotic members of Bcl-2 proteins. The ability of Bax to form homo- or heterodimers[33] could induce Bax to form oligomers when associated with mitochondrial membranes[31,34] or mitochondrial contact sites.[35] This could lead to pore formation and the release of cytochrome *c* and other proapoptotic factors.[36–40] Another possible pathway is Bax interaction with the adenosine nucleotide translocator, which could trigger the opening of PTP.[30,35] Still, Bax could act on antiapoptotic proteins, like Bcl-2 and Bcl-x_L, located at the outer mitochondrial membranes.[39,40]

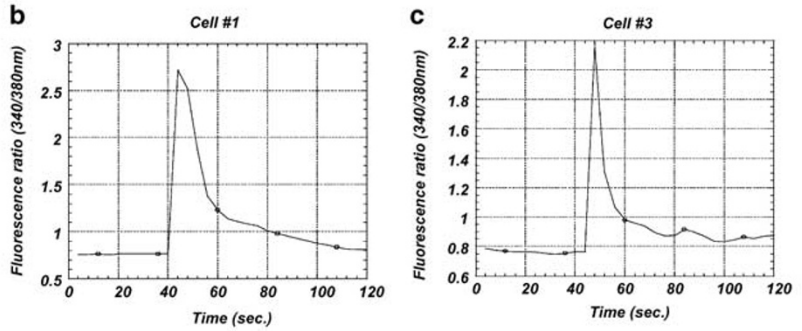
In the present study, under conditions where the plasma membrane was selectively permeabilized, we were able to manipulate precisely the extramitochondrial medium and the use of membrane-impermeable drugs capable of inhibiting specific intracellular components of interest. Since mitochondrial membranes contain low amounts of cholesterol, the lipid for which digitonin presents highest affinity, this experimental condition does not affect the mitochondrial structure or function.[41] Our data indicate that Bax leads to permeabilization of the mitochondrial membranes. This could be a selective permeabilization, which explains the release of cytochrome *c* from the mitochondria, the reduction in mitochondrial respiratory rates and the partial decrease in $\Delta\Psi_m$, since lack of this respiratory chain component causes a deficiency in mitochondrial electron transport and proton pumping.

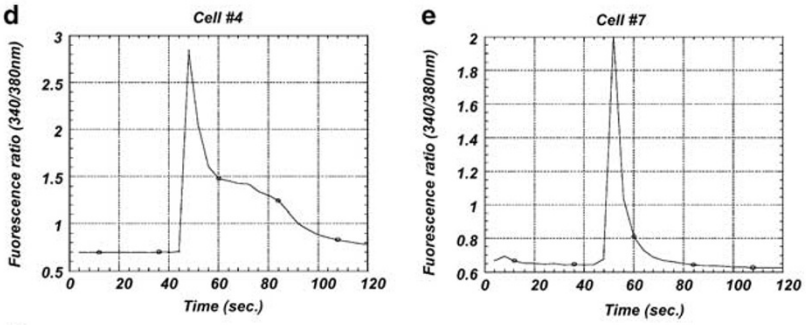
In addition to the release of cytochrome *c* from the mitochondria, we found, using confocal microscopy, that Bax altered the shape of mitochondria with shortening of their elongated tubular aspects. Since Bax affects membrane fission and fusion, this may determine mitochondrial discontinuity of the outer mitochondrial membrane and nonspecific lipid pore formation.[42,43] We show that normal astrocytes present a complex network of elongated mitochondria, which appear physically interconnected, while Bax-treated cells present more rounded mitochondria, which did not exhibit contact points between them (Figure 3b). These results argue in favor of Bax being related to a selective permeabilization of the mitochondrial membranes, leading to a partial release of cytochrome *c* and changes in mitochondrial morphology as described by others.[44] Although we cannot exclude that morphological changes can lead to alterations in fluorescence distribution, the changes in mitochondrial morphology induced by Bax may also be due to the modification in the mitochondrial architecture promoted by some apoptotic proteins.[45,46] This hypothesis is reinforced by the fact that Bax inhibited respiration and this effect was prevented by CSA, indicating a PTP involvement without mitochondrial membrane ruptures and total release of cytochrome *c*.

Respiratory inhibition and $\Delta\Psi_m$ decrease were accompanied by a release of Ca_m^{2+}, which may be explained by the fact that the Ca_m^{2+} uptake against the concentration gradient is driven by $\Delta\Psi_m$,[28] and will not be well maintained during some decrease or loss of $\Delta\Psi_m$. This was corroborated by our results since RRed strongly inhibited Bax-induced Ca_m^{2+} release. However, in the absence of RRed and presence of PTP, an inhibition was also observed. This result further supports the idea that mitochondrial effects of Bax are primarily related to mitochondrial membrane permeabilization.

While mitochondria are involved in apoptosis[47] and Ca_m^{2+} has clearly been shown to play important roles in physiological signaling processes,[11,12] the role of Ca^{2+} in apoptosis is still unclear. Several recent lines of evidence suggest a coordinating role of Ca^{2+} ions in apoptosis.[48] For example, Ca^{2+} was reported to be required during cell death and was able to cause apoptosis itself under certain circumstances.[49] Depletion of ER Ca^{2+} stores may lead to Bax translocation to the mitochondria,[50] induce apoptotic signals via death receptor pathways[51] and favor cytochrome *c* release from the mitochondria.[52] In addition, Zong *et al*[10] have shown that Bax, and another multidomain protein Bak, can localize to the ER, involving a depletion of Ca^{2+} from this organelle followed by caspase-12 activation. Other studies demonstrated that Bax and Bak deficiency caused a decrease in Ca^{2+} at the ER, which could reduce apoptotic cell death. Therefore, the Ca^{2+} levels at the ER may play a role when apoptotic inducers that release the ion from internal stores are present.[20] Further







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CELL #	LOCATION	BEGINING	TIME TO PEAK
1	MICROINJECTED CELL	40 SEC.	44 SEC.
2	Adjacent to #1	40 SEC.	44 SEC.
3	Adjacent to #1	44 SEC.	48 SEC.
4	Conected to #3	45 SEC.	48 SEC.
5	Conected to #4	45 SEC.	48 SEC.
6	Conected to #4	48 SEC.	50 SEC.
7	Conected to #4	48 SEC.	50 SEC.

Figure 6 Bax microinjected induces Ca^{2+} waves that are propagated throughout the cells**.** Astrocytes plated on coverslips were loaded with Fura-2AM (10 μM) in microscopy buffer. Ca_c^{2+} levels in isolated cells were analyzed using high-resolution digital microscopy with an inverted microscope coupled to a cooled CCD camera and controlled by computer software. For each experiment one of the cells in the field was used for microinjection of rBax (10 ng/ml) that was injected in bolus (Supplementary Figure 6 video 1 and Supplementary Figure 6 video 2). (**a**) Images were collected at 3-s intervals and Bax was injected in cell #1 at 40 s. (**b**) Graphs show that the increase in Ca_c^{2+} after Bax injection occurred not only in the injected cell #1, but also in the adjacent cells #2 (**c**), #3 (**d**) and #4 (**e**) at different intervals. Cells showed a transient peak, which reached maximum response after 2–4 s of the beginning of the effect. (**f**) Cells in the field were numbered accordingly to the location of the microjected cell (#1). Numbers show the location of each cell, the time that Bax response started and the time to peak

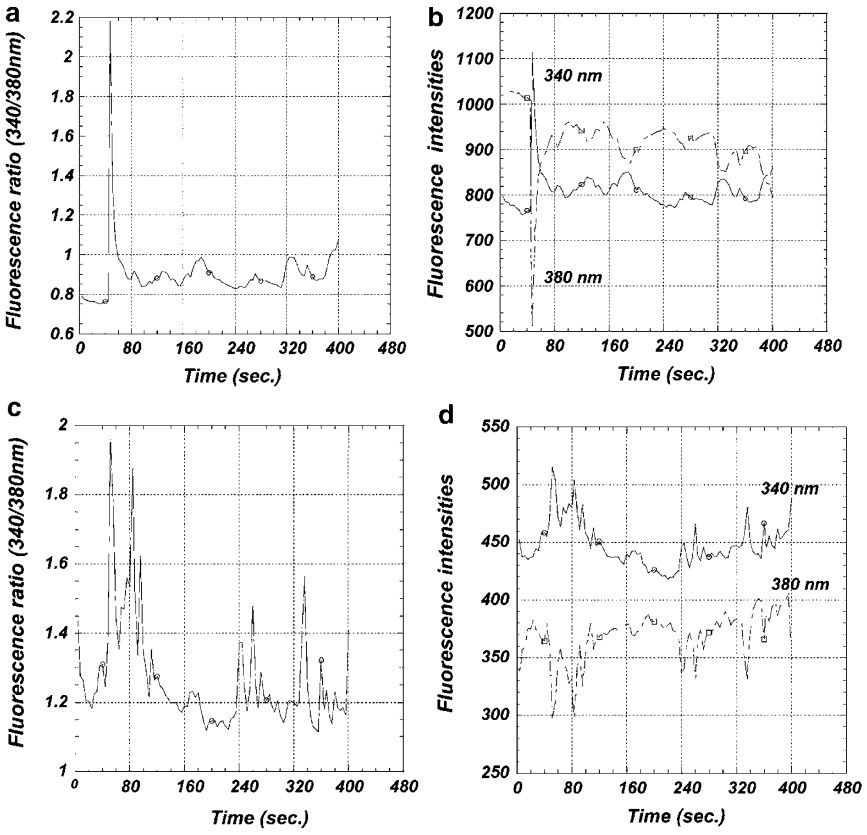

Figure 7 Bax induces waves and oscillations. In certain cells the addition of Bax caused oscillations with different amplitudes and frequency. (**a**) shows a graph of oscillation represented in fluorescence ratio for one cell type and (**b**) the respective intensity traces. (**c**) shows another oscillatory pattern and (**d**) the corresponding intensity lines

evidences that Ca^{2+} dynamics can be a control point show that when Bcl-2, predominantly localized to the ER, is phosphorylated, there is a decrease in its antiapoptotic effect together with an increase in Ca^{2+} discharge from ER.[53] In fact, the antiapoptotic protein Bcl-2 is able to affect ER and mitochondrial Ca^{2+} stores.[19,22] Even though results are still controversial, increasing evidences indicate that either a Ca^{2+} overload or perturbation of the intracellular compartmentalization can trigger apoptotic cell death.[48]

To bring further support to Ca^{2+} signaling in apoptosis, we found that Bax induced Ca^{2+} release mainly from the mitochondria, although we cannot exclude that some release may from other Tap-insensitive stores. This effect might be correlated with its mitochondrial location and apoptotic effect, since Bax mutant W107, which does not translocate from cytosol[2] to mitochondrial and presents limited apoptogenic capacity (Sharpe and Youle, unpublished results), did not promote Ca^{2+}_m release. This extramitochondrially Ca^{2+} increase caused by Bax evoked a Ca^{2+} wave and wave propagation, showing that Bax affects Ca^{2+} signaling as well. Waves are important phenomena not only in physiological cell signaling but also during amplification of apoptotic signals.[54] In addition, several reports have already shown that Ca^{2+} waves can be propagated through cell junctions (e.g. gap junctions).[55] In astrocytes, this propagation contributes to spread death signals between cells and increases apoptosis instead of necrosis.[56] Furthermore, recent reports showed that gap junctions permeate apoptotic signals and mediate cell death in developing retina.[57] In our findings, Bax-induced release of Ca^{2+} of mitochondrial stores. This Ca^{2+} might induce a further release of Ca^{2+} from this organelle generating a calcium-induced calcium release (CICR). It is possible that these calcium events are coordinated with augmented cytochrome *c* release.[58] In this scenario, one can suggest that Ca^{2+} released in one cell that has high levels of Bax may serve to send Bax signals to other cells via Ca^{2+} release. Thus, in addition to its intracellular effects, Ca^{2+} would contribute to propagate signals related to perturbations in cytosolic or stored Ca^{2+} levels. In the presence of more massive stimuli, Ca^{2+} would cause not only the amplification of cell death but also toxicity.

In light of this evidence one could propose two interrelated mechanisms where Ca^{2+} plays different roles depending on the location of the proapoptotic protein in the cytosol or mitochondria (Figure 8). In the former, Bax is present in the cytosol and, in the presence of an apoptotic stimulus, leads to an increase in ER Ca^{2+} levels followed by the release of this Ca^{2+} to the cytosol. This Ca^{2+} could either accumulate in the mitochondria or activate caspase-12. In the latter, Bax translocated to mitochondria would cause release of cytochrome *c*, inhibition of respiration and a dual effect on the mitochondrial membranes leading to Ca^{2+}_m release. In both situations, increase in Ca^{2+}_c induced by Bax may be transmitted to other cells through wave propagation and cell

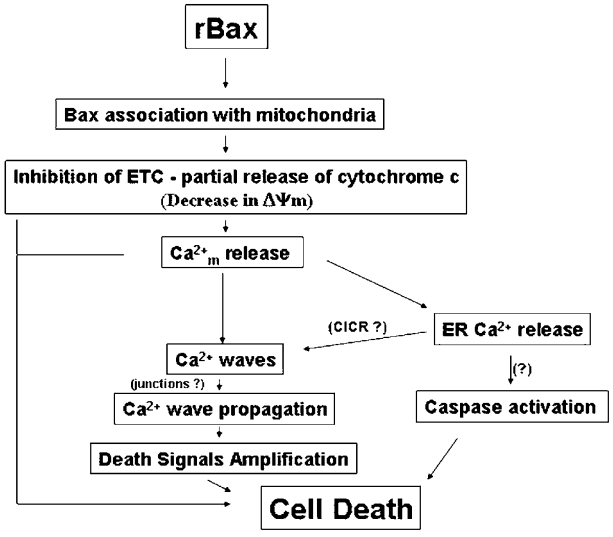

Figure 8 Scheme proposed for rBax effect on mitochondria function and morphology. rBax induces inhibition of mitochondrial electron transport chain (ETC) and $\Delta\Psi_m$ decrease with a permeabilization of the mitochondrial membranes and partial release of cytochrome *c*. This may cause the release of Ca^{2+}_m through the uniporter, working in a reverse mode and/or PTP. The released Ca^{2+}_m may cause a further release and decrease of ER Ca^{2+}, which induces Ca^{2+} waves via a CICR mechanism. The propagation of Ca^{2+} may occur via gap/cell junctions. Ca^{2+} released from ER and mitochondria may be involved in caspases activation. Propagation of Ca^{2+} waves may contribute to further caspases activation and amplification of apoptotic signals and increase in toxicity

junction. In the presence of apoptotic stimuli that release Ca^{2+} from intracellular stores, the result would be an even higher degree of cell death.[59] Although the nature of these changes can differ according to the experimental conditions, the results presented here are original observations showing that proapoptotic activities of Bax protein involves Ca^{2+} signals and homeostasis, which may serve as an important element during apoptosis.

Materials and Methods

Cell cultures

Astrocytes were prepared from cortices of 2-day-old rats, as described previously.[13] Cultures were maintained at 5% CO_2 and 37°C in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% fungizone. When cells were confluent (after 7–10 days), they were trypsinized and suspended in IB (see below), counted and immediately used for the experiments. Cell culture reagents were from Gibco-Invitrogen (Carlsbad, California, USA).

Protein expression and purification

Full-length Bax and the mutant W107 were produced in *Escherichia coli* as a chitin fusion-binding protein using the pTYB1 plasmid and were purified by affinity chromatography followed by ion exchange chromatography. The peak protein fraction was concentrated, aliquoted and stored at −80°C. Bax oligomerization was induced by a 1-h incubation of the protein with 1% octylglucoside.[3,60] Bax mutant W107 present point mutations on Bax sequence where five tryptophans were changed to phenylalanine except the named residue (e.g. W107 was mutated at W139F, W151F, W158F, W170F, W188F and W107 was preserved). These mutations affected mainly the α6, α7, α8, α9 helices. Replacement of Ser^{184} or Trp^{188} (as in the mutant W107) abolished Bax translocation from the cytosol to mitochondria and decreased Bax toxicity.[2,3]

Single cell measurements of $\Delta\Psi_m$

Cells cultured on coverslips were transferred to a thermostatically controlled temperature chamber and incubated with TMRE (20 nM, Molecular Probes, Eugene, OR, USA) for 5–10 min in a microscopy buffer containing (mM): 130 NaCl, 5.36 KCl, 0.8 $MgSO_4$, 1 Na_2HPO_4, 25 glucose, 20 HEPES, 1 Na pyruvate, 1.50 $CaCl_2$, 1 ascorbic acid, pH 7.3. Before permeabilization, cells were washed in IB containing (mM) : 135 KCl, 15 NaCl, 1.2 KH_2PO_4, 10 HEPES, 5 glutamate, 1 pyruvate, 10 U/ml aprotinin and the protease inhibitors leupeptin, antipain and pepstatin A (1 μg/ml each). IB was run through a Chellex (Bio-Rad Laboratories, Hercules, CA, USA) column to decrease Ca^{2+} and contaminating Mg^{2+}. After Chellex treatment, the pH was adjusted to 7.2 and the Ca^{2+} levels in IB medium were estimated to be below 300 nM after measurement with Fura-2 free acid (Molecular Probes, Eugene, OR, USA) in a spectrofluorimeter (Photon Technology International, New Jersey, USA). Before the experiments, cells were washed with IB and permeabilized with digitonin (7 μg/ml) for 2 min and monitored microscopically. After permeabilization, cells were washed with IB free of digitonin, as ATP-regenerating system with 2 mM ATP.Mg, 5 mM phosphocreatinine, 5 U/ml creatinine kinase and TMRE (20 nM) at 37°C. During all experiments, the cells were perfused in the presence of 20 nM TMRE to replace the photobleached dye.[13] TMRE fluorescence (548 nm excitation and 585 nm emission) was acquired using a TE300 Nikon inverted microscope (Nikon Osaka, Japan) and a 16 bit cooled CCD camera MicroMax 512BFT (Roper Sci, Princeton Instruments, USA) controlled by imaging software (Spectralyzer, Philadelphia, PA, USA). Owing to the high resolution, individual mitochondria were localized, especially at the borders of the cells, and the ROI were drawn surrounding each organelle.

Mitochondrial respiration

In these experiments, cells were added to IB buffer containing ATP-regenerating system, as used for all protocols, plus digitonin (7 μg/ml). After the addition of cells to the permeabilization buffer, respiration was measured for 1 min to achieve a steady-state oxygen consumption rate. Thereafter, different concentrations of Bax were added. Some experiments were performed in the presence of CSA (5 μM). Bax was also tested in the presence of ADP (1 mM) to evaluate inhibition in the presence of maximum respiration (not shown). Respiration was measured using a computer-interfaced Clark-type oxygen electrode from Hansatech Instruments (Norfolk, UK) with a water-jacketed chamber kept at 37°C. In some experiments, mitochondrial respiration was uncoupled by the addition of 1 μM FCCP.

Cytochrome *c* immunofluorescence and confocal microscopy

The cells were plated on 12-mm diameter glass coverslips on 24-well cluster plates for 3 days prior to use. Cells were first washed with PBS (0.1 M, pH 7.4) and incubated with 25 nM MTR (Molecular Probes, Eugene, OR, USA) for 10 min. Then the cells were washed three times with PBS (0.1 M pH 7.4) at 4°C and permeabilized with saponin (0.01%, 5 min). Thereafter, cells were washed with PBS and incubated with Bax

(10 ng/ml, 2 min) followed by incubation with primary antibody against cytochrome *c* (Jackson ImmunoResearch, West Grove, PA, USA) in PBS with 1% BSA. At the end of the incubation, the cells were washed three times with cold PBS and fixed for 30 min at room temperature in 2% formaldehyde in the same buffer. Coverslips were then washed five times in PBS and incubated with fluorescently FITC-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min at room temperature, then washed five times in PBS. The cells were incubated with DAPI (1 : 2000) (Molecular Probes; Eugene, OR, USA) for 2 min, washed five times in PBS, mounted in Fluormount-G (EM Sciences; Ft. Washington, PA, USA). Images were collected using a confocal microscope (Zeiss LSM510, Heidelberg, Germany) and Plan-Neofluor $\times 40$ and $\times 63$ oil-immersion 1.3 NA lens, with excitation at the 488 nm laser line argon/krypton, 543 nm laser line He/Ne and two-photon titanium sapphire laser module (Coherent, USA).

Extramitochondrial Ca^{2+} measurements in permeabilized cells

After trypsinization, cells were suspended in IB medium and treated with Chellex as described above. This decreased Ca^{2+} contamination to less than 300 nM. Bax preparation was also measured in terms of Ca^{2+} and did not show a concentration higher than 300 nM. All experiments were performed in the presence of an ATP-regenerating system containing 2 mM ATP.Mg, 5 mM phosphocreatinine, 5 U/ml creatinine kinase at 37°C. Before starting the experiments, cells were permeabilized with 7 μg/ml digitonin for 2 min, then incubated for another 2 min with the low-affinity Ca^{2+} dye Fura-2FF-free acid (5 μM, Molecular Probes Inc., Eugene, OR, USA) and Tap (2 μM) to inhibit ER Ca^{2+} uptake. The low-affinity Ca^{2+} dye Fura-2FF presents a K_d of 35 μM, which allows measurements of intracellular Ca^{2+} concentrations.[61] Fura-2FF was excited at 340 and 380 nm and emission was recorded at 505 nm. Experiments were performed using a spectrofluorometer (Photon Technology International, New Jersey, USA). Recombinant wild-type Bax was added at different concentrations and Ca^{2+}_m release was estimated by the increase in extramitochondrial fluorescence. In some experiments Bax W107 was used as negative control. Calibrations were performed at the end of each experiment to evaluate the minimum and maximum fluorescence of the system by adding ionomycin (8 μg/ml) followed by $MnCl_2$ (1 mM), respectively. These results were analyzed by Student *t*-test and $P<0.05$ was considered to show significant differences.

Microinjection and Ca^{2+} measurements in intact cells

Astrocytes plated on coverslips were loaded with Fura-2AM (10 μM) for 20 min in a regular microscopy buffer as described above. Before the beginning of the experiments, cells were washed with the same buffer free of Ca^{2+} to avoid interference by external Ca^{2+} during the experiments. Ca^{2+}_c levels in isolated intact cells were measured using high-resolution digital microscopy with an inverted microscope coupled to a cooled CCD camera and controlled by computer software. A semiautomatic programmable micromanipulator InjectMan NI 2 (Eppendorf, Hamburg, Germany), especially suitable for microinjection in adherent cells, was placed in same scope stage used for digital imaging. The InjectMan was coupled to the FemtoJet injector (Eppendorf, Hamburg, Germany), which allowed setting the precise parameters for injection in adherent cells. Since the axial movement was very controlled and injection was very rapid (6000 μm/s), a minimum mortality rate was observed. The parameters for microinjection were: 50 hPa for compensatory pressure, 100 hPa for injection pressure of 0.2 s and the injection volume was about 40–70 fl, through glass microcapilar (Femtotips II) with 0.5 μm internal diameter. The precise angles for microinjection were determined as 30° for cytoplasm, respectively. For each experiment one of the cells in the field was microinjected in bolus with rBax (10 ng/ml). In control experiments cells were microinjected with the buffer used for Bax dilution. Images were collected at 3-s intervals for 5–6 min depending on the experiment. Bax was injected in the beginning of the experiment (approximately at 40 s). After the experiments, single cells were analyzed using the ROI tool, fluorescence intensity extracted and ratio calculated and plotted using the Spectralyzer software (Philadelphia, PA, USA).

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Supplementary Information accompanies the paper on Cell Death Differentiation website (http://www.nature.com/CDD)