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Oligomeric BAX induces mitochondrial permeability transition and complete cytochrome *c* release without oxidative stress

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Summary

In the present study, we investigated the mechanism of cytochrome *c* release from isolated brain mitochondria induced by recombinant oligomeric BAX (BAX_{oligo}). We found that BAX_{oligo} caused a complete release of cytochrome *c* in a concentration- and time-dependent manner. The release was similar to those induced by alamethicin, which causes maximal mitochondrial swelling and eliminates barrier properties of the OMM. BAX_{oligo} also produced large amplitude mitochondrial swelling as judged by light scattering assay and transmission electron microscopy. In addition, BAX_{oligo} resulted in a strong mitochondrial depolarization. ATP or a combination of cyclosporin A and ADP, inhibitors of the mPT, suppressed BAX_{oligo}-induced mitochondrial swelling and depolarization as well as cytochrome *c* release but did not influence BAX_{oligo} insertion into the OMM. Both BAX_{oligo}- and alamethicin-induced cytochrome *c* releases were accompanied by inhibition of ROS generation, which was assessed by measuring mitochondrial H_2O_2 release with an Amplex Red assay. The mPT inhibitors antagonized suppression of ROS generation caused by BAX_{oligo} but not by alamethicin. Thus, BAX_{oligo} resulted in a complete cytochrome *c* release from isolated brain mitochondria in the mPT-dependent manner without involvement of oxidative stress by the mechanism requiring mitochondrial remodeling and permeabilization of the OMM.

Keywords

brain; mitochondria; apoptosis; ROS; calcium; BAX

In apoptosis, a release of mitochondrial apoptogenic proteins occurs due to the interaction of mitochondria with pro-apoptotic members of the Bcl-2 family such as activated (truncated) BID (tBID) and BAX [1,2]. Monomeric BAX resides in the cytosol and remains inactive until tBID causes its oligomerization and incorporation into the OMM [3,4]. This leads to permeabilization of the OMM and escape of mitochondrial apoptogenic proteins from mitochondrial intermembrane space [5]. In the experimental conditions, an oligomerization of BAX can be enforced by a low concentration of mild detergents such as octyl glucoside [1,6,

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7]. This oligomerized BAX (BAX_{oligo}) also permeabilizes the OMM and releases cytochrome *c* [1,7].

In early studies, the mitochondrial permeability transition (mPT) was implicated in protein-induced cytochrome *c* release as an essential mechanism leading to mitochondrial swelling and rupture of the OMM [8-12]. However, in our previous study with isolated brain mitochondria, recombinant tBID alone, or in combination either with monomeric BAX lacking C-terminal segment (BAXΔC) or with a full-length monomeric BAX, caused cytochrome *c* release, which was not sensitive to inhibitors of the mPT [13,14]. This suggested an mPT-independent release of cytochrome *c*. This conclusion is consistent with numerous observations from different laboratories, indicating that protein-induced cytochrome *c* release may occur without involvement of the mPT [15-20]. However, it still remains unknown whether BAX_{oligo} causes a release of cytochrome *c* from brain mitochondria in an mPT-dependent or mPT-independent manner.

The massive cytochrome *c* release induced by pro-apoptotic proteins was proposed to occur in two steps including (*i*) cristae remodeling, which eliminates the diffusion barrier for cytochrome *c* and (*ii*) cytochrome *c* escape from the intermembrane space following either pore formation in the OMM or the rupture of the OMM due to matrix swelling [21]. Alternatively, the release of cytochrome *c* induced by BAX_{oligo} from liver mitochondria was hypothesized to occur also in two steps involving loosening of cytochrome *c* binding to the inner mitochondrial membrane (IMM) due to oxidative stress and lipid peroxidation followed by its dissociation from the membrane and escape through the permeabilized OMM [22]. Later, it was proposed that cytochrome *c* release during apoptotic events might occur in a single step requiring only permeabilization of the OMM [23]. In our study, we addressed a question whether mitochondrial remodeling and oxidative stress play an essential role in the BAX_{oligo}-induced cytochrome *c* release from brain mitochondria.

In the present paper, we demonstrate that in isolated brain mitochondria, recombinant BAX_{oligo} induces massive cytochrome *c* release sensitive to a combination of cyclosporin A (CsA) and ADP, the inhibitors of the mPT [24-26]. Moreover, we found that BAX_{oligo} caused large amplitude mitochondrial swelling and depolarization of organelles, which could be suppressed by mPT inhibitors. In addition, we found that an oxidative stress was not required for a complete cytochrome *c* release produced by BAX_{oligo} or by antibiotic alamethicin, which eliminated barrier properties of the OMM [27]. Thus, our data are consistent with the hypothesis that BAX_{oligo} produces complete cytochrome *c* release from isolated brain mitochondria in the mPT-dependent manner by the mechanism involving mitochondrial remodeling but not oxidative stress.

Materials and Methods

Recombinant BAX

Recombinant BAX was prepared and olgomerized in the dialysis buffer containing 25 mM HEPES-NaOH, pH 7.5, 1% (w/v) octyl glucoside, 0.2 mM dithiothreitol, 30% glycerol (v/v) as described previously [6].

Isolation and purification of brain mitochondria

Mitochondria from the brains or livers of male Sprague-Dawley rats, 200–250 g (Harlan, Indianapolis, IN, USA) were isolated in mannitol-sucrose medium according to an IACUC approved protocol and purified on a discontinuous Percoll gradient as described previously [27]. Mitochondrial protein was measured by the Bradford method [28], using BSA as a standard.

Measurements of mitochondrial respiration

Mitochondrial respiration was measured in the standard incubation medium at 37°C under continuous stirring. The standard incubation medium contained 125 mM KCl, 10 mM HEPES, pH 7.4, 0.5 mM $MgCl_2$, 3 mM KH_2PO_4, 10 μM EGTA, 0.1% bovine serum albumin (free from fatty acids) and was supplemented either with 3 mM succinate plus 3 mM glutamate, or with 3 mM succinate plus 1μM rotenone, or with 3 mM pyruvate plus 1 mM malate. The 0.3 ml incubation chamber was equipped with a Clark-type oxygen electrode and a tightly closed lid. The slope of the O_2 electrode trace corresponded to the respiratory rate. All data traces shown are representative of at least three separate experiments.

Monitoring of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi$) was monitored by following the distribution of TPP^+ between the external medium (initially 1.8 μM TPP^+-Cl) and the mitochondrial matrix with a TPP^+-sensitive electrode [29] in the standard incubation medium supplemented with 3 mM succinate plus 3 mM glutamate unless stated otherwise. A decline in the external TPP^+ concentration in the medium corresponded to mitochondrial polarization, whereas a rise in the TPP^+ concentration in the medium corresponded to depolarization. In all experiments with isolated mitochondria, the concentration of mitochondrial protein in the chamber was 0.2 mg/ml. All data traces shown are representative of at least three separate experiments.

Measurements of mitochondrial light scattering

Mitochondrial swelling was evaluated in the standard incubation medium by monitoring the scattering of light directed on mitochondrial suspension under 90° to the axis of the photodetector at 525 nm in a 0.4-ml cuvette under continuous stirring using a PerkinElmer LS-55 luminescence spectrometer unless stated otherwise.

Measurements of ROS Generation

Production of ROS by isolated brain mitochondria incubated in the standard incubation medium was assessed using the Amplex Red assay for H_2O_2 (Invitrogen), as described previously [14].

Transmission electron microscopy

Electron microscopy of isolated brain mitochondria was performed as described previously [30]. Mitochondria were incubated in the standard 125 mM KCl-based medium supplemented with 3 mM succinate plus 3 mM glutamate at 37°C prior to fixation in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M phosphate buffer in the same incubation medium at room temperature for 15 minutes. Samples for transmission electron microscopy (TEM) were taken using a Tecnai G12 BioTwin electron microscope (FEI, Hillsboro, OR) equipped with an AMT 2.6×2.6K digital CCD camera. To quantitatively assess the morphological changes, we used the morphometric analysis described previously [12]. Total mitochondrial population was categorized into three groups according to their morphology as follows: (i) condensed, (ii) mitochondria with tubular cristae, and (iii) swollen. Mitochondria with characteristics bridging morphologic groups were assigned to the lower category. Mitochondria were counted in a blind fashion, and morphological distribution was statistically analyzed using a one-way analysis of variance followed by Bonferroni's posttest (GraphPad Prism, 4.0).

BAX insertion

To determine alkali-resistant fraction of BAX inserted into the OMM the earlier described method was used (Roucou et al. 2002a). Briefly, mitochondria treated with BAX_{oligo} (10.8 μg/ml) at 37°C for 30 min were pelleted at 15,800 g for 5 min, and supernatant was used for

the cytochrome *c* release measurements. Mitochondrial pellets were re-suspended in 0.2 ml of 0.1 Na_2CO_3, pH 11.5, and incubated for 20 min on ice. Samples were centrifuged for 25 min at 100,000 g in Sorvall Ultra Pro® 80 ultracentrifuge. The pellets were solubilized using 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and analyzed by western blotting against BAX and cytochrome oxidase subunit IV (COX IV).

Immunoblotting

The release of cytochrome *c* from isolated brain mitochondria was assessed using western blotting in supernatants obtained through incubation of mitochondria in the standard 125 mM KCl-based incubation medium for 30 min at 37 °C. For electrophoresis, we used 4-12% Bis-Tris MOPS gels (Invitrogen, Carlsbad, CA). Western blotting was performed as previously described [14]. The release of cytochrome *c* from mitochondria treated with alamethicin (30μg/ml) was used as a control for maximal cytochrome *c* release. For detection of Smac/DIABLO, AIF, Omi/HtrA2, and Endo G the supernatants were concentrated 6 fold by using Microcon YM-10 filtering devices (Millipore Corporation, Bedford, MA). Mitochondrial voltage-dependent anion channel (VDAC) or COX IV were used as a loading control for the pellet samples. VDAC was detected with goat polyclonal anti-VDAC antibody, dilution 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA). COX IV was detected with mouse monoclonal anti-COX IV antibody, dilution 1:5000 (Invitrogen, Carlsbad, CA). Following electrophoresis proteins were transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and blots were incubated with primary mouse anti-cytochrome *c* antibody (7H8.2C12, PharMingen, San Diego, CA) at 1:1000, rabbit anti-Smac/DIABLO antibody (Alexis Biochemicals, San Diego, CA) at 1:500, anti-EndoG antibody (Oncogene, San Diego, CA) at 1:1000, rabbit anti-Omi/HtrA2 antibody (R&D Systems, Minneapolis, MN) at 1:1000 or rabbit anti-AIF antibody (R&D Systems, Minneapolis, MN) at 1:2000 dilution for an hour at room temperature in 5% non-fat milk, phosphate-buffered saline, pH 7.2, and 0.15% Triton X-100. In the BAX_{oligo} insertion experiments, BAX was detected with rabbit anti-BAX antibody (Upstate, Lake Placid, NY) used at 1:2000 dilution. Blots were developed using goat anti-rabbit and anti-mouse IgG (1:20000) coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and Supersignal West chemiluminescent reagents (Pierce, Rockford, IL). Molecular weight marker SeeBlue® Plus 2 Standards (5 μl), (Invitrogen, Carlsbad, CA) were used to determine molecular weights of the bands.

Statistics

Statistical analyses of experimental data consisted of a one-way analysis of variance followed by Bonferroni's posttest (GraphPad Prism, version 4.0; GraphPad Software, San Diego, CA). The data represent the mean ± S.E. of at least three separate, independent experiments.

Results

Since during isolation and purification procedure the outer mitochondrial membrane (OMM) could be damaged, we first analyzed the intactness of the OMM in our mitochondrial preparations. Figure 1 shows measurements of mitochondrial respiration performed with and without exogenous cytochrome *c*. The lack of cytochrome *c* effect in these experiments indicated intactness of the OMM in the majority of mitochondria. This was also supported by the fact that isolated brain mitochondria used in our study retained their structural integrity and released minimal amounts of cytochrome *c* during incubation in the standard incubation medium at 37°C for 30 minutes (Fig. 2). An addition of BAX_{oligo} to mitochondria caused cytochrome *c* release in a concentration- and time-dependent fashion. The release of cytochrome *c* became evident with as low as 1.8μg/ml BAX_{oligo} (Fig. 2a). Higher concentrations of BAX_{oligo} produced larger cytochrome *c* release, culminating at 10.8μg/ml

of BAX_{oligo}. At this concentration, BAX_{oligo} released the entire cytochrome *c* similar to alamethicin, an antibiotic which completely eliminated barrier properties of the OMM [27] and induced maximal cytochrome *c* release (Fig. 2a). Consistent with this, the amount of cytochrome *c* remaining in the corresponding mitochondrial pellets appeared to be below the detection limit of western blotting. Here and in other similar experiments, detection of VDAC in the pellets with anti-VDAC antibody ensured equal sample loading. Cytochrome *c* release induced by 10.8μg/ml of BAX_{oligo} occurred in a time-dependent manner and was completed within 30 minutes (Fig. 2b). Figures 2c and 2f show statistical analyses of cytochrome *c* release induced by BAX_{oligo}.

In parallel with cytochrome *c* release, BAX_{oligo} induced a massive release of Smac/DIABLO while Endo G was released neither after BAX_{oligo} nor after alamethicin treatment (Fig. 3). With anti-Omi/HtrA2 antibody we detected faint bands in the supernatants obtained after incubation of mitochondria with BAX_{oligo} or alamethicin (Fig. 3). With anti-AIF antibody, we detected two bands in the supernatants obtained after incubation of mitochondria with BAX_{oligo} and three bands after incubation with alamethicin (Fig. 3). In the experiments with AIF release measurements we incubated mitochondria without BSA because BSA interferes with AIF detection [30]. While the major, thick band detected with the supernatant sample after alamethicin treatment might belong to AIF, the two faint bands detected with the supernatants obtained after incubation of mitochondria with BAX_{oligo} or alamethicin might represent products of AIF cleavage. To estimate the extent of the protein release, the same amount of brain mitochondria used in the “release” experiments was solubilized and analyzed by western blotting. These estimations revealed that the total amount of AIF and Omi/HtrA2 significantly exceeded the amount of these proteins detected in the supernatants after incubation of mitochondria with BAX_{oligo}. Thus, the release of AIF and Omi/HtrA2 induced by BAX_{oligo} was miniscule in comparison with a complete release of cytochrome *c* and Smac/DIABLO.

Replacement of the standard KCl-based incubation medium for the low ionic strength, mannitol-sucrose (MS) medium completely prevented BAX_{oligo}-induced cytochrome *c* release (Fig. 4). Similar results were obtained with alamethicin (not shown). In mannitol-sucrose medium BAX induced mitochondrial swelling and depolarization in CsA, ADP-sensitive manner (Fig. 4b,c). Thus, the lack of cytochrome *c* release in mannitol-sucrose medium cannot be explained by the absence of the mPT under these conditions. These observations suggest that in brain mitochondria, cytochrome *c* attachment to the IMM appears to be primarily due to an electrostatic interaction between cytochrome *c* and the inner mitochondrial membrane (IMM) in agreement with early reports [31,32].

The release of cytochrome *c* from mitochondria incubated with succinate plus rotenone appeared to be smaller than the release from mitochondria fueled with succinate plus glutamate (Fig. 5a). It is known that rotenone suppresses the mPT by maintaining mitochondrial pyridine nucleotides in the reduced state [24,33-35]. Therefore, the mPT might be involved in BAX_{oligo}-induced cytochrome *c* release from brain mitochondria as it was demonstrated earlier for liver mitochondria [9,36]. Indeed, a combination of CsA and ADP, inhibitors of the mPT [24-26], significantly diminished cytochrome *c* release induced by BAX_{oligo} (Fig. 5a). Here and in all other experiments, ADP was used in the presence of 1μM oligomycin to prevent ADP phosphorylation. The time-dependence of BAX_{oligo}-induced cytochrome *c* release in the presence of CsA and ADP is shown in Fig. 5e. In the parallel experiments, CsA and ADP did not influence BAX_{oligo} insertion into the OMM (Fig. 5g) suggesting that BAX_{oligo} insertion did not require the mPT. Of note, the amount of endogenous BAX in naïve brain mitochondria was below the detection limit of western blot (Fig. 5g). ATP, another inhibitor of the mPT [37], also attenuated cytochrome *c* release induced by BAX_{oligo} (Fig. 5b) but did not influence BAX_{oligo} insertion (not shown). Alamethicin-induced release of cytochrome *c* was insensitive

to mPT inhibitors (Fig. 5a-d). Figures 5b, d, and f show statistical analyses of cytochrome *c* release. Thus, suppression of BAX_{oligo}-induced cytochrome *c* release by inhibitors of the mPT suggested involvement of the mPT in this process.

In addition to cytochrome *c* release, BAX_{oligo} resulted in a large amplitude swelling of brain mitochondria as judged by the decrease in light scattering of mitochondrial suspension measured at 90° to the incident beam (Fig. 6). Alamethicin produced a maximal decrease in light scattering corresponding to the maximal extent of mitochondrial swelling [27]. To compare different light scattering experiments, we assumed the maximal swelling produced by alamethicin as 100% and estimated the extent of swelling produced by BAX_{oligo} under different conditions as a percentage from the maximal swelling (X%) (Fig. 6b). An aliquot of the dialysis buffer used for oligomerization of BAX_{oligo} and containing 1% octyl glucoside failed to produce significant change in light scattering (Fig. 6a). BAX_{oligo} caused the largest decrease in light scattering when mitochondria were fueled with succinate plus glutamate (Fig. 6b), while with succinate plus rotenone the decrease in light scattering was smaller (Fig. 6c). Importantly, a combination of cyclosporin A and ADP suppressed the mitochondrial swelling induced by BAX_{oligo} (Fig. 6d). Similar effect was obtained with 1mM ATP (Fig. 6e). Figure 6f summarizes the results of the light scattering measurements. Thus, BAX_{oligo} induced a large amplitude mitochondrial swelling sensitive to the mPT inhibitors, suggesting mPT involvement.

To further examine mitochondrial morphological changes, we performed transmission electron microscopy (TEM) with isolated brain mitochondria treated with BAX_{oligo} (Fig. 7). All mitochondria were divided in three morphological classes including *condensed* (C), *swollen* (S), and *mitochondria with tubular cristae* (T) shown in Figure 7a, b, and c respectively. The results of morphometric analysis performed in a blind manner are shown in Figure 7g. A vast majority of organelles treated with the vehicle appeared to be in the condensed state with a significant vacuolization of matrices (Fig. 7d) typical for the isolated brain mitochondria [13, 27,38]. Treatment of mitochondria with BAX_{oligo} caused swelling of organelles (Fig. 7e). A few mitochondria had particular matrix structures, which we defined as tubular cristae (Fig. 7c). Pretreatment of mitochondria with mPT inhibitors prevented mitochondrial swelling (Fig. 7f). However, mitochondria did not retain their initial morphology. With mPT inhibitors, the tubular configuration of cristae appeared to be prevalent (Fig. 7f). Thus, BAX_{oligo} caused a dramatic mitochondrial remodeling, which was sensitive to mPT inhibitors and, therefore, might involve the mPT.

The release of cytochrome *c* (Fig. 2d-f) occurred much longer after the onset of the mPT induced by BAX_{oligo} (Fig. 6b). To examine whether cytochrome *c* release correlated with the time course of tubular cristae formation, we performed additional electron microscopy evaluation of mitochondrial morphology over time following BAX_{oligo} addition (Fig. 8). We found that tubular cristae were formed already after 2 minutes of incubation with BAX_{oligo} (Fig. 8c). Then, over time the number of mitochondria with tubular cristae declined and number of swollen mitochondria increased. Thus, BAX_{oligo}-induced cytochrome *c* release did not correlate with the time course of tubular cristae formation and rather paralleled mitochondrial swelling. However, this does not rule out an important role of tubular cristae formation as a step in structural rearrangement of mitochondria leading to complete cytochrome *c* release.

In addition to the release of cytochrome *c* and large amplitude swelling, BAX_{oligo} resulted in mitochondrial depolarization in the concentration-dependent manner (Fig. 9a,d,g). In contrast to depolarization induced by a combination of tBID and monomeric BAX [13,14], depolarizations induced by BAX_{oligo} were abrupt and profound. At the end of the experiments, mitochondria were treated with Ca^{2+} to induce the Ca^{2+}-dependent mPT and completely depolarize organelles. Pretreatment of mitochondria with CsA and ADP (Fig. 6b,e,h) or with

ATP suppressed depolarizations induced by BAX_{oligo} (Fig. 6c,f,i). The mPT inhibitors also protected against Ca^{2+}-induced sustained depolarization, but only in the experiments in which Ca^{2+} was added after low or moderate BAX_{oligo} (3.6 or 7.2μg/ml). With a high BAX_{oligo} (10.8μg/ml), the inhibitors of the mPT failed to preclude sustained depolarization induced by Ca^{2+} (Fig. 6e,f), probably due to massive loss of cytochrome *c* (Fig. 2) and impaired capacity of the respiratory chain. Thus, in addition to the cytochrome *c* release and mitochondrial swelling, brain mitochondria responded to BAX_{oligo} by depolarization, which appeared to be sensitive to mPT inhibitors and, therefore, associated with the induction of the mPT.

The large amplitude swelling of isolated brain mitochondria produced by BAX_{oligo} might lead to the rupture of the OMM, which subsequently would result in a cytochrome *c* escape from the intermembrane space [27,39]. Alternatively, BAX_{oligo} could specifically permeabilize the OMM. In order to evaluate the role of mitochondrial swelling in the OMM permeabilization, we compared mitochondrial swelling and the release of cytochrome *c* induced by BAX_{oligo} or a bolus of Ca^{2+}. Previously, we have shown that in the standard 125 mM KCl-based incubation medium, isolated brain mitochondria undergo large amplitude swelling without substantial release of cytochrome *c* [27]. Similar observation has been made by other investigators with mitochondria isolated from *Xenopus* eggs [16]. It was concluded that under these circumstances the extent of swelling appeared to be insufficient to rupture the OMM and release cytochrome *c* [16,27]. We confirmed these findings in the present study. Indeed, with all tested oxidative substrates, Ca^{2+} produced a significant decrease in light scattering of mitochondrial suspension, indicative of mitochondrial swelling, which was comparable with a decrease in light scattering produced by BAX_{oligo} (compare Figs. 6b,c and 10a-c). This suggested similar swelling of organelles treated with BAX_{oligo} or Ca^{2+}. Indeed, TEM confirmed that a significant fraction of mitochondria treated with Ca^{2+} appeared to be swollen similar to mitochondria treated with BAX_{oligo} (compare Figs. 7e and 10f). However, Ca^{2+}, in contrast to BAX_{oligo}, did not produce a detectable cytochrome *c* release (Fig. 10h) while BAX_{oligo} caused a massive release of cytochrome *c* (Fig. 2). Thus, it seems likely that in addition to mitochondrial swelling and possible rupture of the OMM, which we cannot rule out, BAX_{oligo} causes dramatic permeabilization of the OMM by another as yet unidentified mechanism.

The results presented so far indicate that in isolated brain mitochondria BAX_{oligo} induces cytochrome *c* release that parallels an induction of the mPT. Ca^{2+} is the most prominent inducer of the mPT [21]. Without additional testing, we could not rule out that calcium might contaminate BAX_{oligo} preparations used in our experiments. To address this issue we assessed calcium contamination in our BAX_{oligo} preparation using the Ca^{2+}-selective electrode (Fig. 11a). These experiments revealed that BAX_{oligo} preparations used in our experiments did not contain appreciable amounts of Ca^{2+}. Nevertheless, we examined the cytochrome *c* release induced by BAX_{oligo} in the presence of 1 mM EGTA and did not find any difference with experiments where we used 10μM EGTA (Fig. 11b). Thus, all data obtained with recombinant BAX_{oligo} could be attributed to the action of this protein and to Ca^{2+} contamination.

Earlier, it was proposed that oxidative stress and lipid peroxidation could contribute to BAX_{oligo}-induced cytochrome *c* release from isolated liver mitochondria [22]. In the following experiments, we addressed the question of whether the intensity of oxidative stress, judged as the rate of ROS generation by mitochondria, correlated with the release of cytochrome *c* induced by BAX_{oligo} or alamethicin. In mitochondria, superoxide radical $O_2^{-\cdot}$, a primary reactive oxygen species, is converted by Mn-superoxide dismutase into H_2O_2 which can be easily followed with Amplex Red assay [40]. With succinate as a substrate, mitochondrial generation of ROS is linked to the reverse electron flow from Complex II to Complex I of the respiratory chain and can be effectively inhibited by mild mitochondrial depolarization [41, 42]. In our experiments, BAX_{oligo} decreased the rate of ROS generation in a concentration-dependent manner (Fig. 12), according to its ability to depolarize mitochondria (Fig. 9). FCCP

and alamethicin produced even stronger suppression of ROS generation. CsA and ADP attenuated inhibition of ROS generation by BAX_{oligo} (Fig. 12), but not by FCCP or alamethicin (not shown). A combination of CsA and ADP attenuated the inhibition of ROS generation by BAX_{oligo} presumably due to protection of $\Delta\psi$ and, therefore, preservation of the reverse electron flow in the respiratory chain. In the presence of mPT inhibitors, ROS generation was high (Fig. 12), but the release of cytochrome *c* was significantly diminished (Fig. 5). On the other hand, mPT inhibitors failed to affect the inhibition of ROS generation induced by alamethicin (not shown). Thus, in our experiments with isolated brain mitochondria the intensity of oxidative stress and the release of cytochrome *c* induced by BAX_{oligo} or alamethicin had an inverse correlation. Therefore, it seems unlikely that lipid peroxidation associated with the oxidative stress contributed to the release of cytochrome *c* from isolated brain mitochondria.

Discussion

The release of mitochondrial intermembrane proteins plays a key role in execution of the apoptotic program [43]. The cell-free experimental model of isolated mitochondria in conjunction with the use of recombinant pro-apoptotic proteins proved to be a very helpful tool in the elucidation of these mechanisms. However, most studies have been performed with liver mitochondria [1,9,12,22,36,44] whereas the interaction of pro-apoptotic proteins with isolated brain mitochondria has been less studied [13,14,19,45]. As with any model system, results obtained with isolated mitochondria have some limitations and cannot be applied directly to situation in the cell. However, the experiments with whole cells often suffer from ambiguity based on less than ideal experimental approaches, difficulty to control intracellular environment, and enormous complexity of cell organization at structural and biochemical levels. Therefore, we strongly believe that preparations of isolated purified mitochondria represent an excellent model system, which allows precise control over experimental conditions and, thus, significantly facilitates detailed dissection of molecular mechanisms of mitochondrial involvement into apoptotic program.

The main finding of our study with isolated brain mitochondria is that recombinant BAX_{oligo} induces massive cytochrome *c* release by the mechanism associated with the mPT but without involvement of oxidative stress. Several different lines of experimental evidence support this conclusion. First, BAX_{oligo} produced cytochrome *c* release, which was suppressed by inhibitors of the mPT. Second, BAX_{oligo} produced large amplitude mitochondrial swelling, which was also inhibited by mPT inhibitors. Third, BAX_{oligo} caused abrupt and profound mitochondrial depolarization that was antagonized by mPT inhibitors. Fourth, BAX_{oligo} led to the inhibition of ROS generation and mPT inhibitors attenuated inhibition of ROS generation by BAX_{oligo}. Finally, cytochrome *c* release induced by BAX_{oligo} had an inverse correlation with the rate of ROS generation. Thus, it appeared that in the experiments with brain mitochondria BAX_{oligo} resulted in both the mPT and complete cytochrome *c* release, which was accompanied by dramatic mitochondrial remodeling and did not require oxidative stress.

In early studies with isolated liver mitochondria, BAX_{oligo} was found to be able to cause cytochrome *c* release associated with the mPT [9,11,36,46]. Correspondingly, it was hypothesized that cytochrome *c* release occurred as a result of mitochondrial swelling and the rupture of the OMM. Some investigators proposed that the mPT played a regulatory role in facilitating BAX translocation into the membrane [47] or in promoting a protein-induced efflux of cytochrome *c* into the intermembrane space by remodeling mitochondrial cristae [12]. On the other hand, a large group of investigators failed to demonstrate a link between the protein-induced cytochrome *c* release and the mPT [1,13,14,16,19,44,48]. For instance, in rat liver mitochondria, BAX_{oligo} neither induced the mPT nor preventing the mPT with EGTA attenuated cytochrome *c* release induced by BAX_{oligo} [44]. Later, another group of investigators showed independence of BAX_{oligo}-induced cytochrome *c* release from the mPT

using isolated liver mitochondria from cyclophilin D-knockout mice [49]. Thus, in isolated liver mitochondria, BAX_{oligo} did not induce the mPT, and BAX_{oligo}-induced cytochrome *c* release from liver mitochondria appeared to be mPT-independent.

The reasons for discrepancies in results from different groups are not clear but variations in the properties of mitochondrial preparations, in the experimental protocols and in the activity of recombinant BAX_{oligo} might contribute to this controversy. The elusive nature of CsA inhibition of the mPT [50] as well as our limited knowledge about molecular composition and the mechanisms of induction of the mPT [51,52] obviously also contribute to this problem. To identify mPT induction in our experiments, we used ATP or a combination of CsA and ADP, which were previously found to be the most effective ways to inhibit the mPT [25,27]. With the use of these inhibitors, we clearly demonstrated that BAX_{oligo} induces mPT in isolated brain mitochondria and that mPT is involved in cytochrome *c* release induced by BAX_{oligo}.

The mPT is considered a process of induction/activation of the huge proteinaceous pore that increases the permeability of the IMM up to 1.5kDa [37]. Ca^{2+} is the most prominent inducer of the pore. Although it is known that Ca^{2+} has to enter mitochondria in order to induce the mPT, the precise Ca^{2+} targets and the mechanisms leading to the mPT is still not quite clear [21,52]. Ca^{2+}, however, is not the only inducer of the mPT. There are number of agents and factors that can also lead to induction of the mPT in the absence of Ca^{2+} [10,12,46,53]. In our experiments, BAX_{oligo} produced mitochondrial depolarization and large amplitude swelling of organelles, both typical features of the mPT [54]. These effects of BAX_{oligo} were sensitive to mPT inhibitors. Based on these observations, we concluded that BAX_{oligo} induced mPT in isolated brain mitochondria. Since these experiments were performed in the absence of Ca^{2+}, we concluded that BAX_{oligo}-induced mPT was Ca^{2+}-independent.

An inhibition of the mPT strongly attenuated cytochrome *c* release induced by BAX_{oligo} in our experiments. Thus, induction of the mPT and mitochondrial remodeling appeared to be important for BAX_{oligo}-induced cytochrome *c* release. The most straightforward model posits the mPT-associated mitochondrial swelling as a mechanism of rupture of the OMM, leading to escape of cytochrome *c* from the intermembrane space [39,55,56]. In our experiments, Ca^{2+} also induced swelling of brain mitochondria, but failed to release cytochrome *c*. It is conceivable that under these experimental conditions Ca^{2+}-induced swelling was insufficient to rupture the OMM. Contrariwise, BAX_{oligo} produced mitochondrial swelling similar to Ca^{2+}, but resulted in a complete cytochrome *c* release. This suggested either a larger amplitude of swelling or an additional, more specific mechanism of OMM permeabilization, independent from swelling. Since TEM images of BAX_{oligo}- and Ca^{2+}-treated mitochondria look strikingly similar, the latter explanation seems more likely.

If BAX_{oligo} can permeabilize the OMM independently from swelling, then, the next question is how could an inhibition of the mPT and suppression of swelling diminish the release of cytochrome *c*? One plausible explanation consists in the assumption that BAX_{oligo} induces mPT-dependent remodeling of mitochondria, manifested in unfolding of mitochondrial cristae, providing opening of the closed spaces limited by cristae and, thus, facilitating escape of cytochrome *c*. This could be better understood by keeping in mind that intra-cristae regions may contain up to 85% of the total cytochrome *c*, whereas only about 15% is contained in the intermembrane space [57]. Thus, by wrapping matrix regions, cristae could restrict free diffusion of cytochrome *c*. This hypothesis was proposed earlier for interaction of tBID with isolated liver mitochondria [12]. In this study, tBID caused distinct mitochondrial remodeling, which could be attenuated by CsA and therefore linked to the mPT [12]. Interestingly, tBID applied to mouse liver mitochondria led to a prevalent appearance of mitochondria with tubular cristae similar to those observed in our experiments with BAX_{oligo} and mPT inhibitors. In our experiments, most of the brain mitochondria treated with BAX_{oligo} in the absence of mPT

inhibitors appeared to be swollen and only a few had tubular cristae. It is conceivable that in our experiments an inhibition of the mPT stopped mitochondrial remodeling at the intermediate stage characterized by tubular cristae. Thus, our results argue in favor of the essential role of mitochondrial remodeling in cytochrome *c* release induced by BAX_{oligo}. Therefore, it seems likely that different factors, which promote the mPT (e.g. Ca^{2+} and oxidative stress) and hence favor mitochondrial remodeling, could facilitate BAX_{oligo}-induced cytochrome *c* release while factors, which inhibit the mPT could impede the release of cytochrome *c*.

Previously, it was hypothesized that cytochrome *c* bound to the outer surface of the IMM forms two distinct pools [22]. The “loosely bound” cytochrome *c* appeared to be electrostatically attached to the IMM via interaction with anionic lipids, mainly cardiolipin [58]. In addition, it has been proposed that some cytochrome *c* molecules are anchored to the lipid membrane due to hydrophobic interactions and, thus, form a pool of “tightly bound” cytochrome *c*, which represents only about 10% of the total cytochrome *c* [32]. Peroxidation of cardiolipin might disrupt the interaction between cytochrome *c* and cardiolipin, increasing the fraction of “loosely bound” cytochrome *c* [59,60]. It was proposed that “loosening” of cytochrome *c* might serve as a first step in the process of cytochrome *c* release from isolated liver mitochondria [22]. Hence, oxidative stress could amplify the release of cytochrome *c* by increasing its detachment from the membrane. However, oxidative stress could also promote the mPT [37], which we found to be associated with the cytochrome *c* release induced by BAX_{oligo} in brain mitochondria. The release of cytochrome *c* induced in our experiments either by BAX_{oligo} or by alamethicin was not accompanied by the increased generation of ROS. On the contrary, the generation of ROS, which could potentially cause lipid peroxidation, was drastically diminished. Nevertheless, alamethicin as well as BAX_{oligo} resulted in a complete cytochrome *c* release. Since this release proceeded without activation of ROS generation, oxidative stress appeared to play a dispensable role in the BAX_{oligo}-induced release of cytochrome *c* from brain mitochondria. The experiments with replacement of the standard KCl-based medium for the low ionic strength mannitol-sucrose medium indicated that the attachment of cytochrome *c* to the IMM is governed primarily by weak electrostatic interactions which could be easily interrupted in high ionic strength KCl-based medium [31,32]. Consequently, it seems likely that the massive release of cytochrome *c* induced by BAX_{oligo} proceeds by a mechanism involving permeabilization of the OMM accompanied by mPT-dependent mitochondrial remodeling without necessity for oxidative stress-dependent loosening of cytochrome *c* attachment to the IMM.

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Abbreviations

mPT

mitochondrial permeability transition

OMM
outer mitochondrial membrane

IMM
inner mitochondrial membrane

$\Delta\psi$
mitochondrial membrane potential

TPP^+
tetraphenylphosphonium cation

CsA
cyclosporin A

VDAC
voltage-dependent anion channel

COX IV
cytochrome oxidase subunit IV

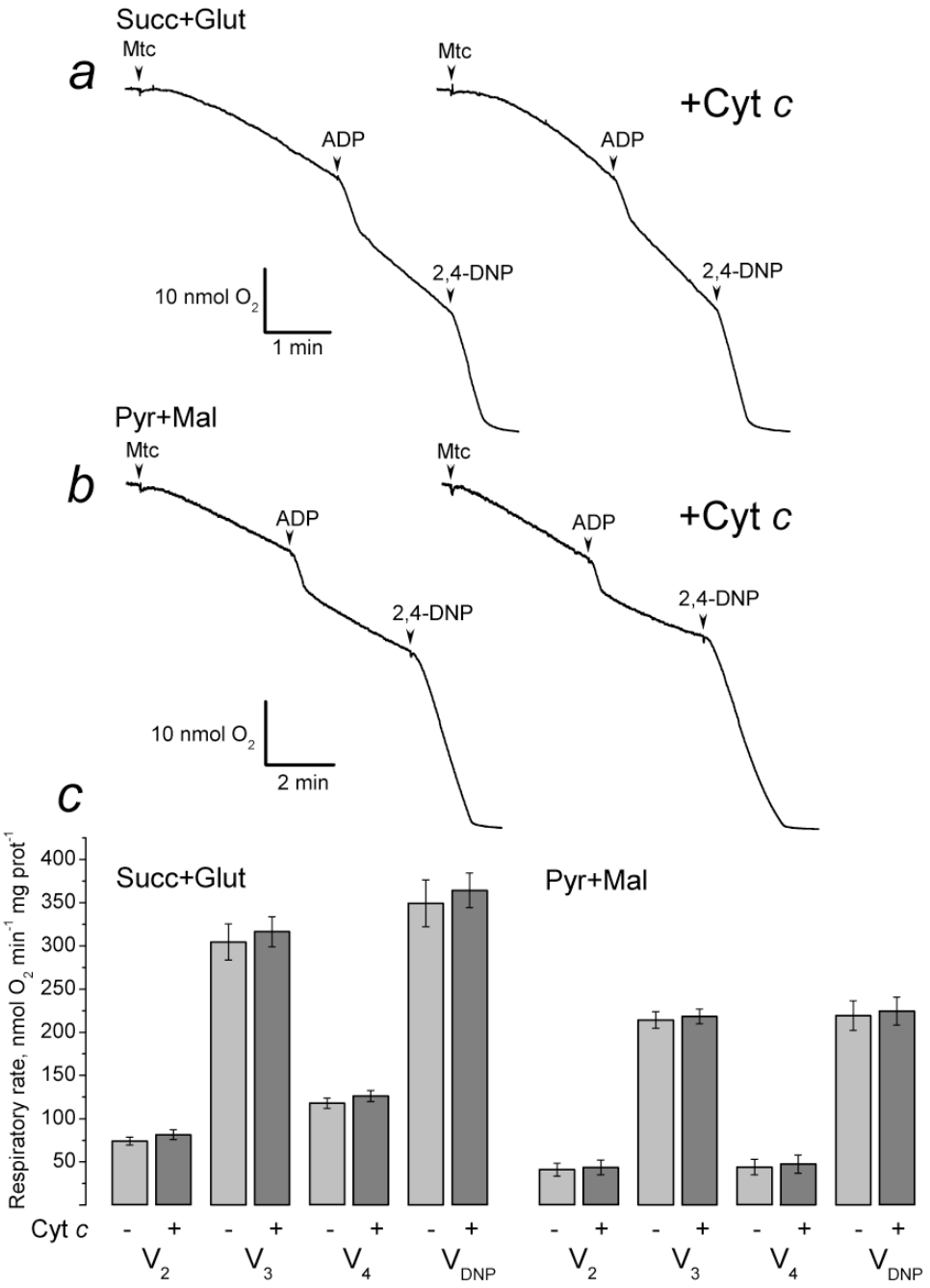

Figure 1. Exogenous cytochrome *c* did not influence respiration of isolated brain mitochondria

Mitochondria were incubated in the standard incubation medium supplemented either with 3 mM succinate plus 3 mM glutamate (***a***) or with 3 mM pyruvate plus 1 mM malate (***b***). Cytochrome *c* (+Cyt *c*, 50μM) was present in the media prior to addition of mitochondria. Where indicated, 200μM ADP or 60μM 2,4-dinitrophenol (2,4-DNP) were added. Panel ***c*** shows statistical analysis of respiratory rates obtained with and without 50μM cytochrome *c*.

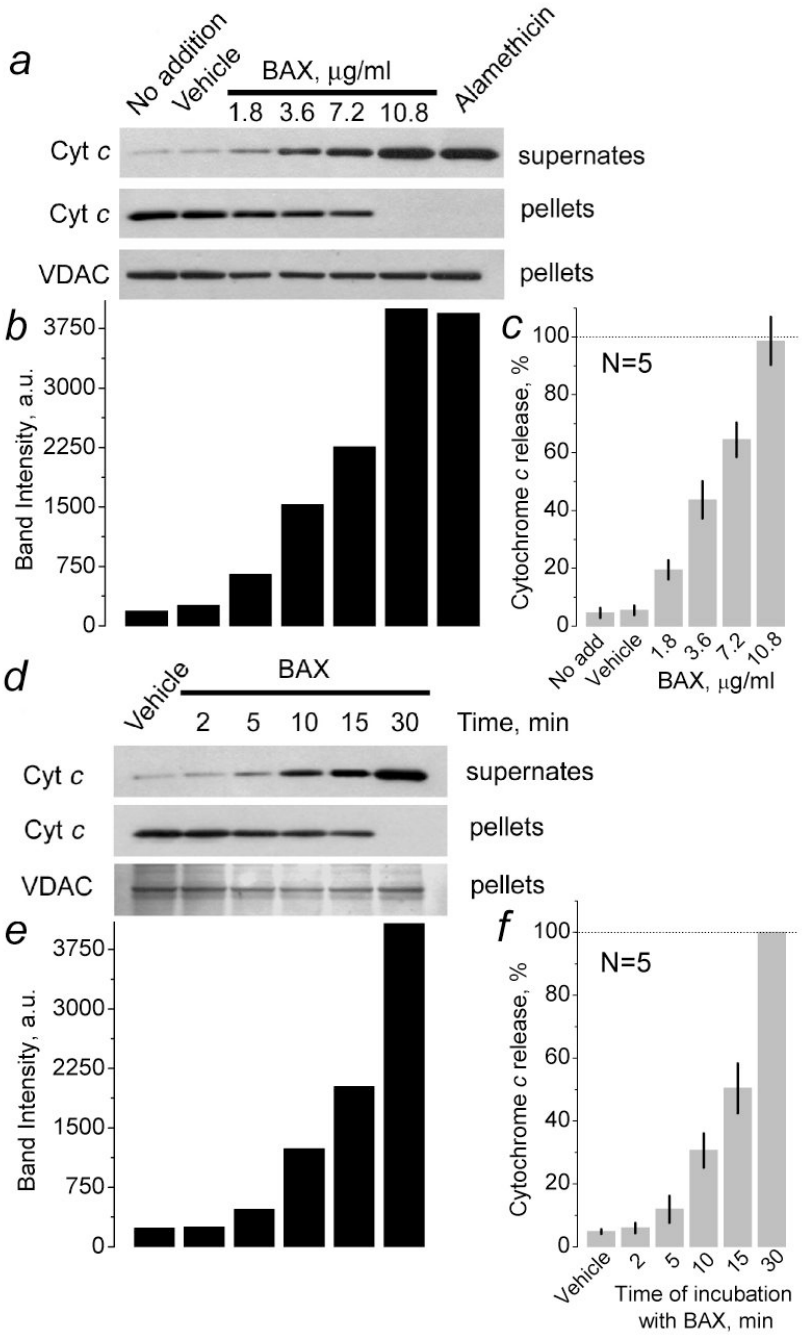

Figure 2. Recombinant oligomeric BAX (BAX_{oligo}) triggered cytochrome *c* release from isolated brain mitochondria in a time- and concentration-dependent manner

In ***a***, a concentration-dependence of BAX_{oligo}-induced cytochrome *c* release is shown. Mitochondria were incubated under very gentle shaking for 30 minutes at 37°C with the indicated concentrations of BAX_{oligo}. Here and in other experiments, western blots obtained with solubilized pellets and anti-VDAC antibody were used as loading controls. Here and in other similar experiments, an aliquot (6μl added into 300μl of the incubation medium) of the dialysis buffer containing 1% octyl glucoside was used as a vehicle. In ***d***, a time-dependence of BAX_{oligo}-induced cytochrome *c* release is shown. 10.8μg/ml BAX_{oligo} was applied to isolated brain mitochondria for the time indicated. In ***b*** and ***e***, columns show the results of

densitometry produced with Quantity One® software (Bio-Rad Laboratories, Hercules, CA) and expressed in arbitrary units (a.u.) for the corresponding bands. In ***c*** and ***f***, the statistical analyses of densitometry results are shown as percentage of cytochrome *c* release. In ***c***, cytochrome *c* release with alamethicin was taken as 100%. In ***f***, cytochrome *c* release after 30 minutes of incubation with BAX_{oligo} was taken as 100%.

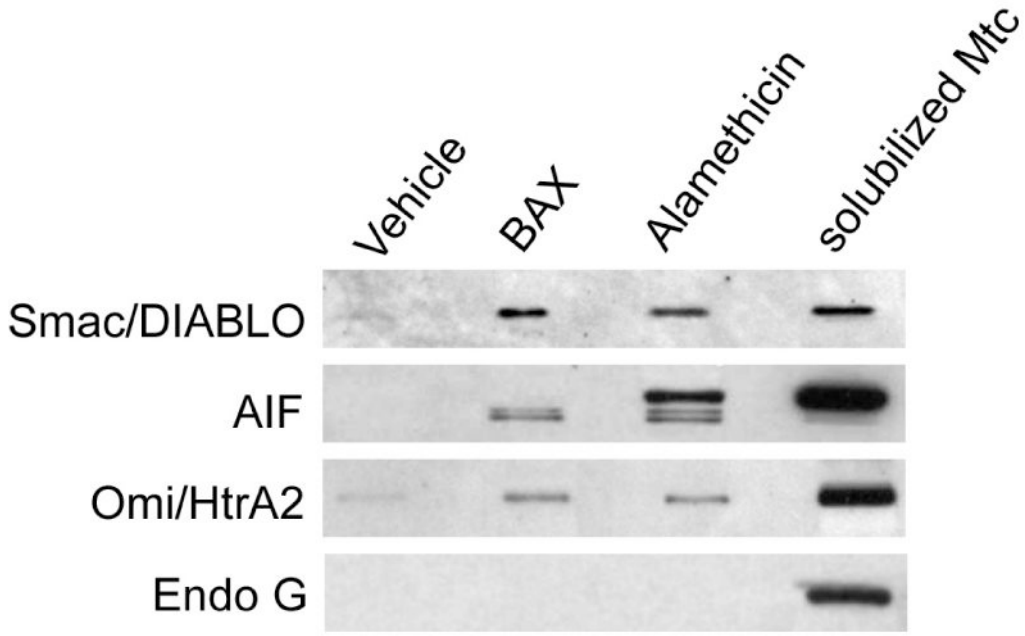

Figure 3. Detection of Smac/DIABLO, AIF, Omi/HtrA2 and Endonuclease G (Endo G) in the supernatants obtained after incubation of brain mitochondria with BAX_{oligo}

Mitochondria were incubated under very gentle shaking for 30 minutes at 37°C with 10.8μg/ml BAX_{oligo} or with 30μg/ml alamethicin. Endo G was not detected in the supernatant even after incubation of mitochondria with alamethicin. The total amount of Smac/DIABLO, AIF, Omi/HtrA2 and Endo G was determined in the same amount of mitochondria (solubilized Mtc) used in the "release" experiments.

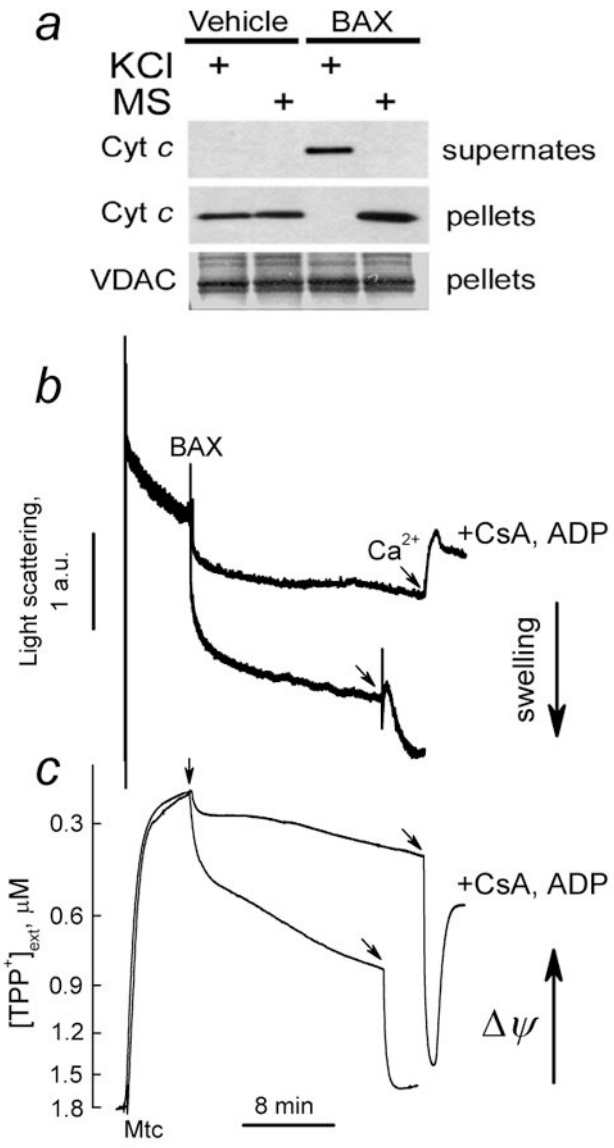

Figure 4. BAX_{oligo} caused complete cytochrome *c* release in high-ionic strength KCl-based medium but not in low-ionic strength mannitol-sucrose incubation medium. BAX_{oligo} produced mitochondrial swelling and depolarization in mannitol-sucrose medium

In ***a***, the results of western blotting. Brain mitochondria were incubated with 10.8μg/ml BAX_{oligo} for 30 minutes at 37°C either in the standard 125 mM KCl-based incubation medium or in the incubation medium where KCl was replaced for 215 mM mannitol plus 70 mM sucrose (MS). In ***b*** and ***c***, in mannitol-sucrose medium BAX_{oligo} (10.8μg/ml) induced mitochondrial swelling and depolarization in CsA, ADP-sensitive manner. In these experiments mitochondrial swelling and membrane potential were monitored simultaneously by following changes in light scattering at 180° (*thick traces*) and distribution of TPP^+ between external medium and mitochondrial matrix (*thin traces*). Two pairs of light scattering traces, indicative of changes in mitochondrial volume, and TPP^+ traces, indicative of changes in mitochondrial membrane potential, obtained with and without 1μM CsA and 100μM ADP (plus 1μM oligomycin) are overlapped for comparison. At the end of the experiments 267μM Ca^{2+} was added to induce Ca^{2+}-dependent permeability transition.

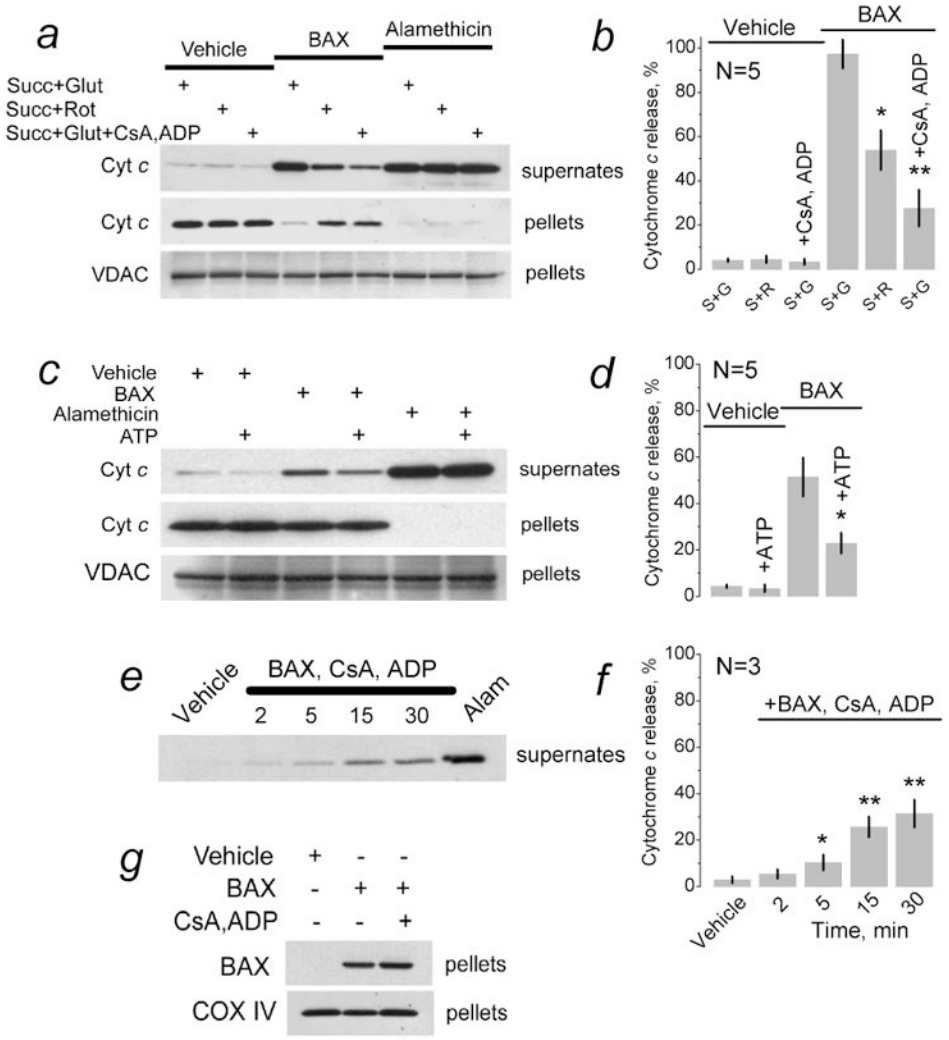

Figure 5. Rotenone, a combination of cyclosporin A and ADP, or ATP inhibited cytochrome *c* release induced by BAX_{oligo} but did not influence BAX insertion into the outer membrane

In ***a***, ***c***, and ***e***, brain mitochondria were treated with vehicle (6μl of dialysis buffer for BAX_{oligo} solubilization added into 300μl of the standard incubation medium), with 10.8 (***a,e***) or 7.2 μg/ml BAX_{oligo} (***c***), or with 30μg/ml alamethicin (Alam) under very gentle shaking for 30 minutes at 37°C. The incubation medium was supplemented either with 3 mM succinate and 3 mM glutamate (S+G) or with 3 mM succinate and 1μM rotenone (Rot, S+R) as indicated. In addition, where indicated the incubation medium was supplemented with 1μM cyclosporin A (CsA) and 100μM ADP (plus 1μM oligomycin) (*panel* ***a,e***) or with 1 mM ATP (*panel* ***c***). In ***e***, numbers show the time of incubation with BAX_{oligo}, CsA, ADP and oligomycin in minutes. Panels ***b***, ***d***, and ***f*** show statistical analyses of cytochrome *c* release in different conditions. In all cases, cytochrome *c* release with alamethicin was taken as 100%. In ***b***, **p*>0.05 between the BAX_{oligo}-induced cytochrome *c* release in the experiments with succinate plus glutamate versus succinate plus rotenone, N=5; ***p*>0.01 between the BAX_{oligo}-induced cytochrome *c* release with and without CsA, ADP, and oligomycin. In ***d***, **p*<0.05 between BAX_{oligo}-induced cytochrome *c* release with and without ATP, N=5. In ***f***, **p*<0.05, ***p*<0.01 between the effect of vehicle and BAX_{oligo}, N=3. In ***g***, mitochondria were incubated under very gentle shaking for 30 minutes at 37°C with 10.8μg/ml BAX_{oligo}. Then, BAX_{oligo} insertion was evaluated as described in Materials and Methods. Where indicated, mitochondria were

incubated with 1μM cyclosporin A (CsA) and 100μM ADP (plus 1μM oligomycin). COX IV immunoblots were used as a control for equal loading.

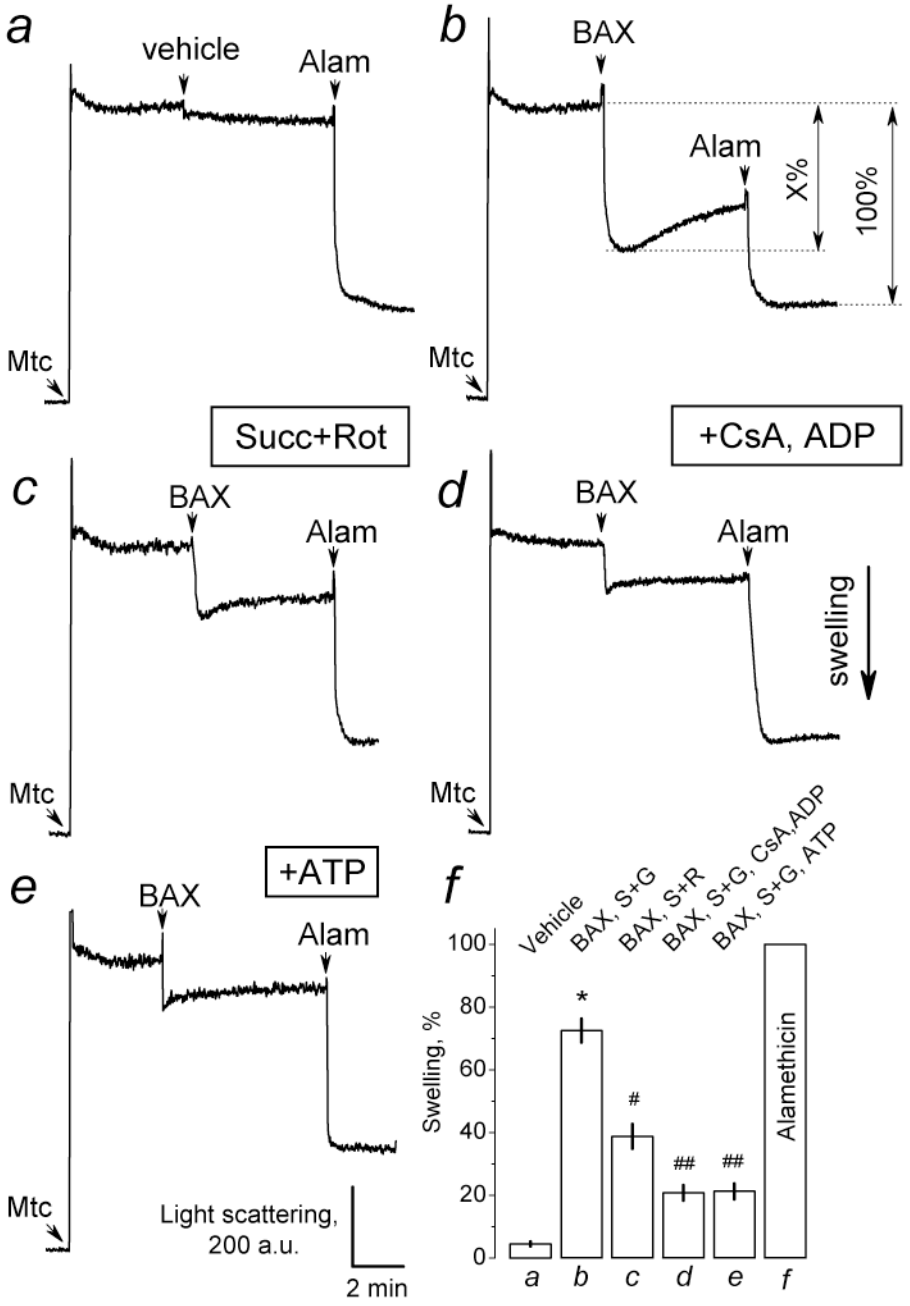

Figure 6. In brain mitochondria BAX_{oligo} induced large amplitude mitochondrial swelling sensitive to the inhibitors of the mPT

In ***a***, ***b***, ***d***, and ***e***, the standard incubation medium was supplemented with 3 mM succinate plus 3 mM glutamate; in ***c*** - with 3 mM succinate plus 1μM rotenone (Rot). In ***a***, 8μl of the dialysis buffer for BAX_{oligo} solubilization (vehicle) were added as indicated into a cuvette with a total volume of 400μl of the incubation medium. In ***b-e***, 10.8μg/ml BAX_{oligo} was added as indicated. In all experiments, 30μg/ml alamethicin was added at the end of recordings to obtain maximal swelling. In ***d***, the incubation medium was supplemented with 1μM cyclosporin A (CsA), 100μM ADP, and 1μM oligomycin. In ***e***, the incubation medium was supplemented with 1 mM ATP. In ***f***, a graph summarizing assessments of mitochondrial swelling is presented. The maximal amplitude of a decrease in light scattering was assumed to reflect the maximal

mitochondrial swelling taken as 100% (see in *Panel **b***). The extent of mitochondrial swelling induced by BAX_{oligo} was calculated as a percentage of the maximal swelling. $^{*}p>0.01$ between the effect of vehicle and BAX_{oligo} in the experiments with succinate plus glutamate; $^{\#}p>0.05$ between the effect of BAX_{oligo} with succinate plus glutamate and with succinate plus rotenone; $^{\#\#}p>0.01$ for the effect of BAX_{oligo} with or without CsA, ADP, and oligomycin, and for the effect of BAX_{oligo} with or without ATP (N=3).

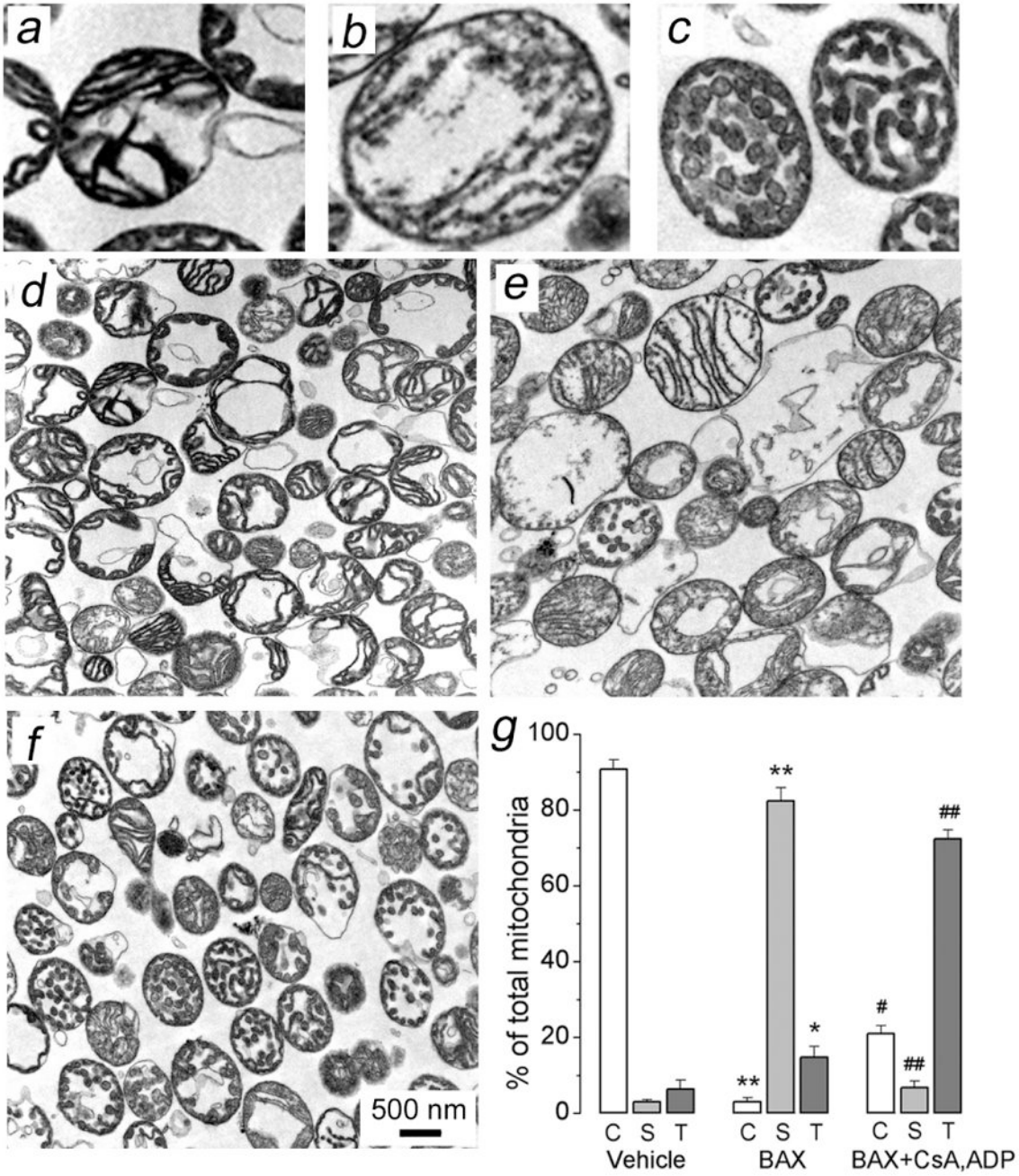

Figure 7. The changes in mitochondrial morphology induced by BAX_{oligo} visualized by transmission electron microscopy with isolated brain mitochondria. The effect of cyclosporin A and ADP

Three distinct types of mitochondrial morphology were identified: condensed (**C**, representative mitochondria are shown in *Panel **a***), swollen (**S**, representative mitochondria are shown in *Panel **b***), and mitochondria with tubular cristae (**T**, representative mitochondria are shown in *Panel **c***). In ***d***, mitochondria were treated with a vehicle (6μl of the dialysis buffer for BAX_{oligo} in 300μl of incubation medium) under very gentle shaking for 30 minutes at 37° C. In ***e***, mitochondria were treated with 10.8μg/ml BAX_{oligo}. In ***f***, mitochondria were pre-treated with 1μM cyclosporin A (CsA) and 100μM ADP plus 1μM oligomycin for 3 minutes

prior to the addition of 10.8μg/ml BAX_{oligo}, to which mitochondria were incubated for the next 30 minutes. In ***g***, the results of morphometric analysis are expressed as a percentage from the total number of analyzed mitochondria. Mitochondria were counted in a blind manner, the total number of mitochondria per condition was 550-600. *p>0.05, **p>0.01 for the corresponding types of mitochondria after treatment with vehicle versus BAX_{oligo}; $^{\#}p$>0.05, $^{\#\#}p$>0.01 for the corresponding types of mitochondria after treatment with BAX_{oligo} in the presence or absence of CsA, ADP, and oligomycin.

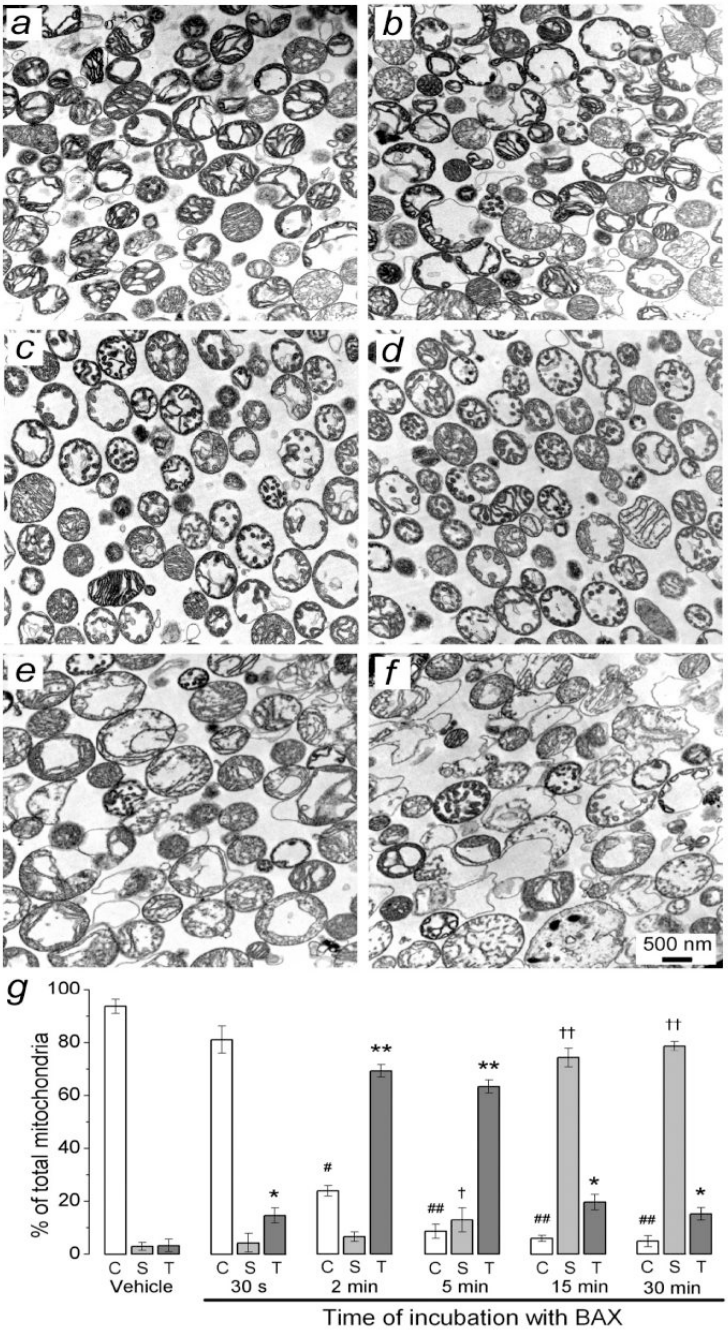

Figure 8. Time-dependence of BAX_{oligo}-induced morphological changes in brain mitochondria

In ***a***, mitochondria were treated with a vehicle (6μl of the dialysis buffer for BAX_{oligo} in 300μl of incubation medium) under very gentle shaking for 30 minutes at 37°C. In ***b-f***, brain mitochondria were fixed after 30 seconds, 2, 5, 15 and 30 minutes of incubation with 10.8μg/ml BAX_{oligo}, respectively. In ***g***, the results of morphometric analysis are expressed as a percentage from the total number of analyzed mitochondria. **C**, condensed mitochondria; **S**, swollen mitochondria; **T**, mitochondria with tubular cristae. Mitochondria were counted similar as described in the legend to Fig. 7. $^{*}p>0.05$ and $^{**}p>0.01$, $^{\#}p>0.05$ and $^{\#\#}p>0.01$, $^{\dagger}p>0.05$ and $^{\dagger\dagger}p>0.01$ for the corresponding types of mitochondria after treatment with vehicle versus BAX_{oligo}.

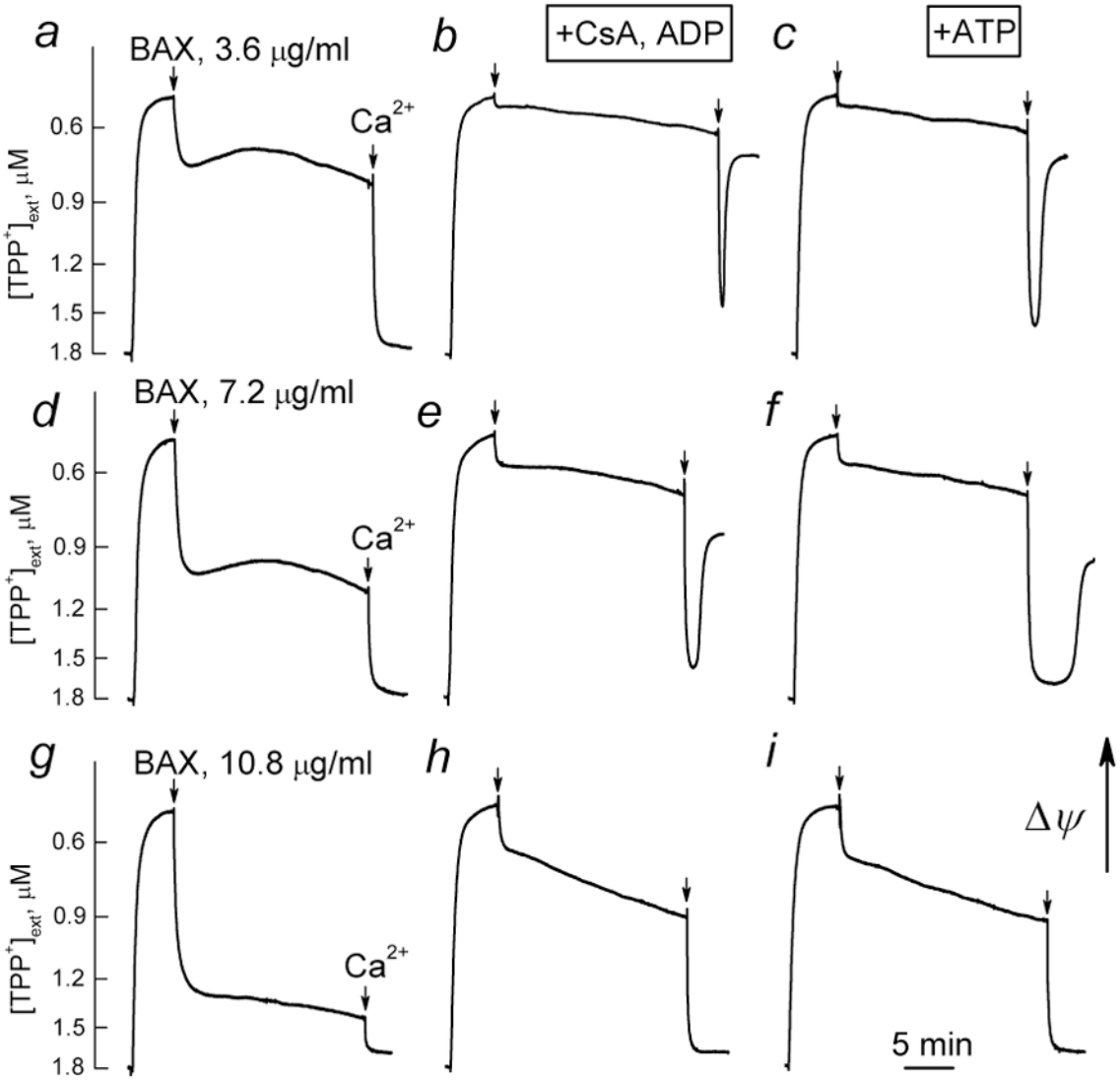

Figure 9. In isolated brain mitochondria BAX_{oligo} caused mitochondrial depolarization sensitive to the inhibitors of the mPT

Different concentrations of BAX_{oligo} were added as indicated. At the end of the experiments, 267μM Ca^{2+} was added to completely depolarize mitochondria. In ***b***, ***e***, and ***h***, the incubation medium was supplemented with 1μM cyclosporin A (CsA), 100μM ADP, and 1μM oligomycin. In ***c***, ***f***, and ***i***, the incubation medium was supplemented with 1 mM ATP. In the control experiments, an aliquot of the dialysis buffer for BAX_{oligo} with 1% octyl glucoside (6μl added into a 300μl of the incubation medium) failed to affect mitochondrial membrane potential (not shown).

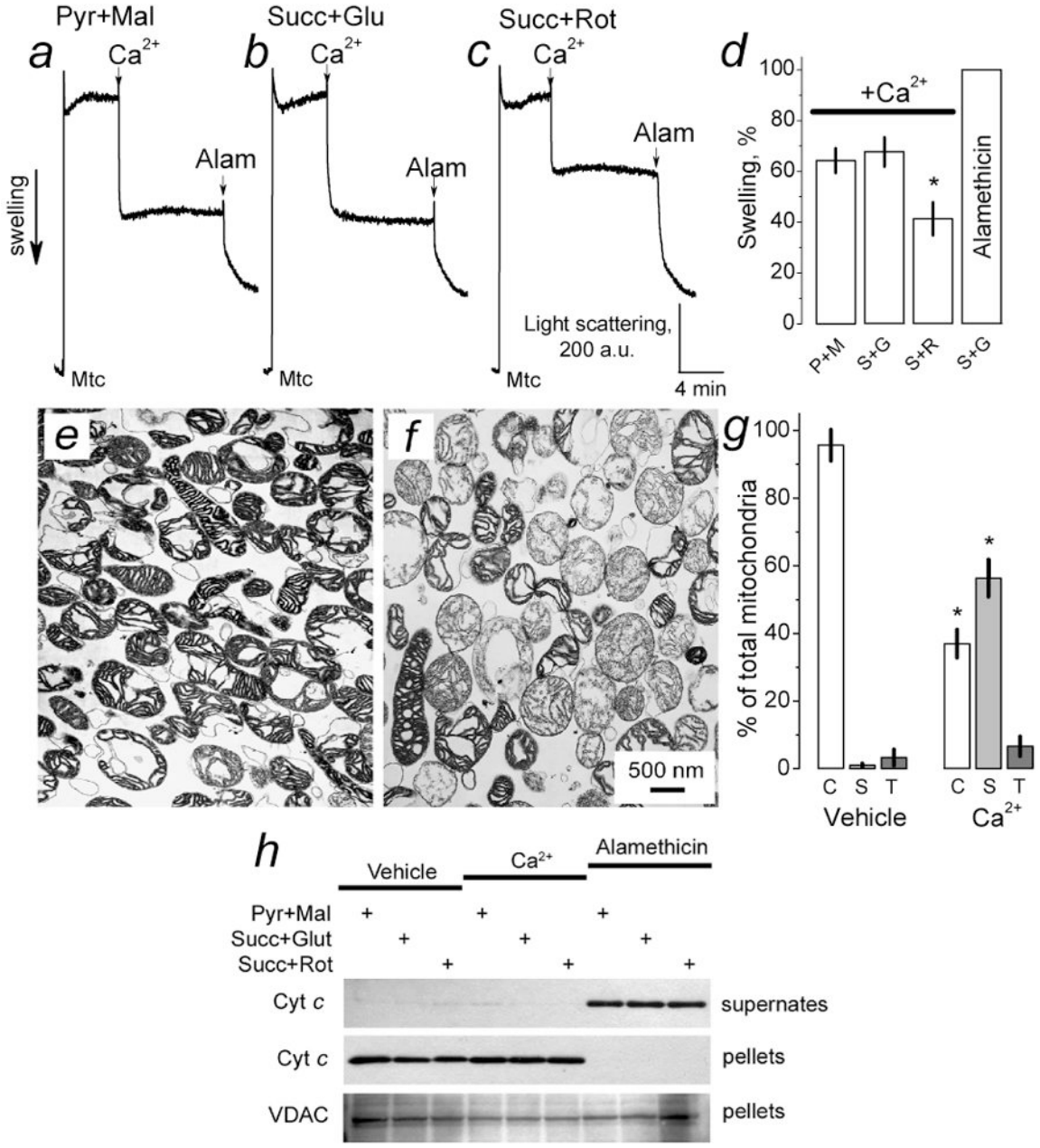

Figure 10. In the standard 125 mM KCl-based incubation medium, Ca^{2+} induced mitochondrial swelling but not the release of cytochrome *c*

In ***a-d***, the results of light scattering assays are shown. Mitochondria were incubated in the media with different oxidative substrates as indicated. 267μM Ca^{2+} and 30μg/ml alamethicin were added as indicated. The extent of mitochondrial swelling was assessed as described in the legend to Figure 3. In ***d***, **p*>0.05 for the effect of Ca^{2+} in the experiments with succinate plus glutamate and with succinate plus rotenone, N=3. In ***e-g***, the results of transmission electron microscopy are shown. In these experiments, mitochondria were incubated in the standard incubation medium supplemented with 3 mM succinate and 3 mM glutamate. In ***e*** and ***f***, mitochondria were incubated under very gentle shaking for 30 minutes at 37°C. In ***f***, mitochondria were treated with 267μM Ca^{2+}. In ***g***, a graph summarizing estimations of mitochondrial swelling similar to those described in the legend to Figure 7. **p*>0.01 for the

corresponding types of mitochondria prior to and after treatment with Ca^{2+}. In ***h***, western blot analysis of cytochrome *c* release from mitochondria treated with 267μM Ca^{2+} or 30μg/ml alamethicin. In ***i***, the statistical analysis of cytochrome *c* release induced by Ca^{2+}. Cytochrome *c* release with alamethicin was taken as 100%.

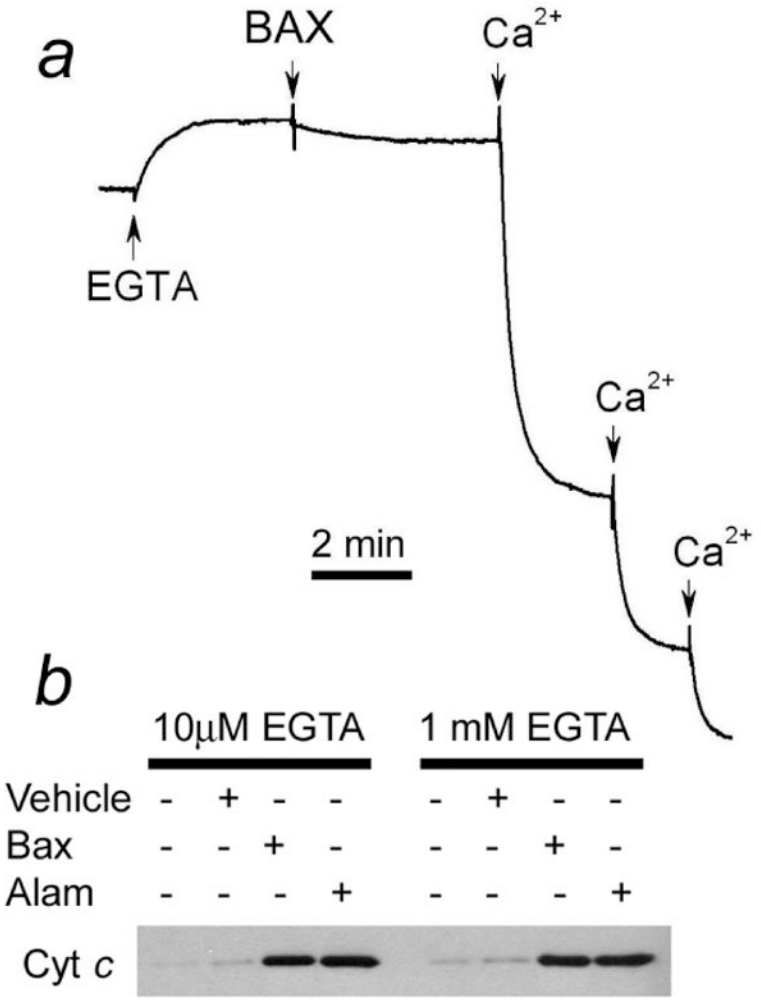

Figure 11. Preparation of BAX_{oligo} did not contain appreciable amounts of Ca^{2+}. BAX_{oligo} induced similar cytochrome *c* release in the eincubation media supplemented with 10μM or 1 mM EGTA

In ***a***, a representative trace obtained with a Ca^{2+}-sensitive electrode. Where indicated, 10μM EGTA, 10.8μg/ml BAX_{oligo} or 25μM Ca^{2+} were added. In ***b***, cytochrome *c* release from brain mitochondria induced by 10.8μg/ml BAX_{oligo} or 30μg/ml alamethicin in the incubation media supplemented either with 10μM or with 1 mM EGTA. Respiratory substrates: 3 mM succinate plus 3 mM glutamate.

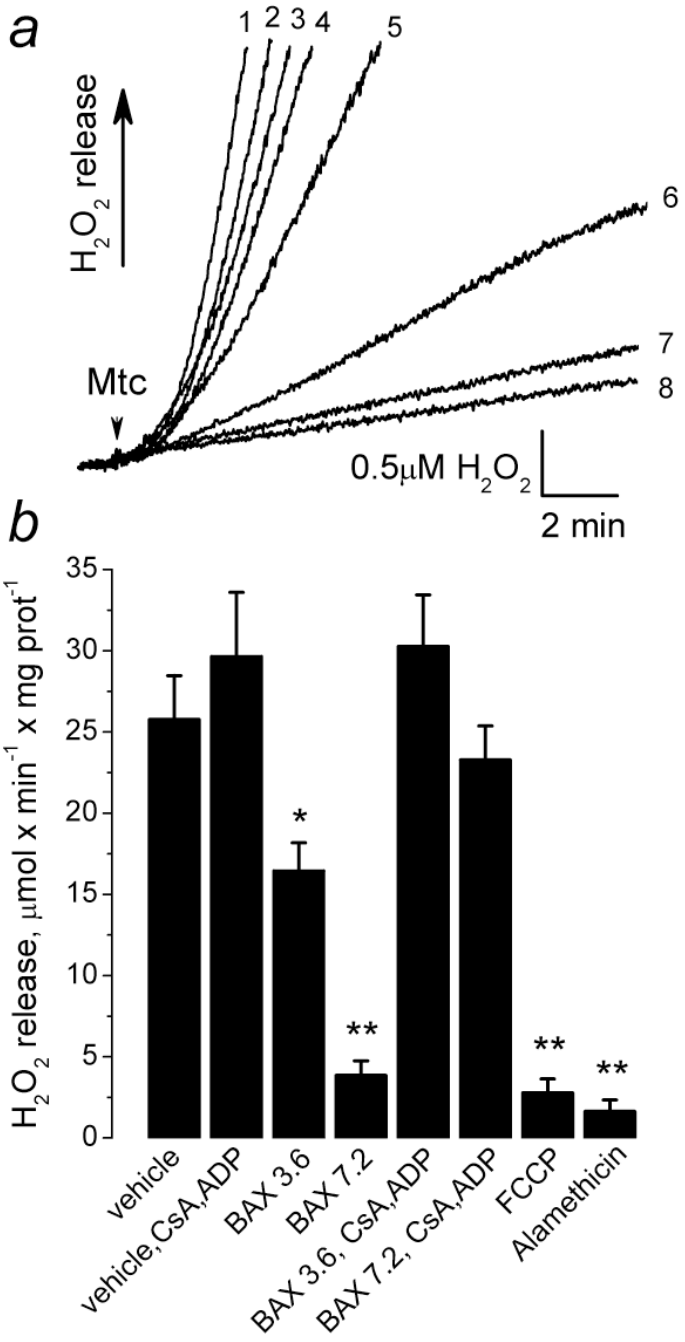

Figure 12. Recombinant BAX_{oligo}, FCCP or alamethicin inhibited ROS generation by isolated brain mitochondria. Cyclosporin A and ADP attenuated the BAX_{oligo}-induced inhibition of ROS generation

In ***a***, the representative traces obtained under different experimental conditions are shown as: 1 – vehicle plus 1μM cyclosporin A (CsA), 100μM ADP, and 1 μM oligomycin (Oligo); 2 – 3.6μg/ml BAX_{oligo}, CsA, ADP, and Oligo; 3 – vehicle; 4 – 7.2μg/ml BAX_{oligo}, CsA, ADP, Oligo; 5 – 3.6μg/ml BAX_{oligo}; 6 – 7.2μg/ml BAX_{oligo}; 7 – 1μM FCCP; 8 – 30μg/ml alamethicin. All additions were made prior to the mitochondria. 5.5μl of the dialysis buffer for BAX_{oligo} solubilization was used as a vehicle. In ***b***, a graph summarizes the data obtained with the Amplex Red assay under different experimental conditions. The concentration of BAX_{oligo} is

given in μg/ml. *p>0.05, **p>0.01 for H_2O_2 generation after treatment with vehicle versus treatment with BAX_{oligo} or alamethicin, N=3.