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Dissimilar mechanisms of cytochrome *c* release induced by octyl glucoside-activated BAX and by BAX activated with truncated BID

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ABSTRACT

In the present study, we compared alkali-resistant BAX insertion into the outer mitochondrial membrane, mitochondrial remodeling, mitochondrial membrane potential changes, and cytochrome *c* (Cyt *c*) release from isolated brain mitochondria triggered by recombinant BAX oligomerized with 1% octyl glucoside (BAX_{oligo}) and by a combination of monomeric BAX (BAX_{mono}) and caspase 8-cleaved C-terminal fragment of recombinant BID (truncated BID, t^cBID). We also examined whether the effects induced by BAX_{oligo} or by BAX_{mono} activated with t^cBID depended on induction of the mitochondrial permeability transition. The results obtained in this study revealed that t^cBID plus BAX_{mono} produced BAX insertion and Cyt *c* release without overt changes in mitochondrial morphology. On the contrary, treatment of mitochondria with BAX_{oligo} resulted in BAX insertion and Cyt *c* release, which were accompanied by gross distortion of mitochondrial morphology. The effects of BAX_{oligo} could be at least partially suppressed by mitochondrial depolarization. The effects of t^cBID plus BAX_{mono} were insensitive to depolarization. BAX_{oligo} produced similar BAX insertion, mitochondrial remodeling, and Cyt *c* release in KCl- and in *N*-methyl-D-glucamine-based incubation media indicating a non-essential role for K^+ influx into mitochondria in these processes. A combination of cyclosporin A and ADP, inhibitors of the mitochondrial permeability transition, attenuated Cyt *c* release, mitochondrial remodeling, and depolarization induced by BAX_{oligo}, but failed to influence the effects produced by t^cBID plus BAX_{mono}. Thus, our results suggest a significant difference in the mechanisms of the outer mitochondrial membrane permeabilization and Cyt *c* release induced by detergent-oligomerized BAX_{oligo} and by BAX activated with t^cBID.

1. Introduction

The release of cytochrome *c* (Cyt *c*) and other apoptogenic proteins located in the intermembrane space between the outer (OMM) and the inner mitochondrial membranes (IMM) is pivotal for execution of apoptosis [1]. Since mitochondrial apoptogenic proteins are confined within the intermembrane space, their release during apoptosis requires permeabilization of the OMM. Permeabilization of the OMM could result from the rupture of the OMM due to mitochondrial swelling in the process called the mitochondrial permeability transition (mPT) [2–5]. The precise molecular mechanisms of the mPT are still unclear [6,7], but it is known that Ca^{2+} influx into mitochondria is a major factor leading to the mPT [8,9]. Alternatively, OMM permeabilization could be due to interaction of pro-apoptotic proteins such as BAX and BID with the OMM [10–12]. In this case, the release of mitochondrial proteins might occur without overt mitochondrial morphological changes via proteinaceous or lipidic pores in the OMM [13,14]. However, there are several reports indicating that the pro-apoptotic proteins BAX and BID could also trigger mPT-like events leading to mitochondrial swelling and the release of Cyt *c* perhaps associated with the rupture of the OMM [15–17].

Both full-length BID and BAX monomers (BAX_{mono}) are normally located in the cytosol and remain inactive until apoptotic stimulus triggers a cascade of apoptotic reactions [18–20]. Following apoptotic stimulus, BID cleaved by caspase-8 (truncated BID, tBID) interacts with BAX_{mono} causing its oligomerization and insertion of the oligomeric BAX in the OMM [21–23]. In addition, BAX_{mono} can be enforced to oligomerize in the presence of mild non-ionic detergents

Abbreviations: BAX_{mono}, monomeric BAX; BAX_{oligo}, monomeric BAX oligomerized in the presence of 1% octyl glucoside; tBID, truncated BID; t^cBID, C-terminal fragment of BID generated by cutting BID with caspase 8 and subsequently separated from the N-terminal fragment and caspase; mPT, mitochondrial permeability transition; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; $\Delta\psi$, mitochondrial membrane potential; COX IV, cytochrome oxidase subunit IV; TPP^+, tetraphenyl phosphonium cation

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producing artificially oligomerized BAX (BAX_{oligo}) [18,24,25]. The artificially oligomerized BAX_{oligo} as well as a combination of recombinant tBID and BAX_{mono} is widely used to study the mechanisms of OMM permeabilization in experiments with isolated mitochondria [26–29]. While it is known that both BAX_{oligo} and a combination of tBID and BAX_{mono} produce significant Cyt *c* release from brain mitochondria [17,28], it remains unknown whether the mechanism of OMM permeabilization is the same in both cases.

In the present study, we examined Cyt *c* release and morphological remodeling triggered by recombinant, artificially oligomerized BAX_{oligo} and by a combination of BAX_{mono} and C-terminal fragment of recombinant BID (t^cBID) in isolated brain mitochondria. The results obtained in this study revealed that BAX_{mono} activated by t^cBID produced alkali-resistant BAX insertion and Cyt *c* release without overt changes in mitochondrial morphology and independently from $\Delta\psi$. On the contrary, treatment of mitochondria with BAX_{oligo} resulted in BAX insertion and Cyt *c* release accompanied by gross distortion of mitochondrial morphology. All these effects of BAX_{oligo} were at least partially suppressed by mitochondrial depolarization. The combination of cyclosporin A and ADP, efficacious inhibitors of the mPT in brain mitochondria [17], attenuated Cyt *c* release, mitochondrial swelling, and depolarization induced by BAX_{oligo}, but failed to influence the effects produced by t^cBID plus BAX_{mono}. Thus, our results demonstrate significant differences in the effects of artificially oligomerized BAX_{oligo}

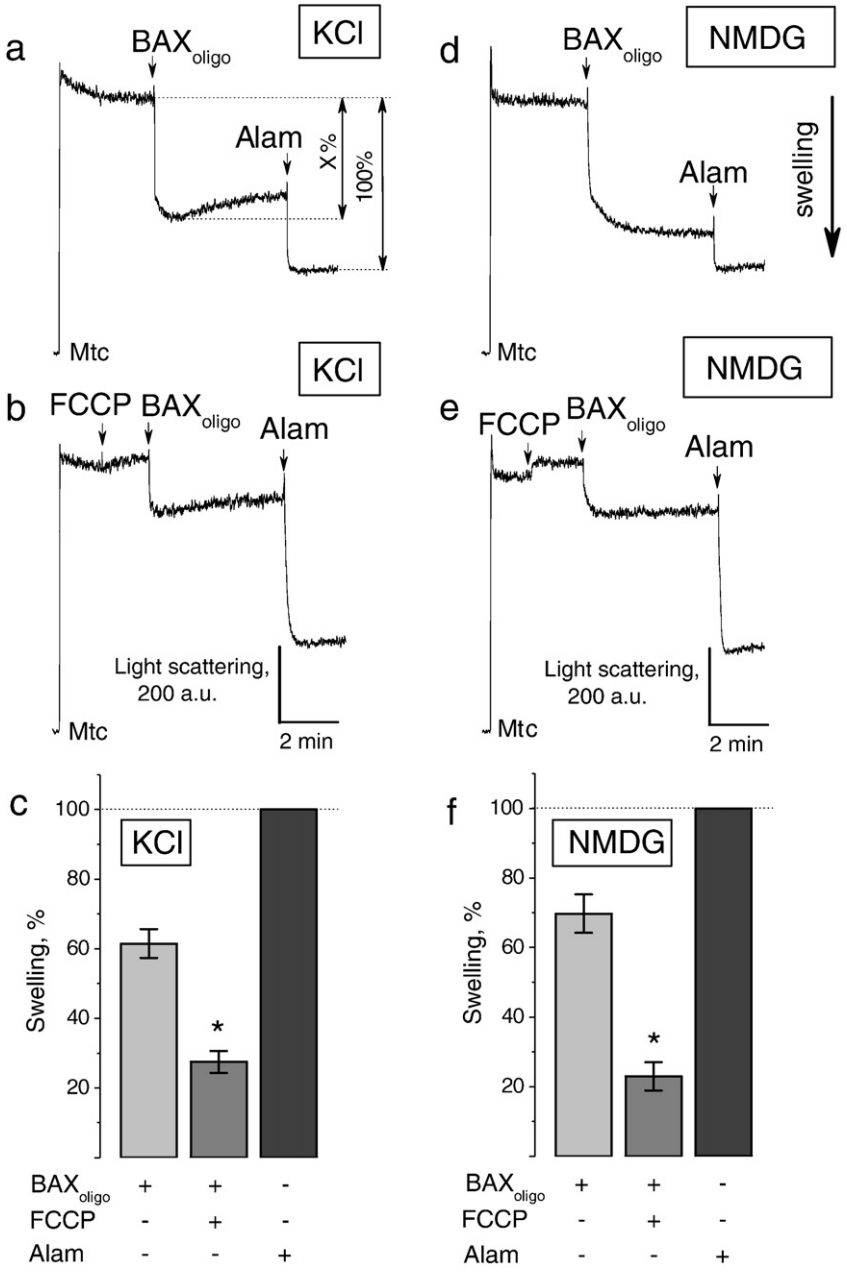

Fig. 1. BAX_{oligo} induced large-amplitude mitochondrial swelling sensitive to mitochondrial depolarization with FCCP in KCl- and in *N*-methyl-D-glucamine (NMDG)-based incubation medium. In a–c, mitochondria were incubated in the KCl-based medium. In d–f, mitochondria were incubated in NMDG-based medium. In a–f, 7.2 μg/ml BAX_{oligo} was added as indicated. In all panels, 30 μg/ml alamethicin was added at the end of the experiments to obtain maximal swelling. In b and e, 1 μM FCCP was added as indicated. In c and f, a summary of the experiments in KCl- and NMDG-medium, respectively. In all experiments, the amplitude of swelling produced by alamethicin was taken as 100% and amplitude of swelling produced by BAX_{oligo} was expressed as a percentage from the maximal alamethicin-induced swelling. $^*p<0.01$ between the effect of BAX_{oligo} alone and BAX_{oligo} in the presence of 1 μM FCCP. Data are mean ± SEM, $N=4$.

and BAX_{mono} activated by $t^{C}BID$ and suggest different mechanisms underlying the OMM permeabilization in these cases.

2. Materials and methods

2.1. Recombinant proteins

Recombinant full-length BAX and active C-terminal fragment of recombinant BID ($t^{C}BID$), generated by cutting BID with caspase 8 and subsequently separated from the N-terminal fragment and caspase, were prepared as described earlier [30,31]. Monomeric full-length BAX was oligomerized in the dialysis buffer containing 25 mM HEPES–NaOH, pH 7.5, 1% (w/v) octyl glucoside, 0.2 mM dithiothreitol, and 30% glycerol (v/v) as described previously [30].

2.2. Isolation and purification of brain mitochondria

Mitochondria from the brains 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 [5]. Mitochondrial protein was measured by the Bradford method [32] using BSA as a standard. In all experiments with isolated mitochondria, the concentration of mitochondrial protein in the chamber was 0.2 mg/ml.

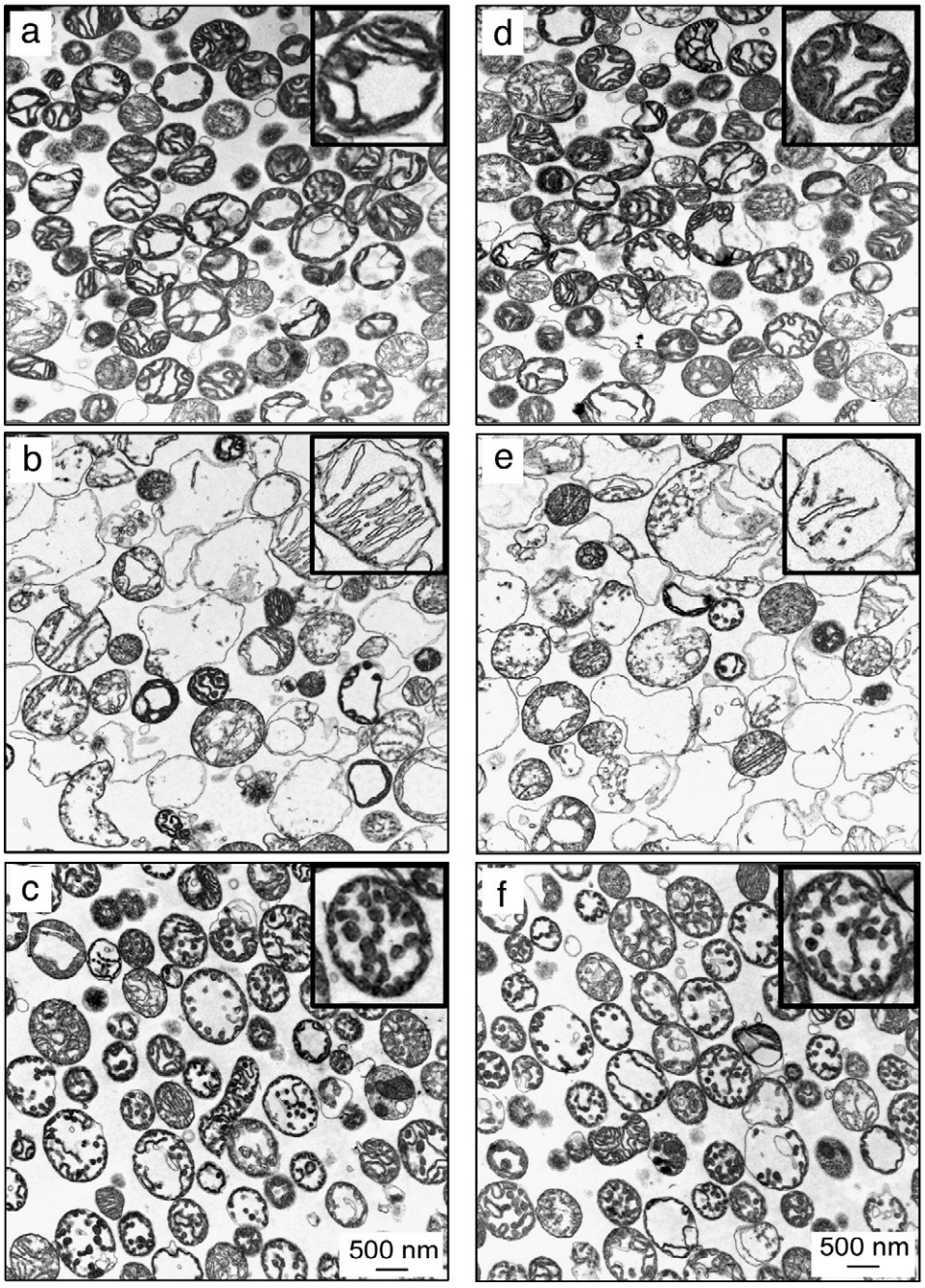

Fig. 2. The electron micrographs of isolated brain mitochondria treated with BAX_{oligo} with and without FCCP. In a–c and d–f, mitochondria were incubated under very gentle shaking for 30 min at 37 °C in KCl- or NMDG-based medium, respectively. In a and d, mitochondria were incubated without additions. In b and e, mitochondria were incubated for 30 min at 37 °C with 7.2 μg/ml BAX_{oligo}. In c and f, mitochondria were pre-treated with 1 μM FCCP for 2 min, and then 7.2 μg/ml BAX_{oligo} was added for the next 30 min. FCCP (1 μM) was in the incubation medium during the experiment.

2.3. Assessment of mitochondrial swelling, $\Delta\psi$, and Ca^{2+} concentration in the incubation medium

Mitochondrial swelling was evaluated by monitoring the light scattering of 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. The incubation medium contained 125 mM KCl or 125 mM *N*-methyl-D-glucamine (NMDG), 10 mM HEPES, pH 7.4, 0.5 mM $MgCl_2$, 3 mM KH_2PO_4, 0.1% bovine serum albumin (free from fatty acids), 3 mM succinate, 3 mM glutamate, and 10 μM EGTA unless stated otherwise. In the case of NMDG-based medium, all precautions were taken to avoid K^+ in the medium. KH_2PO_4 was substituted for H_3PO_4, and pH in all solutions was adjusted with Tris–HCl. Alternatively, mitochondrial swelling was evaluated simultaneously with $\Delta\psi$ by following changes in light scattering of the mitochondrial suspension at 525 nm with an incident beam under 180° in a 0.3 ml chamber at 37 °C and continuous stirring. $\Delta\psi$ was monitored by following the distribution of tetraphenylphosphonium cation (TPP^+) between the external medium (initially 1.8 μM TPP^+–Cl) and the mitochondrial matrix with a TPP^+-sensitive electrode [33] in the standard KCl-based incubation medium. A decline in the external TPP^+ concentration ($[TPP^+]_o$) corresponded to mitochondrial polarization, while a rise in the $[TPP^+]_o$ in the medium corresponded to depolarization. Ca^{2+} concentration in the incubation medium was assessed with a miniature Ca^{2+}-selective electrode in a 0.3 ml chamber at 37 °C and continuous stirring. In all figures, all data traces shown are representative of at least three separate experiments.

2.4. Transmission electron microscopy

Electron microscopy of isolated brain mitochondria was performed as described previously [17,34]. Briefly, mitochondria were incubated in the standard 125 mM KCl- or 125 mM NMDG-based medium with or without recombinant BAX_{oligo} (7.2 μg/ml) or t^cBID (0.33 μg/ml) or a combination of t^cBID (0.33 μg/ml) and monomeric BAX (BAX_{mono}, 1 μg/ml) for 30 min 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 min. Electron micrographs were taken using a Tecnai G12 BioTwin electron microscope (FEI, Hillsboro, OR) equipped with an AMT 2.6×2.6 K digital CCD camera.

2.5. BAX insertion

Alkali-resistant BAX insertion into the OMM was assessed as described earlier [35]. Briefly, mitochondria treated with either BAX_{oligo} or t^cBID or a combination of t^cBID and BAX_{mono} 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 a 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, loading control).

2.6. Immunoblotting

The release of cytochrome *c* from isolated brain mitochondria was assessed as described previously [17] using western blotting in supernates obtained through incubation of mitochondria in the 125 mM KCl- or 125 mM NMDG-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 [28]. The release of cytochrome *c* from mitochondria treated with alamethicin (30 μg/ml) was used as a control for maximal cytochrome *c* release. COX IV was used as a loading control for the pellet samples. COX

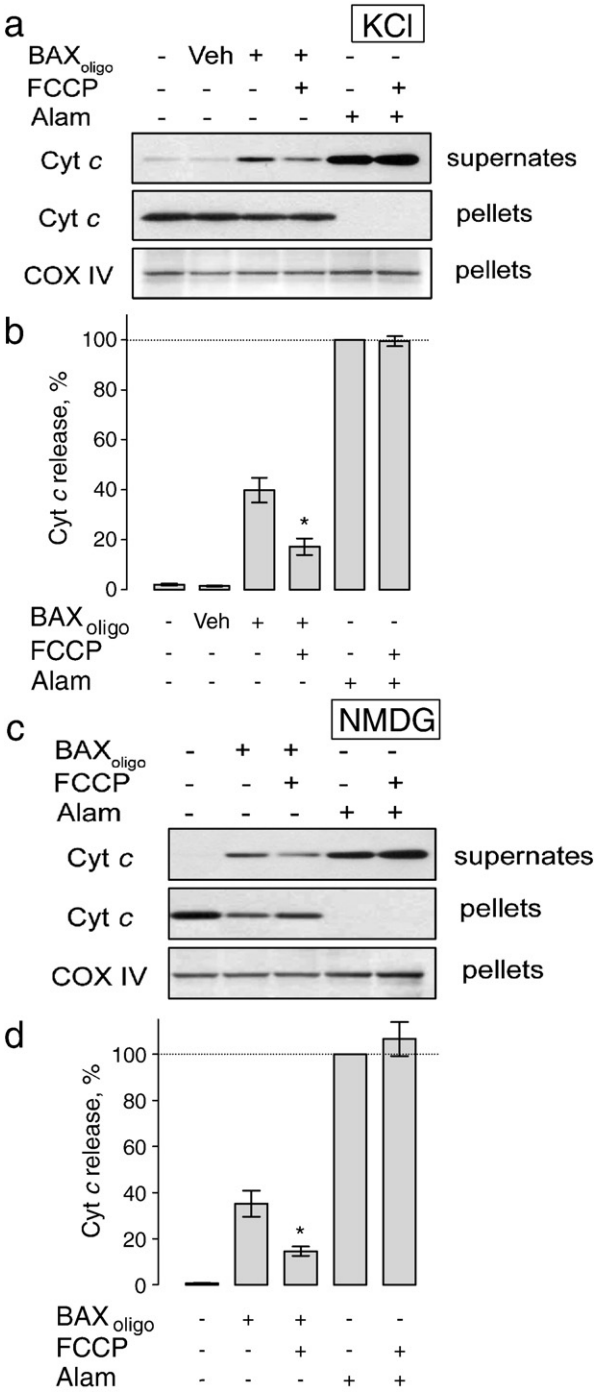

Fig. 3. Mitochondrial depolarization with FCCP inhibited cytochrome *c* release induced by BAX_{oligo} but did not influence cytochrome *c* release induced by alamethicin. In a, mitochondria were incubated in KCl-based medium; in c, mitochondria were incubated in NMDG-based medium. In a and c where indicated, mitochondria were treated with 7.2 μg/ml BAX_{oligo} for 30 min at 37 °C under very gentle shaking. 0.01% octyl glucoside was used as a vehicle. Where indicated, prior to addition of BAX_{oligo} mitochondria were pre-treated with 1 μM FCCP for 2 min and FCCP (1 μM) was kept in the incubation medium during the experiment. To completely release cytochrome *c* (Cyt *c*), 30 μg/ml alamethicin was applied to mitochondria for 30 min at 37 °C. COX IV immunoblots were used as a control for equal loading. Panels b and d show summaries of cytochrome *c* release in different conditions. In all cases, cytochrome *c* release with alamethicin (Alam) was taken as 100%. In b, $^*p<0.001$ between the BAX_{oligo}-induced cytochrome *c* release with and without 1 μM FCCP; in d, $^*p<0.05$ between the BAX_{oligo}-induced cytochrome *c* release with and without 1 μM FCCP. Data are mean ± SEM, $N=5$.

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 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 (1:20,000) 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) was used to determine molecular weights of the bands. Band intensities were evaluated using ImageJ software (http://rsb.info.nih.gov/ij/).

2.7. Statistics

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

3. Results

In our previous paper, we found that BAX_{oligo} caused Cyt *c* release accompanied by strong mitochondrial swelling [17]. We confirmed our previous observations in the present study. Addition of BAX_{oligo} to mitochondria resulted in large-amplitude mitochondrial swelling as judged by light scattering assay (Fig. 1a). In these experiments, the antibiotic alamethicin was used to produce maximal mitochondrial

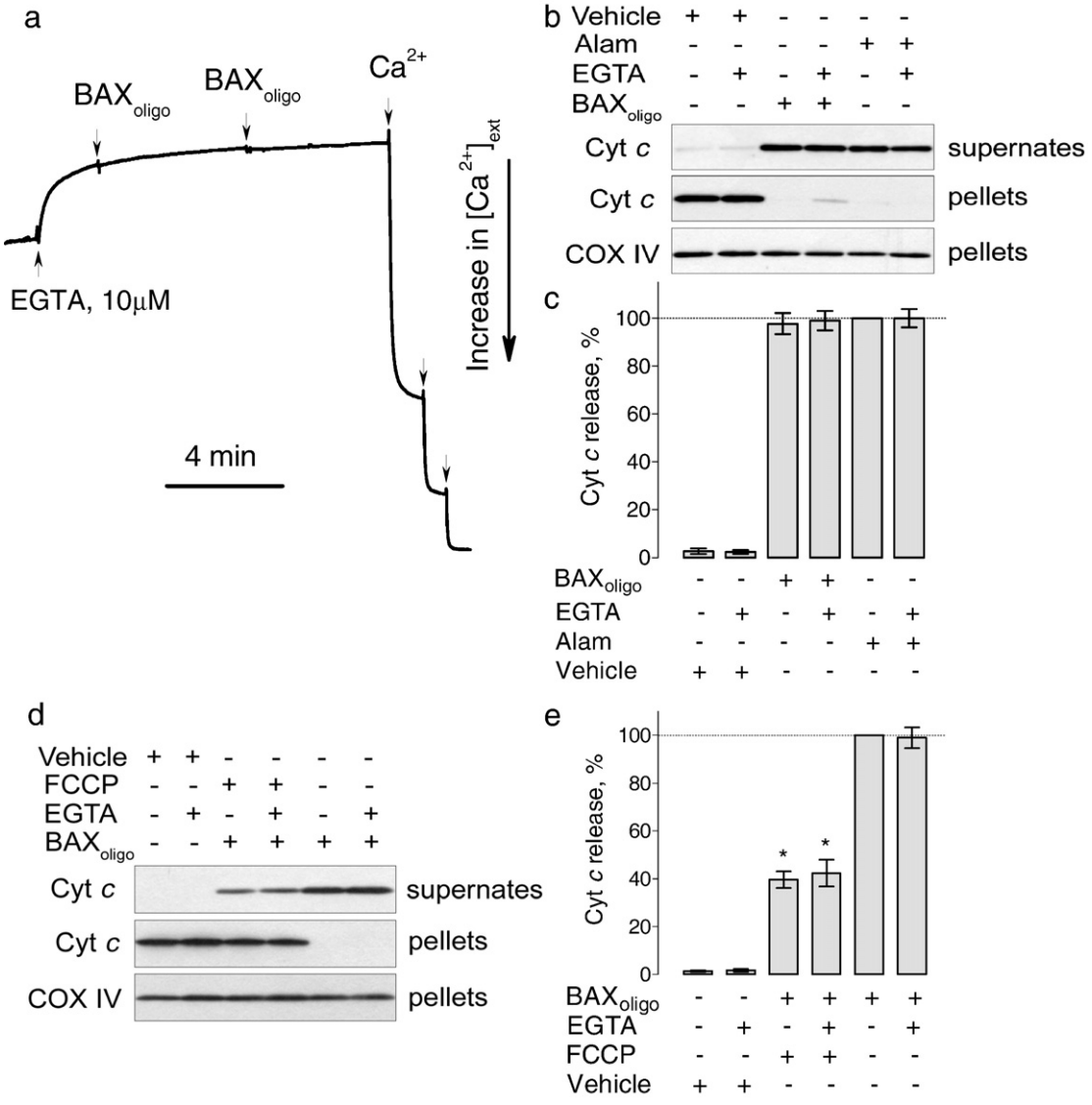

Fig. 4. BAX_{oligo} solution did not contain detectable amounts of Ca^{2+}. BAX_{oligo} induced similar cytochrome *c* release in the incubation media with 10 µM or 1 mM EGTA. FCCP attenuated cytochrome *c* release induced by BAX_{oligo} regardless of EGTA concentration. In a, a representative trace obtained with a Ca^{2+}-sensitive electrode. Where indicated, 10 µM EGTA, 10.8 µg/ml BAX_{oligo} or 25 µM Ca^{2+} was added. In b, cytochrome *c* release from brain mitochondria induced by 10.8 µg/ml BAX_{oligo} or 30 µg/ml alamethicin in the incubation media supplemented either with 10 µM (standard incubation medium, EGTA -) or with 1 mM EGTA(EGTA +). In c, a summary of cytochrome *c* release under different conditions. Cytochrome *c* release with alamethicin (Alam) was taken as 100%. In d, 1 µM FCCP decreased cytochrome *c* release from brain mitochondria induced by 10.8 µg/ml BAX_{oligo} in the incubation media with 10 µM (EGTA -) or with 1 mM EGTA(EGTA +). In e, a summary of cytochrome *c* release under different conditions. Here, cytochrome *c* release with BAX_{oligo} was taken as 100%. $^{*}p<0.01$ between the BAX_{oligo}-induced cytochrome *c* release with and without 1 µM FCCP. In all these experiments, 0.015% octyl glucoside was used as a vehicle.

swelling. Taking the amplitude of the maximal alamethicin-induced swelling as 100%, we quantitatively evaluated mitochondrial swelling induced by BAX_{oligo}. Recently, Eliseev et al. reported that in isolated liver mitochondria tBID stimulated K^+ influx accompanied by osmotically obliged water leading to mitochondrial swelling, the rupture of the OMM, and Cyt *c* release [36]. In our study, we addressed whether BAX_{oligo} could also cause K^+ influx into isolated brain mitochondria leading to mitochondrial swelling and Cyt *c* release. Based on the assumption that mitochondrial membrane potential ($\Delta\psi$) is the driving force for K^+ influx [37], we depolarized mitochondria with FCCP, a potent protonophore, to assess whether BAX_{oligo}-induced mitochondrial swelling occurred due to K^+ influx into mitochondria. In our experiments 1 μM FCCP caused complete mitochondrial depolarization as judged by tetraphenylphosphonium cation (TPP^+) re-distribution in the experiments with a TPP^+-sensitive electrode (not shown). Mitochondrial depolarization with FCCP resulted in a slight increase in light scattering of mitochondrial suspension, suggesting mitochondrial contraction (Fig. 1b). This could be due to an imbalance of K^+ efflux and influx in mitochondria [37]. BAX_{oligo} added after FCCP produced much smaller changes in light scattering of mitochondrial suspension suggesting a decreased amplitude of mitochondrial swelling, or a diminished subpopulation of mitochondria experiencing swelling, or both (Fig. 1b). Overall, this suggested that K^+ influx could be the mechanism of BAX_{oligo}-induced swelling. However, BAX_{oligo} also induced large-amplitude mitochondrial swelling in K^+-free incubation medium where K^+ was substituted for a large organic cation *N*-methyl-D-glucamine (NMDG) (Fig. 1d). This strongly argued against K^+ influx into mitochondria as a mechanism of BAX_{oligo}-induced mitochondrial swelling. Similarly to KCl-based medium, in NMDG-medium, mitochondrial depolarization decreased BAX_{oligo}-induced swelling (Fig. 1e). Fig. 1c and f summarize the results obtained in light scattering assay with BAX_{oligo} and FCCP in KCl- and NMDG-based incubation media, respectively.

While the light scattering assay provides a valuable tool for express-analysis of morphological changes in the entire mitochondrial population, transmission electron microscopy (TEM) helps to visualize morphological changes in individual mitochondria and corroborate the results obtained in light scattering assay. Fig. 2 illustrates mitochondrial remodeling following application of BAX_{oligo} in the absence and in the presence of FCCP in KCl- (Fig. 2a–c) and NMDG-based medium (Fig. 2d–f), respectively. The inserts in the upper right corner of each panel demonstrate a representative type of mitochondrial morphology for the particular experimental conditions under larger magnification. Prior to BAX_{oligo} addition, mitochondria incubated in both KCl- and NMDG-medium were in condensed state (Fig. 2a and d), typical for isolated brain mitochondria [17,27,28]. BAX_{oligo} caused significant mitochondrial remodeling that could be defined as a large-amplitude swelling. In both KCl- and NMDG-medium, the majority of mitochondria appeared to have grossly distorted morphology following 30 min of incubation with BAX_{oligo} (Fig. 2b and e). Depolarization with FCCP inhibited mitochondrial remodeling, presumably stopping mitochondrial morphological changes at an early stage characterized by the appearance of dark circular structures in the matrix which we tentatively define as tubular cristae (Fig. 2c and f). Previously, we found similar inhibition of BAX_{oligo}-induced mitochondrial remodeling evoked by a combination of cyclosporin A and ADP, inhibitors of the mPT [17]. Similar changes in the appearance of mitochondrial cristae in mouse liver mitochondria treated with tBID were reported earlier [38]. In this study, treatment of isolated mouse liver mitochondria with recombinant tBID resulted in the appearance of the circular, electron-dense matrix structures, apparently cristae which, depending on the orientation of the thin section, could be assembled to look like an "intestinal" or "sausage-shaped" electron-dense region.

Thus, TEM confirmed significant morphological changes in individual mitochondria treated with BAX_{oligo}. In addition, TEM revealed a transition to the tubular configuration of mitochondrial cristae following combined application of FCCP and BAX_{oligo}.

In our previous paper, we showed a correlation between the extent of mitochondrial swelling and the BAX_{oligo}-induced Cyt *c* release [17]. Since FCCP inhibited BAX_{oligo}-induced mitochondrial swelling, in the following experiments, we addressed the question whether mitochondrial depolarization diminishes BAX_{oligo}-induced Cyt *c* release. Prior to our experiments we did not know whether mitochondrial depolarization would influence BAX_{oligo}-induced Cyt *c* release, or if it did, in which direction. Therefore, we intentionally used a sub-optimal concentration of BAX_{oligo} (7.2 μg/ml) to provide room for a possible increase in Cyt *c* release following BAX_{oligo} application to depolarized mitochondria. The incubation of naïve mitochondria without additions or with a vehicle (0.01% octyl glucoside) for 30 min at 37 °C produced negligible Cyt *c* release (Fig. 3). Addition of BAX_{oligo} in both KCl- and NMDG-medium caused a significant Cyt *c* release that could be attenuated, but not completely prevented, by mitochondrial depolarization with 1 μM FCCP. Alamethicin produced complete Cyt *c* release that was insensitive to mitochondrial depolarization. Thus, the FCCP-induced decrease in BAX_{oligo}-triggered mitochondrial swelling correlated with the diminished Cyt *c* release in depolarized mitochondria regardless of the composition of the incubation medium.

To test whether BAX_{oligo}-induced Cyt *c* release and mitochondrial swelling could be due to Ca^{2+} contamination in the BAX_{oligo} stock solution, we measured Ca^{2+} in the incubation medium following BAX_{oligo} addition. Fig. 4a shows that adding 10.8 μg/ml BAX_{oligo}, the maximal BAX_{oligo} concentration used in our experiments, failed to change the Ca^{2+} concentration in the medium. On the other hand, addition of Ca^{2+} was easily detected. Thus, it is highly unlikely that the effects of BAX_{oligo} in our experiments were due to Ca^{2+} contamination in

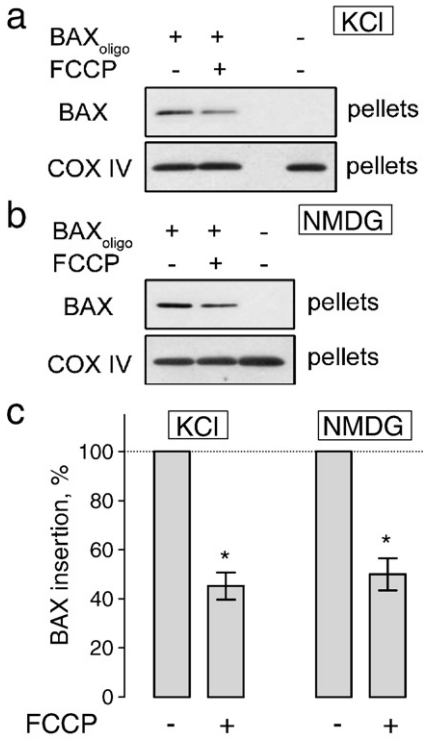

Fig. 5. Mitochondrial depolarization with FCCP inhibited alkali-resistant BAX insertion into the outer membrane. In a, mitochondria were incubated in KCl-based medium; in c, mitochondria were incubated in NMDG-based medium. In a and b, mitochondria were incubated under very gentle shaking for 30 min at 37 °C with 7.2 μg/ml BAX_{oligo}. Then, BAX insertion was evaluated as described in Materials and methods. Where indicated, mitochondria were pre-treated with 1 μM FCCP for 2 min and then incubated with 7.2 μg/ml BAX_{oligo} for 30 min at 37 °C. COX IV immunoblots were used as a control for equal loading. In c, a summary of BAX insertion experiments with and without 1 μM FCCP. In both KCl- and NMDG-medium, BAX insertion without FCCP was taken as 100%. $^*p<0.001$ between the BAX insertion with and without 1 μM FCCP. Data are mean ± SEM, $N=5$.

the BAX_{oligo} stock solution. Nevertheless, in the following experiments we assessed Cyt *c* release induced by 10.8 μg/ml BAX_{oligo} in the incubation medium supplemented with 1 mM EGTA to ensure the lack of residual Ca^{2+}. Similar to alamethicin, BAX_{oligo} at this concentration produced complete Cyt *c* release within 30 min regardless of the presence of 1 mM EGTA (Fig. 4b,c). Likewise, FCCP attenuated Cyt *c* release induced by 10.8 μg/ml BAX_{oligo} regardless of the presence of 1 mM EGTA (Fig. 4d,e). Thus, the effects of BAX_{oligo} were not due to Ca^{2+} contamination in the BAX_{oligo} stock solution, and depolarization could attenuate BAX_{oligo}-induced Cyt *c* release even in the presence of 1 mM EGTA.

The inhibition of mitochondrial remodeling and the decrease in Cyt *c* release from BAX_{oligo}-treated depolarized mitochondria could be due to hindered BAX_{oligo} insertion into the OMM. To test this hypothesis, we evaluated alkali-resistant incorporation of BAX_{oligo} into the OMM in polarized versus depolarized mitochondria. In both KCl- and NMDG-medium, we found a significant decrease in alkali-resistant BAX_{oligo} insertion in the OMM of depolarized mitochondria (Fig. 5). Of note, the amount of endogenous BAX in isolated brain mitochondria was below the detection limit of western blotting. Thus, the decrease in BAX_{oligo}-induced mitochondrial remodeling and Cyt *c* release could be due to the decrease in BAX_{oligo} insertion into the OMM of depolarized mitochondria.

In contrast to BAX_{oligo}, neither t^cBID nor BAX_{mono} nor their combination caused mitochondrial swelling monitored by following light scattering of mitochondrial suspension (Fig. 6a,b). TEM confirmed this conclusion. Neither t^cBID alone nor a combination of t^cBID and BAX_{mono} caused mitochondrial remodeling of individual organelles (Fig. 6c–e). t^cBID alone produced small but statistically significant Cyt *c* release that was not sensitive to mitochondrial depolarization (Fig. 7a,b). On the other hand, mitochondrial depolarization with FCCP caused Cyt *c*

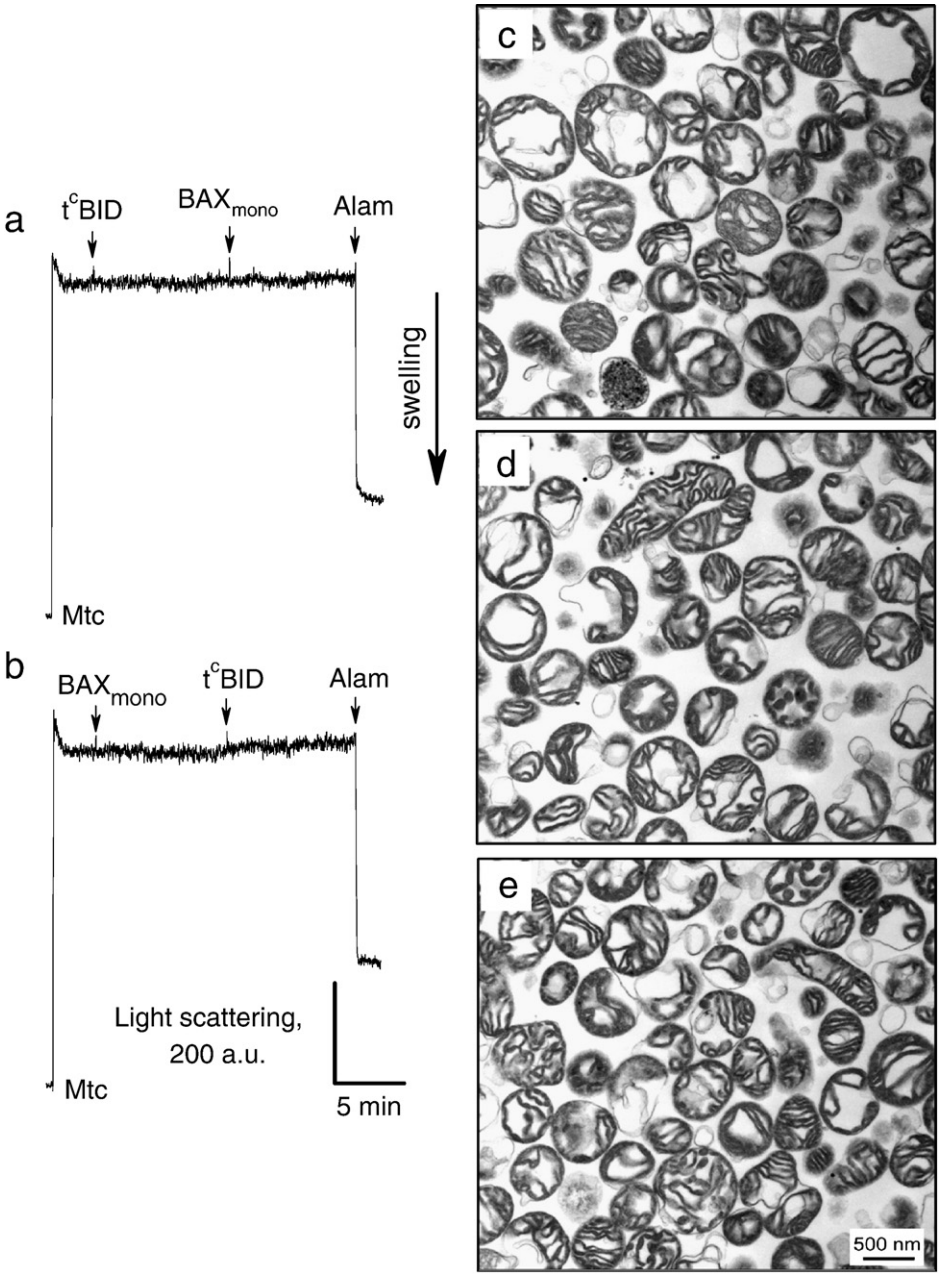

Fig. 6. Neither tcBID nor monomeric BAX nor their combination induced mitochondrial remodeling in isolated brain mitochondria. In a and b, the representative experimental traces obtained in light scattering assay with isolated brain mitochondria. Where indicated 0.33 μg/ml tcBID, 1 μg/ml monomeric BAX (BAX_{mono}) and 30 μg/ml alamethicin were added. In c–e, electron micrographs of isolated brain mitochondria without treatment (c), after 30 min of incubation with 0.33 μg/ml tcBID (d), and after 30 min of incubation with 0.33 μg/ml tcBID plus 1 μg/ml BAX_{mono} (e). In these experiments, mitochondria were incubated in KCl-based medium at 37 °C.

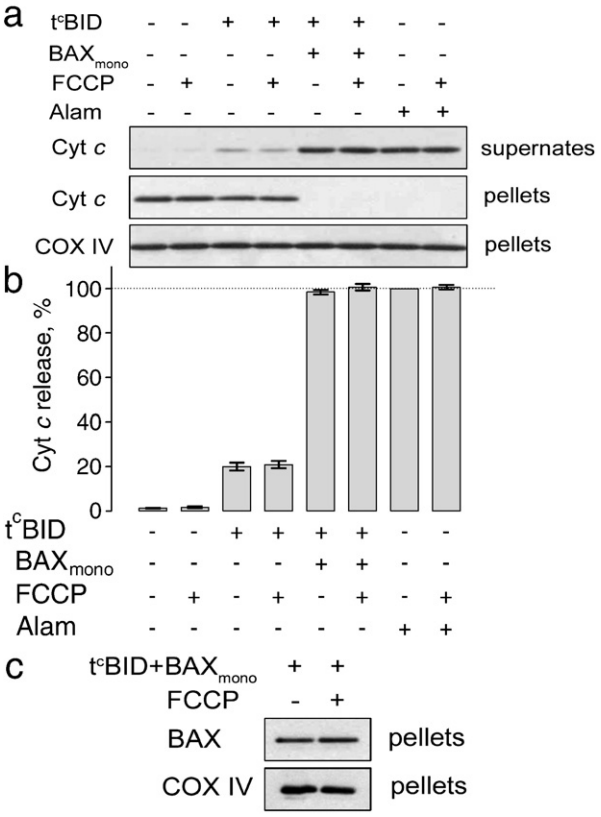

Fig. 7. Neither cytochrome *c* release induced by tcBID and by a combination of tcBID with monomeric BAX nor alkali-resistant BAX insertion appeared to be sensitive to mitochondrial depolarization. In a, cytochrome *c* (Cyt *c*) release induced by 0.33 μg/ml tcBID and a combination of 0.33 μg/ml tcBID and 1 μg/ml monomeric BAX (BAX_{mono}) with and without 1 μM FCCP. In these experiments, mitochondria were incubated in KCl-based medium at 37 °C. To completely release cytochrome *c*, 30 μg/ml alamethicin was applied to mitochondria for 30 min at 37 °C. COX IV immunoblots were used as a control for equal loading. Panel b shows a summary of cytochrome *c* release under different conditions. The cytochrome *c* release with alamethicin (Alam) was taken as 100%. Data are mean ± SEM, $N = 5$. In c, alkali-resistant BAX insertion in the outer mitochondrial membrane with and without mitochondrial depolarization with 1 μM FCCP. Mitochondria were incubated for 30 min at 37 °C with 0.33 μg/ml tcBID and 1 μg/ml BAX_{mono}. Then, BAX insertion was evaluated as described in Materials and methods. Where indicated, mitochondria were pre-treated with 1 μM FCCP for 2 min and then incubated with t^cBID and BAX_{mono} for 30 min at 37 °C. COX IV immunoblots were used as a control for equal loading.

release below detection limit of western blotting. A combination of t^cBID and BAX_{mono} resulted in a complete Cyt *c* release comparable with the alamethicin-induced Cyt *c* release (Fig. 6a,b). As with t^cBID alone, the Cyt *c* release induced by a combination of t^cBID and BAX_{mono} was insensitive to mitochondrial depolarization. The alkali-resistant BAX insertion in the OMM following addition of t^cBID plus BAX_{mono} was depolarization-insensitive as well (Fig. 7c). Thus, in contrast to BAX_{oligo}, t^cBID plus BAX_{mono}-induced alkali-resistant BAX insertion into the OMM and Cyt *c* release appeared to be independent from mitochondrial membrane potential.

Finally, we compared the effects of BAX_{oligo} and a combination of t^cBID and BAX_{mono} in regard to their sensitivity to the inhibitors of the mPT (Fig. 8). In these experiments we measured Cyt *c* release (Fig. 8a) and, in addition, we monitored mitochondrial swelling following light scattering of mitochondrial suspension and mitochondrial membrane potential ($\Delta\psi$) following the distribution of TPP^+ with a TPP^+-sensitive electrode (Fig. 8b–e). Mitochondrial swelling and $\Delta\psi$ were monitored simultaneously. BAX_{oligo} caused abrupt depolarization and pronounced mitochondrial swelling (Fig. 8b) while neither t^cBID nor BAX_{mono} resulted in mitochondrial swelling but produced gradual depolarization (Fig. 8d). In the latter case, we used Ca^{2+} (150 μM) as a positive control to induce the mPT accompanied by mitochondrial swelling and sustained depolarization. The combination of cyclosporin A (CsA) and ADP (in the presence of 1 μM oligomycin to prevent ADP phosphorylation), efficacious inhibitors of the mPT in brain mitochondria [17], attenuated Cyt *c* release, mitochondrial swelling, and depolarization of the organelles induced by BAX_{oligo} (Fig. 8a,c) but failed to influence the effects produced by t^cBID plus BAX_{mono} (Fig. 8a,e). This suggested that the mPT was involved in the effects caused by BAX_{oligo} but not in the effects produced by t^cBID plus BAX_{mono}. Of note, CsA and ADP prevented Ca^{2+}-induced swelling and sustained depolarization (Fig. 8e). Under these conditions, the transient depolarization might be due to Ca^{2+} uptake by mitochondria whereas an increase in light scattering might reflect formation of calcium-phosphate precipitate in the mitochondrial matrix [39]. Thus, taken together, our results argued strongly in favor of different mechanisms of OMM permeabilization by artificially oligomerized BAX_{oligo} and by monomeric BAX activated by t^cBID.

4. Discussion

In the present study, we investigated the mechanisms of the OMM permeabilization by pro-apoptotic proteins BAX_{oligo}, t^cBID, and BAX_{mono}. We clearly demonstrated that (i) BAX_{oligo}-induced Cyt *c* release could be inhibited by mitochondrial depolarization; (ii) BAX_{oligo}-induced mitochondrial swelling and Cyt *c* release did not depend on K^+ influx into mitochondria; and (iii) BAX_{oligo} effects but not the effects of t^cBID plus BAX_{mono} could be inhibited by inhibitors of the mitochondrial permeability transition. These data and our results published previously [17,27,28] strongly suggest that the mechanisms of Cyt *c* release induced by BAX_{oligo} and by a combination of t^cBID plus BAX_{mono} are most likely different.

In our experiments, we found that mitochondrial depolarization with FCCP attenuated mitochondrial remodeling and Cyt *c* release induced by BAX_{oligo}. While the mechanisms of BAX_{oligo}-induced mitochondrial remodeling and its inhibition by FCCP remain obscure, the inhibition of Cyt *c* release most likely was due to a decrease in BAX insertion into the OMM. On the other hand, neither BAX insertion nor Cyt *c* release induced by a combination of BAX_{mono} and t^cBID was affected by mitochondrial depolarization. Moreover, in contrast to BAX_{oligo}, neither t^cBID nor its combination with BAX_{mono} altered mitochondrial morphology. Inhibition of the mPT by CsA and ADP attenuated BAX_{oligo}-induced Cyt *c* release, mitochondrial swelling, and depolarization but failed to influence the effects of t^cBID and BAX_{mono}. Thus, the main finding in our study is that OMM permeabilization induced by artificially oligomerized BAX_{oligo} significantly differs from the permeabilization induced by monomeric BAX_{mono} activated by t^cBID. Our conclusion is consistent with earlier observations that channels formed by artificially oligomerized BAX_{oligo} produced much larger conductance in the planar lipid membranes than BAX_{mono} activated in the presence of caspase 8-cleaved BID [40].

One possible explanation for the difference seen with the detergent-oligomerized BAX_{oligo} compared to the BAX oligomerized with t^cBID might be that the natural BAX channel is composed not only of BAX, but it contains additional proteins [35]. When oligomeric BAX isolated from mitochondria of apoptotic cells was compared to the detergent-oligomerized BAX_{oligo}, a difference in the molecular weights of the oligomers on SDS-PAGE was observed and not all bands correlated with molecular weights of BAX multimers, suggesting that there are additional components associated with BAX in the apoptotic cell [41]. Thus, the effects of detergent-oligomerized recombinant BAX_{oligo} observed in *in vitro* experiments might differ from the effects of BAX activated in its natural intracellular environment. Nevertheless, it is well established and widely accepted that BAX_{mono} artificially oligomerized with a mild, non-ionic detergent octyl glucoside represents a valuable experimental tool that allows investigation of the intimate molecular rearrangements of BAX and its consequences for the barrier properties of

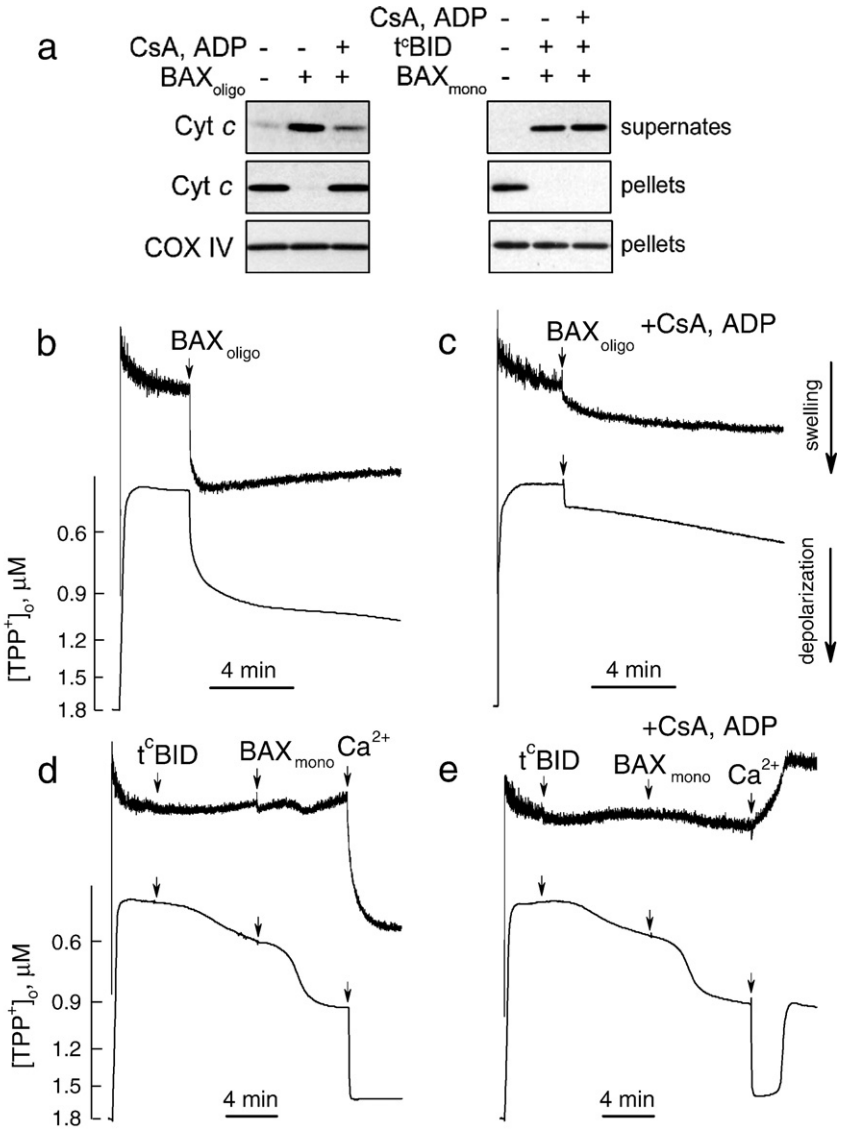

Fig. 8. Cyclosporin A and ADP, inhibitors of the mitochondrial permeability transition, suppressed cytochrome *c* release, mitochondrial swelling, and depolarization of the organelles induced by BAX_{oligo} but not by a combination of t^{c}BID and BAX_{mono}. In a, 1 µM cyclosporin A (CsA), 100 µM ADP (plus 1 µM oligomycin to prevent ADP phosphorylation), 10.8 µg/ml BAX_{oligo}, 1 µg/ml BAX_{mono}, and 1 µg/ml t^{c}BID were added to mitochondria for 30 min at 37 °C as indicated. COX IV immunoblots were used as a control for equal loading. In b–e, the upper traces show changes in light scattering indicative of mitochondrial remodeling, whereas the lower traces show changes in TPP^+ concentration in the incubation medium reflecting changes in $\Delta\psi$. In b, 7.2 µg/ml BAX_{oligo} was added as indicated. In c and e, 1 µM CsA and 100 µM ADP (plus 1 µM oligomycin) were added to the incubation medium prior to mitochondria. 7.2 µg/ml BAX_{oligo} was added as indicated. In d and e, 1 µg/ml t^{c}BID, 1 µg/ml BAX_{mono}, and 150 µM Ca^{2+} were added as indicated.

the OMM under strictly controlled conditions. The detergent-induced re-arrangement of BAX and its subsequent oligomerization simulate the key processes of BAX activation that take place in the cell following apoptotic stimuli. At the same time, our data indicate that t^{c}BID significantly exceeds octyl glucoside in its ability to activate BAX_{mono}. However, t^{c}BID in addition to BAX might also activate BAK, a normal constituent of the OMM [23,42]. Thus, the detergent-induced activation of BAX might be more selective, and therefore more beneficial, in studies of the mechanisms of BAX-induced OMM permeabilization. Yet, the differences in the effects of detergent-oligomerized BAX_{oligo} and BAX_{mono} activated by t^{c}BID demonstrated in the present study have to be kept in mind.

Another interesting observation made in our experiments is that BAX_{oligo} permeabilizes the OMM in a $\Delta\psi$-dependent manner in parallel with mPT induction while a combination of BAX_{mono} plus t^{c}BID permeabilizes the OMM in a $\Delta\psi$-independent manner without mPT induction. The reason for such a difference is not yet clear. However, it is noteworthy that the concentration of BAX_{oligo} required for complete Cyt *c* release was 10 times higher [17] than the concentration of t^{c}BID-activated BAX_{mono}. A similar difference in the mechanisms of OMM permeabilization was reported recently for low and high concentrations of tBID [43]. At low concentrations, tBID permeabilized the OMM without mPT induction, whereas at high concentrations tBID permeabilized the OMM in parallel with induction of the mPT. The authors proposed that at high concentrations tBID causes cardiolipin transfer from the IMM to the OMM. This could cause depletion of cardiolipin in the IMM, destabilizing the adenine nucleotide translocase (ANT) that might lead to conversion of the ANT into the mPT pore [43]. Indeed, interaction of tBID with cardiolipin was shown to destabilize the ANT [44]. BAX_{oligo} also stimulates transbilayer transfer of lipids including cardiolipin [45]. Therefore, it is possible that a mechanism similar to those proposed to explain the effect of high concentration of tBID [43] could be accountable in our experiments for BAX_{oligo}-induced mPT in brain mitochondria. However, whether such concentrations of activated tBID or oligomerized BAX could be generated in the apoptotic cell and whether such a scenario could take place *in situ* is not yet clear. Regardless of the mechanism of OMM permeabilization, our findings strongly suggest the difference between artificially

oligomerized BAX_{oligo} and BAX_{mono} activated by t^cBID in regard to their ability to induce the mPT and permeabilize the OMM.

The inhibition of BAX insertion and Cyt *c* release induced by mitochondrial depolarization is another key observation made in the present study. The inhibition of BAX_{oligo}-induced Cyt *c* release from depolarized mitochondria has been reported previously. Recently, the release of Cyt *c* and Smac/DIABLO induced by BAX_{oligo} in non-energized (depolarized) mitochondria from BAX/BAK double knock-out mouse embryonic fibroblasts was found to be significantly diminished in comparison with the protein release from energized (polarized) mitochondria [29]. Interestingly, in contrast to Cyt *c*, Smac/DIABLO is not electrostatically attached to cardiolipin in the IMM [46] and does not require detachment from the OMM due to cardiolipin oxidation. Nevertheless, the release of Smac/DIABLO was also significantly decreased in de-energized (depolarized) mitochondria [29] suggesting an essential role of $\Delta\psi$ in the OMM permeabilization by BAX_{oligo}. In another study, investigators showed that the ability of yeast mitochondria to respire is a key determinant of BAX toxicity [47]. Mutations that yield respiration incompetent yeast strains (and, obviously, with depolarized mitochondria) resulted in resistance to BAX toxicity. Pucci et al. [48] presented data indicating that mitochondrial depolarization inhibited BAX translocation to mitochondria in HeLa cells treated with TNF [48]. Correspondingly, Cyt *c* release was strongly attenuated in cells with depolarized mitochondria. In another study with mitochondria derived from HeLa cells, treatment with phospholipase A_2 (PLA_2), which led to phosphatidylethanolamine and cardiolipin hydrolysis, hindered BAX insertion in the OMM [49]. The authors proposed that the changes in membrane properties due to hydrolysis of phospholipids might control the process of BAX insertion in the OMM. However, treatments with PLA_2 also resulted in accumulation of free fatty acids [49], which are natural protonophores that depolarize mitochondria [50]. Thus, it seems conceivable that mitochondrial depolarization due to FFA accumulation contributed to PLA_2-induced inhibition of BAX insertion in mitochondria from HeLa cells. The mechanisms of depolarization-induced inhibition of BAX_{oligo} insertion into the OMM and BAX_{oligo}-induced Cyt *c* release remain unclear. However, it is obvious that in contrast to BAX_{oligo}, BAX insertion and Cyt *c* release induced by a BAX_{mono} activated by t^cBID do not depend on $\Delta\psi$. t^cBID-induced release of Cyt *c* was also $\Delta\psi$-independent in our experiments. This is consistent with the earlier findings that mitochondrial depolarization with CCCP did not inhibit tBID-induced Cyt *c* release from isolated heart mitochondria [51]. Thus, dependence on $\Delta\psi$ is another distinct feature that differentiates the effects of BAX_{oligo} from the effects of BAX_{mono} activated by t^cBID. This conclusion once again emphasizes the likelihood that different mechanisms underlie the effects of BAX_{oligo} and the effects of t^cBID or its combination with BAX_{mono} on the barrier properties of the OMM.

Acknowledgements

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