The Dynamics of Bax Channel Formation: Influence of Ionic Strength

Vidyaramanan Ganesan, Timothy Walsh, Kai-Ti Chang, and Marco Colombini

University of Maryland, College Park, Maryland

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Preparation of cytochrome c

Horse heart cytochrome *c* (44 mg) was reduced with 11 mg ascorbate in 1 mL of 0.2 M Tris-HCl pH 7.5. The reduced cytochrome *c* was separated from the ascorbate by gel filtration through a Sephadex G-10 column equilibrated with 0.2M Tris-HCl pH 7.5.

Isolation of rat liver mitochondria

Fresh mitochondria were purified from rat liver as described (1). The BSA was removed by sedimenting mitochondria in BSA free isolation buffer and the final pellet resuspended in 300 mM mannitol, 5 mM HEPES and 0.1 mM EGTA pH 7.4 (FH-buffer) to a final concentration of 10-20 mg mitochondrial protein/mL. The mitochondrial stock was always kept cold and dilutions were made periodically from the stock for experiments.

Purification of recombinant proteins

Recombinant human Bax was produced as described (2) but the purification procedure was modified. The Bax eluted from the chitin column was dialyzed (12000 MW cutoff) at 4°C for 24 hrs against 3L of 1mM EDTA, 10mM Tris-HCl, pH 8. Then it was dialyzed again for 24 hrs against 5L of 10 mM Tris-HCl pH 8 to remove all remaining DTT. Recombinant tBid was purified as described (3). Bcl-xL was purified as described (4 - 6). All proteins were filter-sterilized (0.2 μm filter), glycerol added (10% final), rapidly flash-frozen with ethanol and dry-ice, and stored at -80 ^{o}C. Protein concentration was determined after dialysis by the BCA method.

Western blot analysis

For all western blotting experiments, three 1mL mitochondrial suspensions (960 μg/mL) were incubated at 30^{o}C under the conditions and for the times indicated in the figure legends. The triplicates were centrifuged (10,000 RCF for 5 min) and each resuspended into 200 μL of FH buffer. These were pooled together, mitochondria re pelleted and subjected to sodium carbonate treatment as described (7). The pellet was resuspended in 50 μL of 2% SDS and incubated for 1 hr on ice. Then, the detergent solubilized fraction was separated from the detergent-insoluble fraction by centrifugation and 30 μL used for PAGE/blotting analysis. VDAC served as loading control. Bax was probed with rabbit anti-Bax (Ab7977: 1:1000) and VDAC was probed with rabbit anti-VDAC anti-serum (1:1,000,000). Both the primary antibodies were probed with HRP tagged anti-rabbit secondary antibodies (1:2500 for Bax and 1:5000 for VDAC). Blots were developed with DAB as described in (8).

Cytochrome c accessibility assay

The cytochrome *c* oxidation or accessibility assay was performed as described previously (6). After appropriate treatment of 96 μg/mL mitochondria with Bcl-2 family proteins, 100 μL of this suspension was dispersed in 600 μL of cytochrome *c* oxidation assay buffer (equivalent to FH buffer (mannitol + KCl (total 300 mOs), 0.1 mM EGTA, 5 mM HEPES pH 7.5) of appropriate ionic strength supplemented with

5 mM DNP and 5 μM antimycin A) and quickly assayed for cytochrome *c* oxidation by adding 10 μL of reduced cytochrome *c* to a final concentration of ~25 μM. Cytochrome *c* oxidation was measured as decline in absorbance at 550 nm. The rate of oxidation was calculated from the slope of the linear region of the absorbance change measured for 2 minutes. Maximal rate of cytochrome *c* oxidation (corresponding to 100% permeabilization) was determined by the rate of cytochrome *c* oxidation by equivalent amount of hypotonically shocked mitochondria (at least 1 volume of mitochondrial suspension to 50 volumes of double distilled H_2O). Hypotonic lysis breaks the MOM and exposes all the cytochrome oxidase to exogenous cytochrome *c*. The % oxidation rate was calculated with maximal oxidation rate as 100% and rate of oxidation by control mitochondria (no treatment) as 0 %. Cytochrome oxidase activity was strongly sensitive to ionic strength, so control and maximal oxidation rates were determined for each salt concentration tested. The actual numerical values of control, Bax-tBid treated and maximal cytochrome *c* oxidation rates at 10 and 90mM KCl are presented (Fig S1A) for comparison.

Adenylate kinase (AK) assay

The assay was performed as described previously (6). This assay measures the release of the 24 kDa protein, adenylate kinase, from the IMS of mitochondria. In this coupled enzyme assay, NADP is reduced to form NADPH which has a strong absorbance at 340 nm. (The oxidized form NADP does not have this absorbance). Prior to assaying, 2.5 μL of a mix of Hexokinase/Glucose-6-Phosphate Dehydrogenase (5 units each) was incubated in 350 μL AK buffer (5 mM $MgSO_4$, 10 mM glucose, 5 mM ADP, 0.2 mM NADP, 50 mM Tris, and pH 7.5) to remove residual ATP. After 2-3 minute incubation, 150 μL of mitochondrial supernatant was added to the AK buffer/ enzyme mix, and absorption was measured for 5 minutes at 340 nm. The initial linear slope of increase in absorption was taken as the rate of AK reaction. Since all other reaction components are in excess, the concentration of AK is the rate-limiting factor. Hence the rate of NADPH formation is a direct measure of amount of AK released from mitochondria. Maximal rate of NADPH formation, corresponding to 100% release of AK from mitochondria, was measured from the activity of supernatant from equivalent amount of hypotonically shocked mitochondria. The actual numerical values of AK activity used as control (0% AK activity or no release of AK), Bax-tBid treated and lysed (100% AK activity or complete release of AK) are presented in Fig S1B for comparison.

Sulfite oxidase (SOX) assay

SOX is a 120 kDa IMS protein and its release from mitochondria was assayed as described (9). SOX reduces cytochrome *c* using sulfite as the electron donor. Prior to the assay, a one-to-one solution of 40 mM Na_2SO_3 and 50 mg/ml oxidized cytochrome *c* was made. Then 10 μL of this solution (0.4 mM Na_2SO_3 and 0.5 mg oxidized cytochrome *c* final) was added to 500 μL mitochondrial supernatant and the absorbance was quickly measured at 550 nm for 5 minutes. Maximal release was measured from the activity of supernatant from equivalent amount of hypotonically shocked mitochondria. SOX activity was strongly sensitive to ionic strength. Hence, the supernatant was supplemented with suitable amount of salt by dilution from stock salt solution to adjust the ionic strength. The actual numerical values of SOX activity used as control (0% SOX activity or no release of SOX), Bax-tBid treated and lysed (100 % SOX activity or complete release of SOX) are presented in Fig S1C for comparison.

Measurement of fumarase activity

Fumarase is a matrix enzyme that reversibly converts malate to fumarate. Fumarase release was measured by a standard procedure (10). Briefly 96 μg/mL mitochondrial suspension in F H buffer was treated with varying concentrations of digitonin (from 0.0025% to 0.1% in multiples of 2) for 5 minutes at room temperature and centrifuged for 5 minutes at 14000 RCF and supernatant collected. 500 μL of the supernatant was mixed with 500 μL of fumarase assay buffer (50 mM malate, 50 mM Na_2HPO_4 pH 7.3) and change in absorbance measured for 5 minutes at 250 nm in quartz cuvettes to measure formation of fumarate which shows strong absorbance at 250 nm. Maximal fumarase release corresponding to 100% was obtained by treating equivalent amount of mitochondria with 0.2 % Triton-X100.

SUPPORTING REFERENCES

1. Siskind, L.J., R. N. Kolesnick, and M. Colombini. 2002. Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins. J. Biol. Chem. 277:26796-26803.
2. Suzuki, M., R. J. Youle, and N. Tjandra. 2000. Structure of Bax: coregulation of dimer formation and intracellular colocalization. Cell 103:645-654.
3. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman M.H., Latterich M., Schneiter, R. D. R. Green, and D. D. Newmeyer. 2000. Bid, Bax and lipids cooperate to form supramolecular openings in the mitochondrial outer membrane. Cell 111:331-342.
4. Basanez, G., Zhang, J., Chau, B.N., Maksaev, G.I., Frolov, V.A., Brandt, T.A., Burch, J., J. M. Hardwick, and J. Zimmerberg. 2001. Pro-apoptotic cleavage products of Bcl-xL form cytochrome *c* conducting pores in pure lipid membranes. J. Biol. Chem. 276:31083-31091.
5. Thuduppathy, G.R., Terrones, O., Craig, J.W., G. Basanez, and R. B. Hill. 2006. The N-terminal domain of Bcl-xL reversibly binds membranes in a pH dependent manner. Biochemistry 45:14533-14542.
6. Siskind, L.J., Feinstein, L., Yu, T., Davis, J.S., Jones, D., Choi, J., Zuckerman, J. E., Tan, W., Hill, R. B., J.M. Hardwick and M. Colombini. 2008. Anti-apoptotic Bcl-2 proteins disassemble ceramide channels. J. Biol. Chem. 283:6622-6630.
7. Teijido, O., and L. Dejean. 2010. Upregulation of Bcl2 inhibits apoptosis-driven BAX insertion but favors Bax relocalization in mitochondria. FEBS Lett. 584:3305-3310.
8. Ahmed, H. 1959. Principles and Reactions of Protein Extraction, Purification, and Characterization, CRC Press LLC, Boca Raton, FL.
9. Johnson, J.L. and K.V. Rajagopalan. 1976. Purification and properties of sulfite oxidase from human liver. J. Biol. Chem 249:859-866.
10. R. L. Hill, and R. A. Bradshaw. 1969. Fumarase. Methods Enzymol. 13: 91-99.

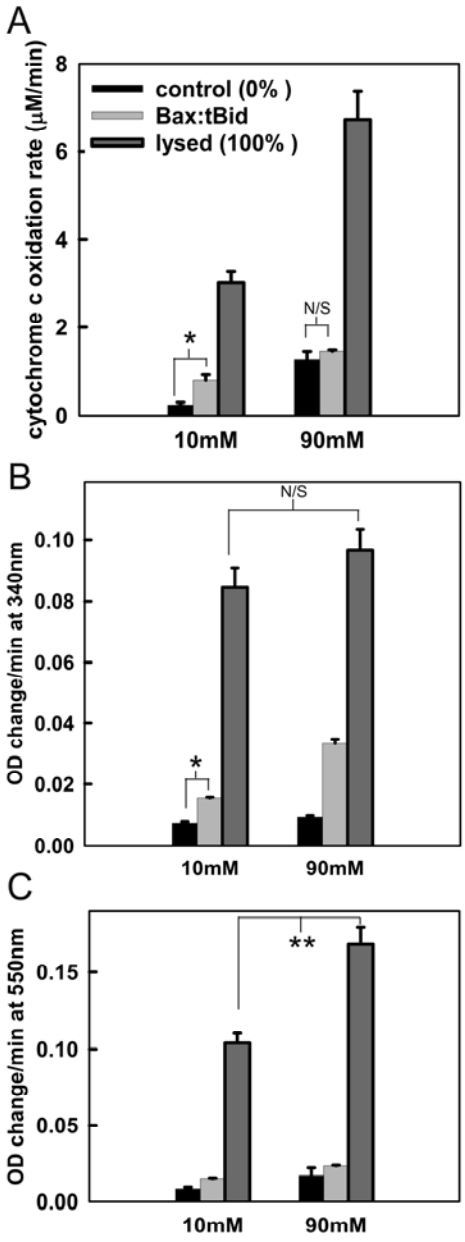

FIGURE S1: Measured rates of enzym atic activity in a set of experim ents showing the rates prior to any treatm ent (control rates), the rate s after tBid/Bax treatment, and the maxim al rates after hypotonic shock. The influence of ionic stre ngth on som e of these rates is illustrated. By subtracting the control r ates and exp ressing the results as a p ercentage of the m aximal rates we correct for background rates a nd effects of i onic strength. Th e figure shows the rates of cytochrome *c* (25 μM) o xidation (A), AK activity as m easured by absorbance (OD) change at 340nm (B) and SOX activity as m easured by abso rbance change at 550nm (C) recorded from 96μg/mL mitochondrial suspensions that were either untreated (c ontrol), or treated with 17nM Bax and 120nM tBid or osm otically lysed (lysed). Incubations (30 m in) were in eithe r 10 o r 90mM KCl buffer. * represents P value < 0. 05 and ** represents P value < 0.01. n= 3.

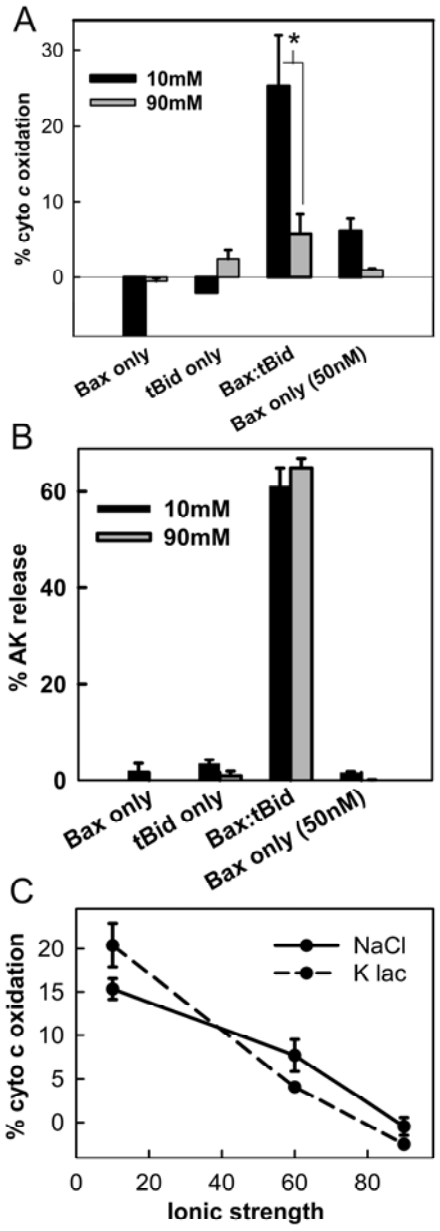

FIGURE S2: Control experiments and evidence of ionic strength dependence. A, B: Bax and tBid alone have little or no effect on MOMP. The rates of cytochrome *c* oxidation (A) or AK release (B) by 96 μg/mL mitochondrial suspension are expressed as a percent of the maximal amount. Except as indicated, mitochondria were incubated for 30 min with 17nM Bax and/or 120nM tBid in the presence of either 10mM (black bars) or 90mM KCl (grey bars) buffer. C. The rates of cytochrome *c* oxidation (expressed as a % of the maximal values) induced by a 30 min incubation with 17nM Bax and 120nM tBid in the presence of varying concentrations of either NaCl (solid line) or potassium lactobionate (K lac, broken line). * represents P value < 0.05. n = 3.

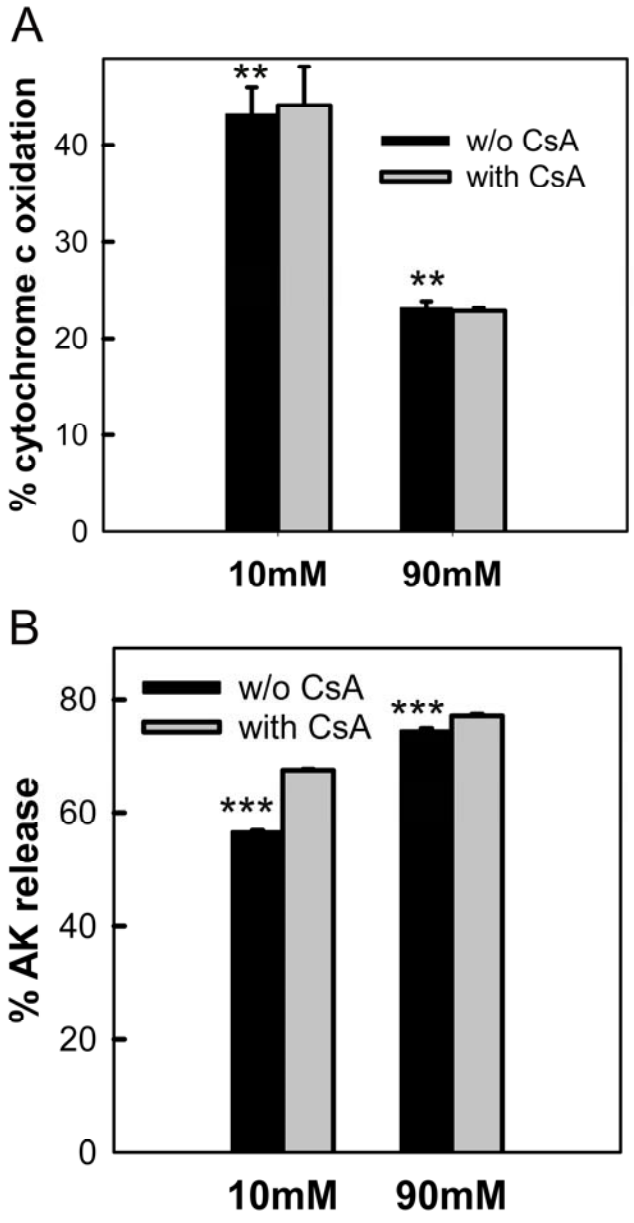

Figure S3: MOMP induced by Bax and tBid is independent of PTP. Mitochondria were incubated with 35nM Bax and 120nM tBid in the presence or absence of 50 μM CsA at either 10mM KCl or 90mM KCl for 30 minutes at 30 °C. Then, the mitochondria from each treatment were assayed for real-time permeabilization using the cytochrome *c* oxidation assay and the supernatant analyzed for extent of AK release. The statistical tests are between the black bars in each panel. Both are significantly different ** P value < 0.01 and *** P value < 0.001 n = 3.

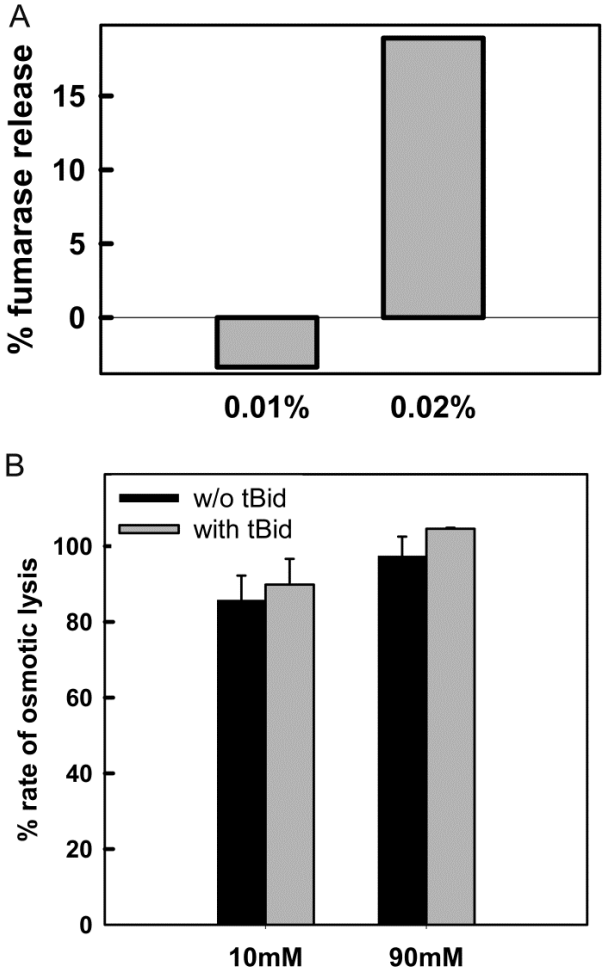

Figure S4: Comparison of maximal cytochrome c oxidation obtained under swelling and non-swelling conditions. A) At 0.01% digitonin, no activity of fumarase, a matrix enzyme, could be detected, but at 0.02% nearly 20% of fumarase was released from 96 µg/mL mitochondrial suspension. B) 96 µg/mL mitochondrial suspension was treated with 0.01% digitonin for 5 minutes either without tBid or after pre-incubation of the mitochondrial suspension with 120nM tBid for 30 minutes at 30 ^{o}C at either ionic strength. Then, the mitochondrial suspension was assayed for the rate of cytochrome *c* oxidation. The mean values are the % of cytochrome *c* oxidation rate observed with an equivalent amount of osmotically lysed mitochondria at the corresponding ionic strengths. No statistically significant difference was found between digitonin treated samples (with or without tBid) and osmotically lysed samples. (P values > 0.15). n = 3.

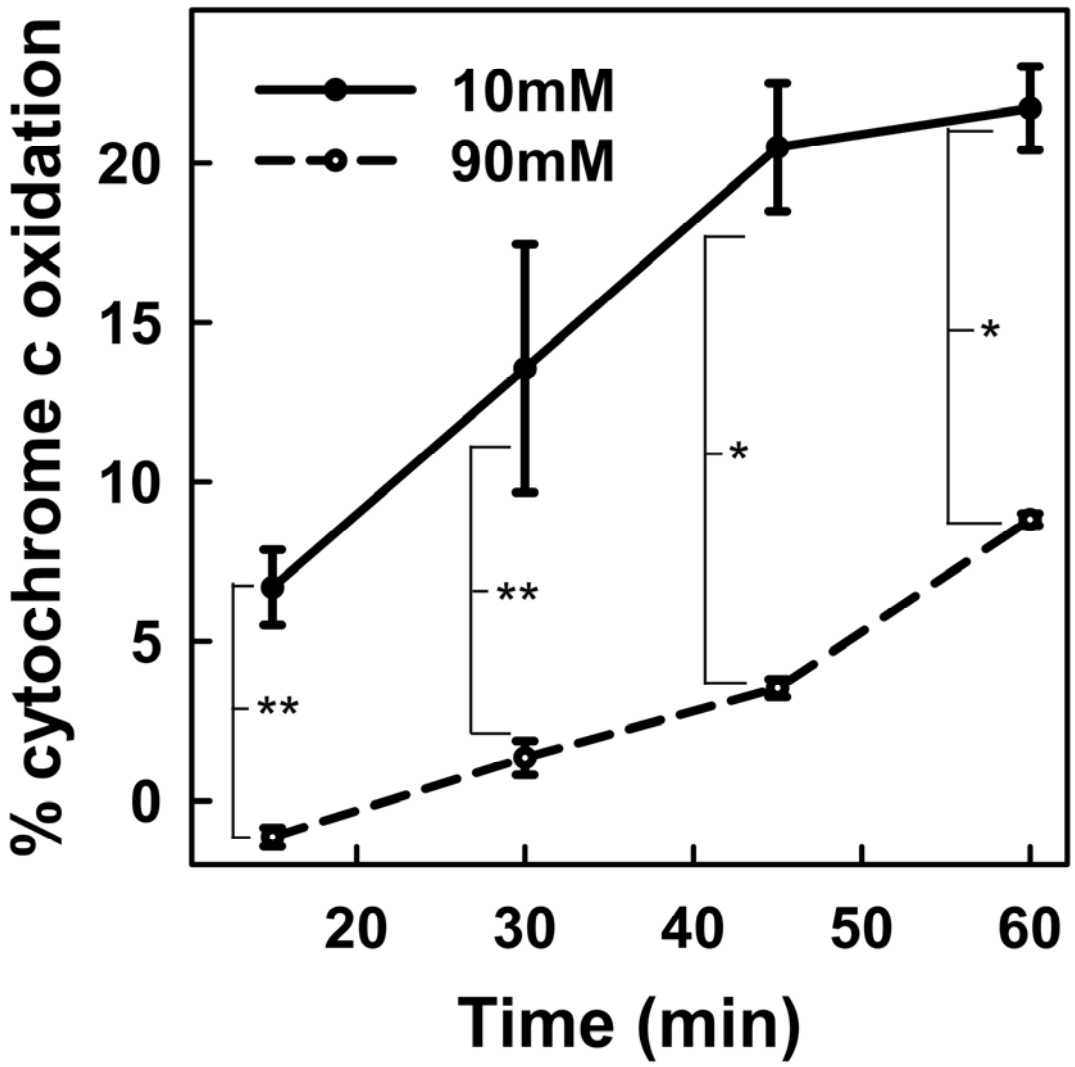

Figure S5: Bax induced MOMP increased at a faster rate at lower ionic strength. Mitochondrial suspensions were treated with 34nM monomeric Bax and 120nM tBid at 30 ^{o}C at either 10mM KCl buffer (solid lines) or 90mM KCl buffer (dashed lines) and the rate of cytochrome *c* oxidation was determined at different time intervals. * represents P value < 0.05, ** - P value < 0.01. n = 3.

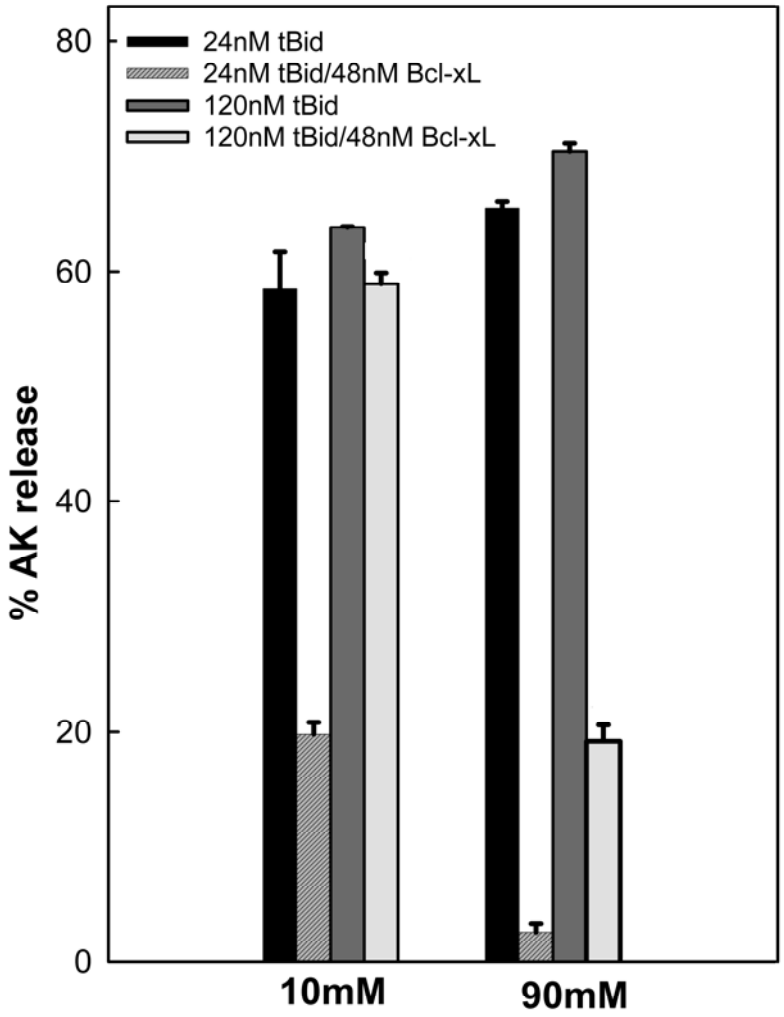

FIGURE S6: Bcl-xL mediated inhibition of MOMP is sensitive to tBid concentration. Mitochondrial suspensions were incubated with 17nM monomeric Bax, either 24nM or 120nM tBid, without Bcl-xL or with 48nM Bcl-xL in 10mM KCl buffer or 90mM KCl buffer for 30 minutes at 30 ^{o}C. Then, the extent of AK release was determined for the different treatments. The differences in the inhibitory effects of Bcl-xL at 10 and 90 mM were statistically significant with $P<0.0001$ at both tBid concentrations. n = 3.

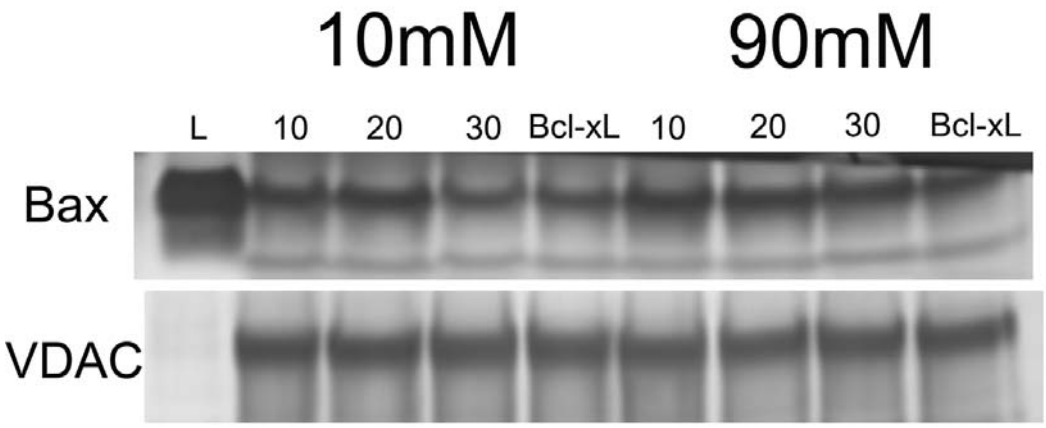

FIGURE S7: Bcl-xL inhibited Bax mediated MOMP without affecting Bax insertion. Mitochondrial suspensions (960µg mitochondrial protein/mL) were treated with 100nM Bax and 240nM tBid for 10, 20 and 30 minutes without Bcl-xL or with 100nM Bcl-xL (for 30 minutes only) in either 10mM KCl buffer or 90mM KCl buffer. The western blots were performed as described in Experimental Procedures. VDAC served as loading control. Band L corresponds to 10% of the total Bax expected to be present if all the added Bax inserted into the mitochondrial outer membrane.