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Bid, but Not Bax, Regulates VDAC Channels*

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During apoptosis, cytochrome *c* is released from mitochondria into the cytosol, where it participates in caspase activation. Various and often conflicting mechanisms have been proposed to account for the increased permeability of the mitochondrial outer membrane that is responsible for this process. The voltage-dependent anion channel (VDAC) is the major permeability pathway for metabolites in the mitochondrial outer membrane and therefore is a very attractive candidate for cytochrome *c* translocation. Here, we report that properties of VDAC channels reconstituted into planar phospholipid membranes are unaffected by addition of the pro-apoptotic protein Bax under a variety of conditions. Contrary to other reports (Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) *Nature* 399, 483–487; Shimizu, S., Ide, T., Yanagida, T., and Tsujimoto, Y. (2000) *J. Biol. Chem.* 275, 12321–12325; Shimizu, S., Konishi, A., Kodama, T., and Tsujimoto, Y. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3100–3105), we found no electrophysiologically detectable interaction between VDAC channels isolated from mammalian mitochondria and either monomeric or oligomeric forms of Bax. We conclude that Bax does not induce cytochrome *c* release by acting on VDAC. In contrast to Bax, another pro-apoptotic protein (Bid) proteolytically cleaved with caspase-8 affected the voltage gating of VDAC by inducing channel closure. We speculate that by decreasing the probability of VDAC opening, Bid reduces metabolite exchange between mitochondria and the cytosol, leading to mitochondrial dysfunction.

The crucial role of mitochondria in the initiation of apoptosis is well established. Triggered by a number of different stimuli, the mitochondrial outer membrane (MOM)[1] becomes permeable to apoptogenic factors such as cytochrome *c* and Smac/DIABLO (4, 5). The release of these factors leads to caspase activation, DNA fragmentation, and other characteristic changes associated with apoptotic cell death. So far, it remains unclear exactly how MOM permeabilization occurs, and published results are often contradictory (for reviews, see Refs. 6–8).

The Bcl-2 family of proteins regulates the permeabilization of MOM. Pro-apoptotic proteins such as Bax and Bid induce the release of apoptogenic factors, whereas anti-apoptotic proteins such as Bcl-2 and Bcl-x_L prevent their release. There are two prevailing theories for the permeabilization of the outer membrane. One is that MOM is nonspecifically ruptured; the other proposes the formation of channels that allow cytochrome *c* release (6, 7).

One of the channel models is based on the ability of Bax to form channels when incorporated into lipid membranes (9, 10). It has been reported that Bax can form oligomers of multiple sizes that have features of ion channels large enough to release cytochrome *c* (11). Soluble monomeric Bax is not capable of forming channels in lipid membranes and does not trigger the release of cytochrome *c* from isolated mitochondria (11). Thus, channel formation is associated with the formation of oligomers. Bax oligomerization has been proposed to be triggered by the BH3 domain-only protein Bid after cleavage by caspase-8 into the truncated form, tBid (12, 13). However, the situation has been complicated by a recent report that tBid itself homopolymerizes, and this process, without the participation of Bax, results in the release of cytochrome *c* from mitochondria (14). In addition, it has been observed that tBid promotes leakage in planar lipid membranes and liposomes in the absence of other proteins (15–17). In all these studies, the channel/pore-forming ability was attributed to Bax and Bid; however, it is still an open question whether such channels are formed *in vivo*.

There are two other channel models that associate the Bcl-2 family proteins with the existing major channel in MOM, the voltage-dependent anion channel (VDAC). This channel is known to be responsible for most of the metabolite flux across this membrane (18–21). Thus, VDAC is a very attractive candidate for a pathway for cytochrome *c* release. When reconstituted into planar membranes, the 30-kDa VDAC monomer forms an aqueous pore 2.5–3 nm in diameter (18, 22, 23) that allows uncharged polymers (such as inulin, dextran, and polyethylene glycol with a molecular mass of ~5000 Da) to cross membranes (24, 25). However, the apparent size of the channel is very different for charged molecules. The channel even discriminates between different molecules based on their three-dimensional structure (26). Negatively charged anions such as ATP are favored due to the net positive charge within the channel. VDAC is permeable to anionic metabolites in the open state, but is essentially impermeable to anions such as ATP and phosphocreatine when the channel adopts a closed configuration, and the channel selectivity is reversed from favoring anions to favoring cations (19, 20, 27). Certainly, the positively

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[1] The abbreviations used are: MOM, mitochondrial outer membrane; BH, Bcl-2 homology domain; tBid, truncated Bid; Bax_{FL}, full-length Bax; t^cBid, 15.5-kDa C-terminal fragment of caspase-8-cleaved Bid; DPhPC, diphytanoylphosphatidylcholine; nS, nanosiemens; PTP, permeability transition pore.

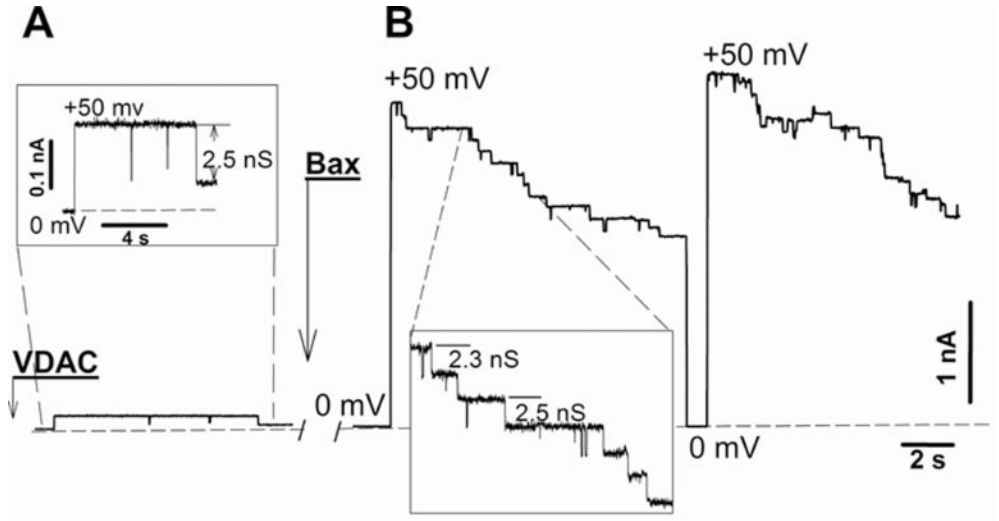

FIG. 1. **Voltage-dependent VDAC channel closure in the absence (*A*) and presence (*B*) of monomeric Bax.** *A*, at a 50 mV applied potential, a single VDAC channel moved from the open state to a low conductance closed state. *B*, after addition of 20 nM monomeric Bax, the conductance increased to 60 nS due to additional insertion of 15 VDAC channels. Application of a 50 mV potential closed the channels. The *inset* shows, at a finer scale, that each step of conductance decrease is ~2.4 nS, similar to the control in *A*. After 0 mV was applied for a short time, the channels reopened and then reclosed at 50 mV. The medium consisted of 1 M NaCl and 1 mM $CaCl_2$ buffered with 5 mM HEPES (pH 7.1). The bilayer membrane was made from DPhPC. Current records were filtered using averaging times of 10 and 1 ms (*insets*). Here and in the other figures, the *dashed line* indicates zero-current level.

charged cytochrome *c* molecules (12 kDa) cannot permeate through this channel under normal conditions. If they did, cytochrome *c* would not be localized within the intermembrane space.

Vander Heiden *et al.* (27, 28) proposed a model of MOM permeabilization that involves regulation of VDAC channels. In this model, VDAC channel closure prevents the efficient exchange of ATP and ADP between the cytosol and the mitochondrial matrix. Loss of outer membrane permeability due to VDAC closure might result in the accumulation of the products of mitochondrial activity within the intermembrane space, generation of an osmotic gradient, and matrix swelling, followed by the rupture of the outer membrane. The anti-apoptotic protein Bcl-x_L restores the ATP/ADP exchange. Experiments with VDAC channels reconstituted into planar phospholipid membranes demonstrated that Bcl-x_L promotes the open configuration of mammalian VDAC channels (27).

Another channel model postulates that the pro-apoptotic protein Bax directly interacts with VDAC, resulting in cytochrome *c* permeation through membranes (1–3). It has been reported that experiments with liposomes and planar phospholipid membranes show that Bax induces a novel VDAC-containing channel that is larger than the channels observed when the proteins are used alone. It was concluded that, although the channels formed by Bax and VDAC alone are unable to allow cytochrome *c* translocation, the new larger pores are permeable to cytochrome *c* (2).

In both models, the ability of Bcl-2 proteins to regulate the state and integrity of VDAC could thus account for their ability to be either anti- or pro-apoptotic. However, the mechanisms of VDAC channel regulation by Bcl-2 proteins proposed in these models are diametrically opposite. In the first model, the closure of VDAC channels leads to MOM rupture, and anti-apoptotic protein helps to maintain VDAC channels in their normal, functional open state. In the second model, the pro-apoptotic protein induces opening of the large VDAC-based channels permeable to cytochrome *c*. The mechanism of MOM permeabilization is undoubtedly very complex and most likely involves more than one protein to form the passages for cytochrome *c* release. Ceramides have also been proposed to form this permeability pathway (29). The role of VDAC in this process is rather controversial; but considering that both BH1–3 proteins such as Bax and BH3 domain-only proteins such as Bid are most likely required for MOM permeabilization, it is important to test the effect of both pro-apoptotic proteins on VDAC channels.

To address this question, we studied the effect of the pro-apoptotic proteins Bax and Bid on the properties of VDAC channels reconstituted into planar phospholipid membranes. We used the full-length Bax protein in the soluble monomeric and oligomeric forms and Bid cut with caspase-8 (referred to as cut Bid). Here, we show that the properties of VDAC channels isolated from mammalian mitochondria are unaffected by addition of Bax. We conclude that Bax does not induce cytochrome *c* release by acting on VDAC. In contrast, we demonstrate that cut Bid induces the closure of VDAC channels. By reducing the permeability of VDAC channels, the pro-apoptotic protein Bid might interfere with metabolite exchange between mitochondria and the cytosol, with the subsequent loss of outer membrane integrity.

EXPERIMENTAL PROCEDURES

Protein Purification—VDAC from rat liver mitochondria was isolated and purified as described previously (30–32). VDAC from *Neurospora crassa* MOMs was obtained using the standard procedure of isolation (31, 33).

Human recombinant wild-type monomeric full-length Bax (Bax_{FL}) was obtained using two different protocols (34, 35). The first sample of monomeric Bax was purified by a previously described procedure (34); *Escherichia coli* BL21(DE3) cells harboring pTYB1-Bax were cultured in LB medium; recombinant proteins were isolated from the cytosol by chitin affinity chromatography according to the protocol provided by the manufacturer (New England Biolabs Inc.) and further purified by ion-exchange chromatography on a mono-Q column (Amersham Biosciences). The second Bax_{FL} sample with a tag of six histidines at the N-terminus was expressed in the pBAD plasmid in *E. coli* (36). Monomeric Bax was recovered in the soluble bacterial fraction and purified by chromatography on nickel-nitrilotriacetic acid-agarose, followed by Q-Sepharose. The protein was stored in 25 mM Tris-HCl, 100 mM NaCl, 0.2 mM dithiothreitol, and 30% (v/v) glycerol (pH 7.5) at −80 °C.

Oligomeric Bax_{FL} with an N-terminal His tag was expressed in and purified from *E. coli* (11). The protein was recovered in the soluble fraction in the presence of 1% Triton X-100 and purified on nickel-nitrilotriacetic acid-agarose, followed by anion-exchange chromatography on a Q-Sepharose column. The purified protein was dialyzed

TABLE I
Summary of the experiments with VDAC channels and Bax

Bax sample	Lipid	Medium	pH	Me^{2+} (1 mM)	Single channel conductance	Selectivity	Voltage gating
Monomeric							
16–3000 nM	DPhPC	1 M NaCl (12)[a] or 0.1 M KCl (1)	7.0 (7), 5.0 (6)	Ca^{2+} (13)	NE[b] (13)		
20–130 nM	Asolectin	1 M NaCl (6) or 0.1 M NaCl (2)	5.5 (8)	Ca^{2+} (8)	NE (1)	NE (1)	NE (7)
Oligomeric with 1% OG[c]							
30–60 nM [~90 nM[d]]	Asolectin	0.25 M NaCl (2), 0.25 M KCl (4), or 0.1 M KCl (1)	5.5 (7)	Mg^{2+} (7)	NE (3)	NE (3)	NE (4)
Oligomeric[e]							
2–10 nM [15–20[d] nM]	Asolectin	0.25/0.05 M NaCl (2), 0.25 M NaCl (1), or 0.25 M KCl (2)	5.5 (4), 7.0 (1)	Mg^{2+} (5)	NE (2)	NE (2)	NE (4)

[a] The number of experiments is indicated in parentheses.
[b] NE, no effect.
[c] Oligomeric Bax was obtained by treating monomeric Bax with 1% octyl glucoside (OG).
[d] The concentration at which Bax addition resulted in membrane rupture.
[e] Oligomeric Bax was obtained by isolating Bax in the presence of detergents.

against 25 mM HEPES-NaOH, 0.2 mM dithiothreitol, 1% (w/v) octyl glucoside, and 30% glycerol (pH 7.5) and stored at −80 °C. Other samples of oligomeric Bax_{FL} were obtained by incubation of monomeric Bax_{FL} with 1% octyl glucoside on ice for 30 min.

Mouse full-length Bid was expressed with an N-terminal His_6 tag in *E. coli*. Caspase-8-cleaved mouse wild-type Bid (cut Bid) was obtained from purified full-length Bid by cleavage with caspase-8 (37, 38). Cut Bid was purified on a nickel affinity column to remove caspase-8 (39). No caspase-8 activity was detected in the purified cut Bid sample. The resulting 15.5-kDa C-terminal fragment (t^{c}Bid, amino acids 60–195) was expressed as an N-terminal His_6-tagged protein in *E. coli* (17).

Electrophysiological Recordings—Planar lipid membranes were formed from monolayers made from 1% (w/v) lipids in hexane on 70–80-μm diameter orifices in the 15-μm-thick Teflon partition that separated two chambers (modified after (40)). The lipid-forming solutions contained diphytanoylphosphatidylcholine (DPhPC); 90% asolectin (soybean phospholipids) and 10% cholesterol; or 42% asolectin, 42% DPhPC, 8% cardiolipin, and 8% cholesterol. DPhPC, asolectin, and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol was purchased from Sigma. The membrane potential was maintained using Ag/AgCl electrodes with 3 M KCl and 15% (w/v) agarose bridges (41). VDAC channels insertion was achieved by adding 0.1–1.5 μl of a 1% Triton X-100 solution of purified VDAC to the 1.5-ml aqueous phase in the *cis*-compartment while stirring. Potential is defined as positive when it is greater at the side of VDAC addition (*cis*). The effect of the pro-apoptotic proteins on the VDAC properties was determined as follows. After VDAC channels were inserted and their parameters were monitored for at least 20–25 min, the pro-apoptotic protein was added to both sides of the membrane (if not indicated otherwise) under constant stirring for 2 min. The currents were recorded for at least 25 min, followed by a series of additions of the protein until a rise in conductance induced by the pro-apoptotic protein was obtained.

Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) in the voltage clamp mode. Data were filtered by a low-pass 8-pole Butterworth filter (Model 9002, Frequency Devices Inc., Haverhill, MA) at 15 kHz, recorded on a chart recorder, and directly saved into the computer memory with a sampling frequency of 50 kHz. The membrane chamber and headstage were isolated from external noise sources with a double metal screen (Amuneal Manufacturing Corp., Philadelphia, PA). The voltage-dependent properties of the VDAC-containing membrane were assessed by applying a symmetrical 5-mHz triangular voltage wave from a Hewlett-Packard 33120A function waveform generator and recording the current using a Digidata 1322A (Axon Instruments, Inc.). Data were acquired at a sampling frequency of 1 Hz and analyzed using pClamp 8 software (Axon Instruments, Inc.).

RESULTS

VDAC and Bax—One of the characteristic properties of VDAC is its voltage gating. The channels are in the high conductance open state at low voltages (<30 mV) and move to low conductance states ("closed" states) at high voltages (Fig. 1*A*, *inset*). The application of 50 mV causes the conductance of the single channel to drop by two-thirds of its open state level. A short exposure to 0 mV reopens the channel. After the conductance and voltage gating properties of the channel were measured, monomeric Bax was added to the membrane-bathing aqueous solutions on one or both sides of the membrane. In most experiments (7 of 10), we observed that monomeric Bax addition caused a 4–6-fold increase in membrane conductance. However, further analysis showed that this increase in membrane conductance was due to the insertion of additional VDAC channels and not to the formation of a novel larger channel. Fig. 1*B* illustrates the stepwise conductance decrease as individual channels closed in the presence of monomeric Bax. The individual steps (Fig. 1*B*, *inset*) are of the same size as in control records (Fig. 1*A*, *inset*). After 0 mV was applied, the channels reopened and closed again under a 50 mV applied potential. All this is normal VDAC behavior. These channels were still sensitive to König polyanion that induced their closure (42) (data not shown). Sizing with polyethylene glycols (43) gave similar results as for single VDAC channels. The conclusion that the observed increase in conductance was indeed the result of new insertion and not formation of larger channels (as previously reported (2)) is supported by the observation that perfusion of the chamber to remove excess VDAC prior to Bax addition resulted in the absence of the Bax-dependent conductance increment.

Other characteristic properties of VDAC such as single channel conductance and selectivity were probed in the presence of monomeric Bax. No significant changes in these properties were observed (Table I). In principle, many additional factors may also influence Bax-VDAC interaction. The possibility that membrane lipids (44), especially charged lipids, might affect the observations was assessed using both the neutral DPhPC and a negatively charged natural mixture, asolectin. No effect of Bax on VDAC was observed with either of these lipids (Table I). The possibility that acidic pH might render the terminal α-helix of Bax accessible for interaction with VDAC was tested by performing the experiments at both neutral and acidic pH values. Ionic strength was also varied, as was the presence of either Ca^{2+} or Mg^{2+}; and no influence of Bax on the properties of VDAC was observed under any of these conditions (Table I).

The possibility that the physical state of Bax (monomeric or oligomeric) is important for interaction with VDAC has been tested using both forms. As reported previously (11), we observed that only Bax oligomers formed channel-like conductances. Examples of the representative current traces induced

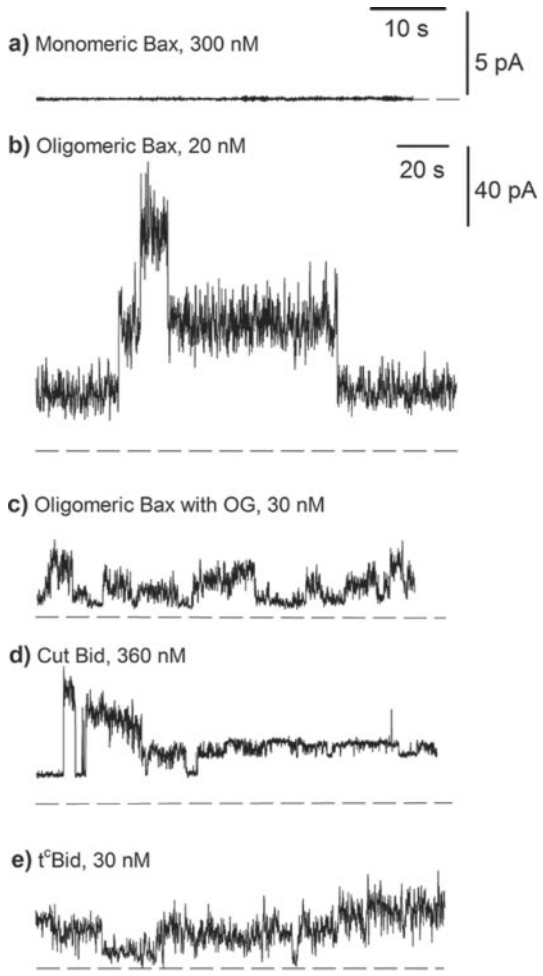

FIG. 2. **Oligomeric Bax and Bid induce channel-like conductances in planar membranes; monomeric Bax is inactive.** The traces show the current in the presence of monomeric Bax (*a*), oligomeric Bax obtained by isolation in the presence of detergent (*b*) and by treatment of monomeric Bax with 1% octyl glucoside (*OG*) (*c*) as described under "Experimental Procedures," cut Bid (*d*), and t^{c}Bid (*e*) through asolectin/DPhPC/cardiolipin/cholesterol (*b*, *d*, and *e*), asolectin/cholesterol (*c*), and DPhPC (*a*) bilayer membranes. The concentrations of the proteins are indicated. The medium consisted of 250 mM KCl and 1 mM $CaCl_2$ (*a*, *d*, and *e*) or $MgCl_2$ (*b* and *c*) buffered with 5 mM HEPES at pH 7.0 (*a*, *d*, and *e*) or MES at pH 5.5 (*b* and *c*). *b–e* are shown at the same time and current scales. The applied potential was +20 mV. Current records were filtered using an averaging time of 30 ms.

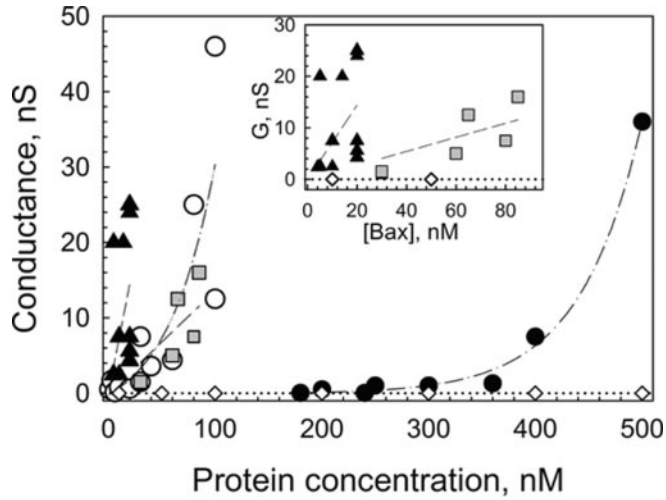

FIG. 3. **Pro-apoptotic proteins increase membrane conductance at different concentrations.** The symbols represent the average conductance values obtained in individual experiments 10–20 min after protein addition. The *inset* shows, at a finer scale, the concentration dependence for oligomeric Bax from two different preparations. Oligomeric Bax obtained by isolation in the presence of detergent (▲) increased membrane conductance at 5–20 nM, whereas oligomeric Bax obtained by treatment of monomeric Bax with 1% octyl glucoside (*shaded boxes*) increased conductance at 30–80 nM. Cut Bid (●) induced membrane conductance at concentrations that were higher than those of t^{c}Bid (○). Monomeric Bax (◇) was inactive at all concentrations. The other experimental conditions were as described in the legend to Fig. 2. The lines were drawn to guide the eye through the experimental points.

by oligomeric Bax are presented in Fig. 2 (*b* and *c*). Monomeric Bax itself was inactive (up to 3 μM) at neutral pH (Fig. 2*a*). Oligomeric Bax was obtained either by isolating Bax in the presence of detergents or by treating monomeric Bax with 1% octyl glucoside (see "Experimental Procedures"). Oligomeric Bax samples from both preparations formed channels in bilayer membranes, but at different concentration ranges (Fig. 3). The former increased membrane conductance at lower concentrations (5–20 nM). The latter required 30–80 nM to increase membrane conductance. The distributions of individual conductance steps produced by oligomeric Bax were very broad, with conductances from hundreds of picosiemens to tens of nanosiemens (nS). In a typical experiment, oligomeric Bax produced channels with a gradual increase in conductance during the experiment (45). Often, after reaching the high total conductance level of 10–20 nS, the membrane ruptured. The decrease in the lifetime of the membranes in the presence of Bax proteins has been described previously (46).

To discriminate between VDAC- and Bax-induced conductances, both oligomeric Bax preparations were used at concentrations below or at the threshold at which they increased the membrane conductance. The concentrations at which oligomeric Bax formed channels were variable (Fig. 3, *inset*). Therefore, in a typical experiment, after the VDAC channel(s) was reconstituted into the membrane and its properties were recorded, oligomeric Bax was added in a series starting with concentrations below those at which Bax was found to form channels until a visible increase in the conductance, due to the formation of Bax channels, was observed. The Bax channels were distinctly different from VDAC channels (compare the noisy current traces produced by Bax in Fig. 2 (*b* and *c*) with the well defined stable currents through VDAC channels in Figs. 1 and 4*A*), which helped to avoid a confusing situation when a total current was actually a sum of two different conductances with different properties. Our task was to find the Bax-induced changes in VDAC properties under the conditions in which Bax did not form channels by itself.

Fig. 4*A* shows an example in which the selectivity of VDAC channel was assessed before and after oligomeric Bax addition. The reversal potential (measured in a 5-fold NaCl salt gradient) was +17 mV before and after Bax addition. The single channel conductance also remained the same with and without Bax. Fig. 4*A* shows no effect of oligomeric Bax on either selectivity or single channel conductance.

The probability of VDAC channels to be open or closed at different potentials in the presence of Bax was estimated by obtaining *G*/V plots as shown in Fig. 4*B*. The data were collected as follows. Slow triangular voltage waves (5 mHz) were applied to a multichannel membrane, and the current was recorded. Because VDAC channels have rapid opening rates (inverse submilliseconds) and slow closing rates (inverse seconds), a near-equilibrium *G*/V plot could be obtained by collecting data when the applied voltage opened the channels, changing from ±60 to 0 mV. In the membranes containing many VDAC channels there was always a possibility that a portion of the conductance was due to Bax channels. The number of VDAC channels could also vary during the recording. To take

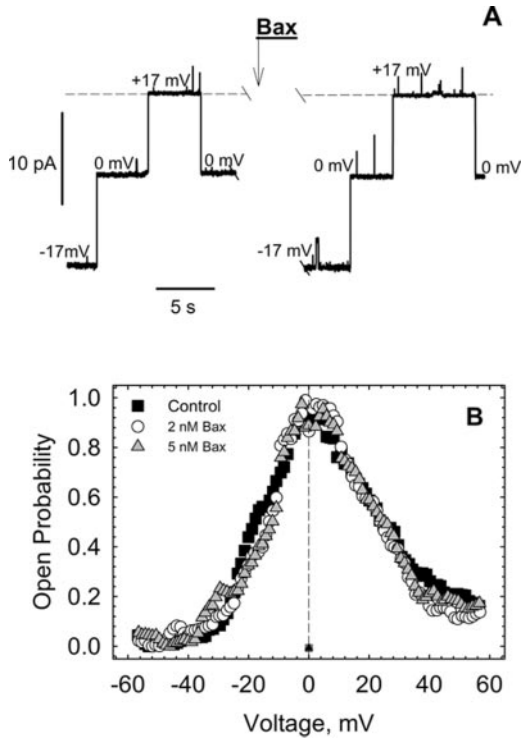

FIG. 4. **Oligomeric Bax has no effect on the conductance, selectivity, or voltage gating properties of VDAC channels.** *A*, the traces show the current through the same single VDAC channel before and after addition of 60 nM oligomeric Bax. Oligomeric Bax was obtained by preincubation of monomeric Bax with 1% octyl glucoside. The reversal potential was equal to +17 mV before and after Bax addition. The single channel conductances were 0.53 and 0.59 nS at positive and negative applied potentials, respectively, and did not change in the presence of Bax. Current records were filtered using an averaging time of 5 ms. *B*, oligomeric Bax (isolated in the presence of detergent) was added to the membrane containing 30 VDAC channels. Each data set is an average of current records in response to five symmetrical 3-mHz triangular voltage waves (±60 mV of amplitude). Open probability is defined as the ratio $(G - G_{min})/(G_{max} - G_{min})$, where G_{max} and G_{min} are the maximum and minimum conductances, respectively. Only the part of the wave during which the channels were reopening was used for subsequent analysis. The membranes were made from asolectin/cholesterol. The medium consisted of 250 mM NaCl (*B*) or 250 mM NaCl in the *cis*-side and 50 mM NaCl in the *trans*-side (*A*) and 1 mM $MgCl_2$ buffered with 5 mM MES (pH 5.5).

these effects into account, the *G*/V plot is expressed as a normalized conductance with the voltage-independent portion subtracted (this would include any Bax conductance). This plot is essentially an expression of the voltage dependence of the open probability. Fig. 4*B* shows the results of a typical experiment. There was no effect of Bax on VDAC voltage dependence. It should be mentioned that, in the experiment shown in Fig. 4*A*, oligomeric Bax was obtained by preincubation of monomeric Bax with 1% octyl glucoside and that, in the experiment presented in Fig. 4*B*, the oligomeric Bax sample was isolated in the presence of detergent. That is why the concentration of Bax used in the experiment shown in Fig. 4*A* was much higher (60 nM) than those used in the *G*/V plot analysis (2 and 5 nM Bax) (Fig. 4*B*). As summarized in Table I, neither monomeric nor oligomeric Bax had any detectable effect on VDAC voltage dependence.

VDAC and Cut Bid—It was recently reported that cardiolipin is required for the binding of tBid to mitochondria (47). We found that, in the presence of cardiolipin (8%), cut Bid in the concentration range of 200–500 nM could induce stable channel-like conductances of ~1–7 nS (in 40% of the experiments). In some experiments, addition of cut Bid caused membrane rupture, as described previously (17). An example of the current traces induced by cut Bid is shown in Fig. 2*d*. It should be noted that cut Bid formed channels at concentrations ~10 times higher than oligomeric Bax (Fig. 3, *closed circles*). Bid was proteolytically cut with caspase-8 (37, 38), resulting in two fragments: a 15.5-kDa C-terminal fragment (t^{c}Bid) and a 6.5-kDa N-terminal fragment. We found that, in membranes containing cardiolipin, t^{c}Bid formed channels at concentrations that were lower (20–100 nM) (Fig. 3, *open circles*) than the concentrations at which cut Bid channels were observed. This is consistent with the fact that cut Bid contains the N-terminal Bid fragment, which has been shown to have an inhibitory effect on the ability of t^{c}Bid to increase membrane conductance (17). Thus, the t^{c}Bid fragment possesses channel-forming activity (Fig. 2*e*). To verify that the C-terminal fragment of cut Bid is also responsible for the effect of cut Bid on VDAC channel, a few experiments were done with only the t^{c}Bid fragment.

Fig. 5*A* illustrates the typical current recording from a single VDAC channel before and after addition of cut Bid. After 20 min of continuous recording of the single VDAC channel, cut Bid was added to the *cis*-side starting with 10 nM. The single channel current was recorded for 20 min, followed by the second addition of cut Bid at a final concentration of 20 nM. Twenty minutes after the second addition of cut Bid, channel conductance dropped by approximately two-thirds of its open state level. Application of 0 mV or a change in the polarity of the applied potential did not reopen the channel. The channel was "locked" in its low conductance state. The same effect is clearly illustrated by another experiment shown in Fig. 5*B*, in which a triangular voltage wave was applied to a single channel membrane. Sample traces show the conductances of open and closed states (slope of the trace, indicated by *dotted lines* in Fig. 5*B*) and transitions between states as the voltage was varied between ±60 mV. Fifteen minutes after addition of 10 nM cut Bid to both sides of the membrane, channel conductance dropped, and the channel did not gate in response to the applied voltage.

Addition of cut Bid to a membrane containing many VDAC channels usually increased the voltage-independent portion of the conductance. This is most likely the result of the formation of cut Bid channels. This often gave an increase in overall conductance that tended to overshadow the effect of cut Bid on VDAC. Addition of cut Bid increased membrane conductance (both G_{min} and G_{max}), but decreased the difference between the conductance at a given potential (G) and the minimum conductance (G_{min}) (Fig. 6*A*). This observation is analogous to what was observed with single channels. The closure of the channels in the presence of cut Bid was observed in 18 of 23 experiments both on single and multichannel membranes. The analysis of the effect of different cut Bid concentrations on the relative portion of closed VDAC channels is presented in Fig. 6*B*. The difference $(G - G_{min})$ obtained from the *G*/V plots as in Fig. 6*A* was normalized over G_{min}. The difference between the voltage-dependent portion of the conductance with and without cut Bid in the same experiment is expressed as a percentage of change in $(G - G_{min})/G_{min}$. Bid increased the voltage-independent portion of the conductance in a dose-dependent manner (Fig. 6*B*).

In parallel to the closing effect, cut Bid stimulated insertion of additional VDAC channels when it was added to multichannel membranes (in 75% of the experiments with multichannel membranes); and vice versa, the presence of VDAC enhanced cut Bid insertion. This effect is illustrated in Fig. 7 with the t^{c}Bid fragment. The initial current-voltage dependence of the membrane containing 20 VDAC channels was sublinear (Fig. 7, *curve 1*) as a result of channel closure at high voltages. After addition of 20 nM t^{c}Bid to the both sides of the membrane, the

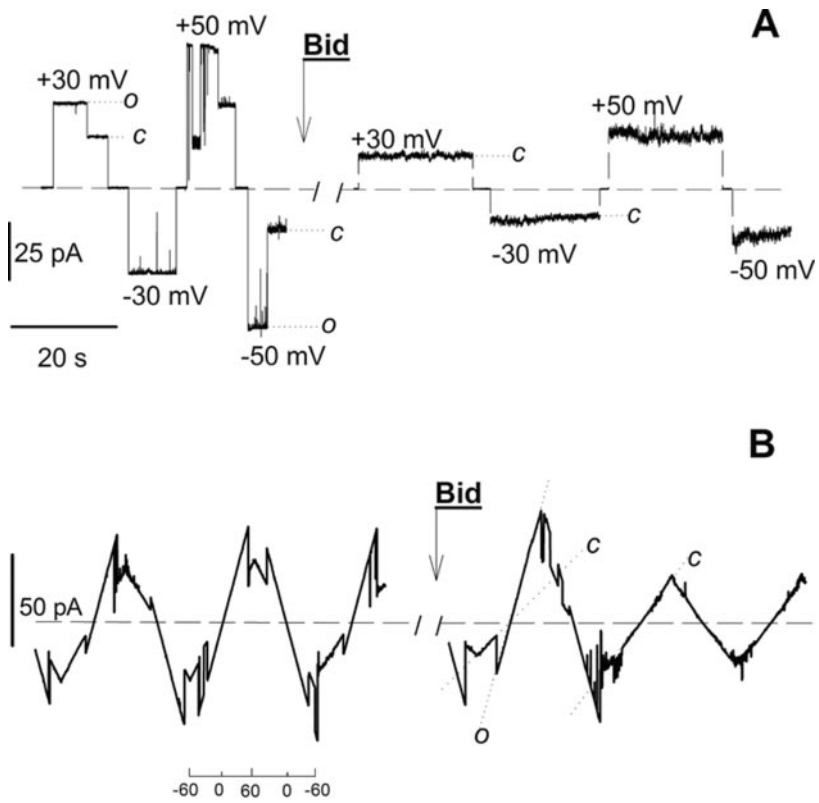

FIG. 5. **Bid induces the closure of a single VDAC channel.** *A*, the traces show the current through the same single VDAC channel in the absence and presence of 20 nM cut Bid in the *cis*-side. Cut Bid was added in 10 nM steps, and the *arrow* indicates the second step addition. Current records were filtered using an averaging time of 2 ms. *B*, triangular voltage waves (±60 mV, 5 mHz) were applied to the membrane containing a single VDAC channel before and after addition of 10 nM cut Bid. The medium consisted of 250 mM KCl and 5 mM $CaCl_2$ buffered with 5 mM HEPES (pH 7.2). The membranes were made from asolectin/DPhPC/cardiolipin/cholesterol. The *dotted lines* indicate the conductances of VDAC channel in its open (*o*) and closed (*c*) states.

current rapidly increased by ~8-fold (Fig. 7, *curve 2*), and the current-voltage dependence became almost linear. The rise in membrane conductance could be due to the insertion of additional VDAC channels and/or to the formation of Bid channels. It should be noted that, in control experiments with t^cBid alone, 20 nM t^cBid increased the conductance by only ~1–2 nS (Fig. 3), which is low compared with the increase of 70 nS in Fig. 7. Cut Bid channels do not possess voltage gating properties; therefore, superimposition of VDAC and cut Bid channels and the closing effect of cut Bid on VDAC are expected to yield a more linear *I*/V dependence. In an attempt to separate the two types of conductances, we inhibited the Bid-induced channels with 100 μM La^{3+}. Although the mechanism of La^{3+} blockage is not understood, this trivalent ion has been used to inhibit ceramide channels (48) and VDAC channels isolated from mammalian mitochondria (49). We found that micromolar La^{3+} inhibited Bid-induced conductances and that EGTA reversed this effect (data not shown). In contrast with VDAC channels from rat liver mitochondria, VDAC channels isolated from *N. crassa* were not closed by La^{3+}, and their open probability was even increased (data not shown). After addition of 100 μM $LaCl_3$ to the membrane containing *N. crassa* VDAC and t^cBid (Fig. 7, *curve 2*), the current abruptly dropped, and the gating properties were almost restored (*curve 3*). The reduced conductance after La^{3+} addition was still significantly higher than in control. This residual conductance was most likely induced by additionally inserted VDAC channels, and Bid was responsible for the portion of the conductance inhibited by La^{3+}. Addition of 400 μM EGTA resulted in a fast rise in membrane current, loss of voltage gating behavior, and rapid membrane rupture.

DISCUSSION

VDAC and Bax—Our experiments suggest that Bax does not form a new large pore with VDAC. Moreover, a detailed analysis of the characteristic properties of VDAC channels such as voltage gating, ion selectivity, and single channel conductance did not show any change after Bax addition regardless of lipid composition, medium pH, or ionic content (Table I). We conclude that there is no functionally detectable interaction between VDAC channels isolated from mammalian mitochondria and either monomeric or oligomeric Bax.[2] This is consistent with the failure to detect Bax-VDAC interaction by immunoprecipitation or cross-linking (50) and with the insensitivity of the effect of Bax expressed in yeast cells to the knockout of VDAC (51).

Thus, in contrast to the publications of Shimizu *et al.* (1–3), we did not observe the formation of a new large pore when monomeric full-length Bax was added to mammalian VDAC channels reconstituted into planar phospholipid membranes. The high membrane conductance *per se* is not sufficient evidence of the formation of a large pore. As we show, the conductance increase is a result of many smaller pores opening due to insertion of additional VDAC channels following monomeric Bax addition. It should be mentioned here that, contrary to what has been reported (2), it is well documented now that monomeric full-length Bax has a highly compact conformation (34), does not penetrate into the membrane, and does not form channels (11, 45, 52).

However, in some cells, monomeric Bax has been found to be loosely attached to MOM (53). A recent study has shown that purified monomeric Bax_{FL} undergoes a reversible conformational change upon interaction with the lipid surface of liposomes in the absence of other proteins (54). Neither insertion into membranes, oligomerization, nor pore formation by monomeric Bax was found. Therefore, it is possible that monomeric Bax could be adsorbed on the surface of planar phospholipid membranes. Monomeric Bax molecules adsorbed or attached to the membrane surface might stimulate the insertion of additional VDAC channels. The acceleration of insertion of VDAC channels by themselves and by other channel formers was

[2] From two separate laboratories (R. J. Y. and B. A.).

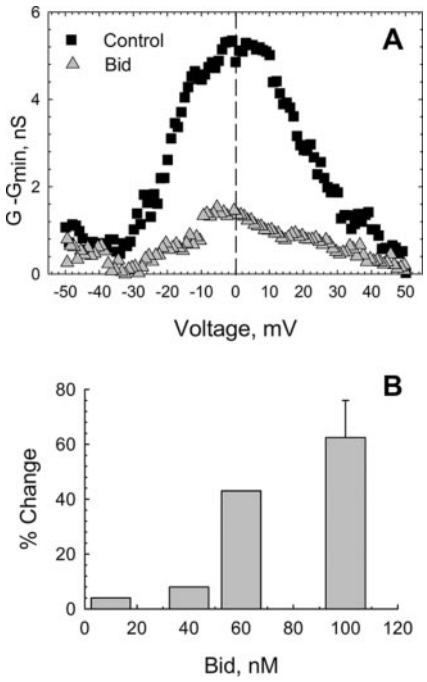

FIG. 6. **Bid promotes the closed configuration of VDAC channels.** *A*, 100 nM cut Bid was added to the membrane containing 10 VDAC channels. Each data set is an average of currents recorded in response to 6–10 symmetrical triangular voltage waves (±50 mV, 5 mHz). The difference between the conductance (*G*) and the minimum conductance (G_{min}) is graphed in the absence (*Control*) and presence of Bid. The experimental conditions were the same as described in the legend to Fig. 4. *B*, the voltage-independent portion of the conductance increased with the cut Bid concentration. The data were obtained from individual experiments with different concentrations of Bid. *G*/V plots were expressed as described for *A* and normalized over G_{min}. The percentage of change is a normalized difference $((G - G_{min}) \times 100/G_{min})$ obtained with and without Bid in the same experiment.

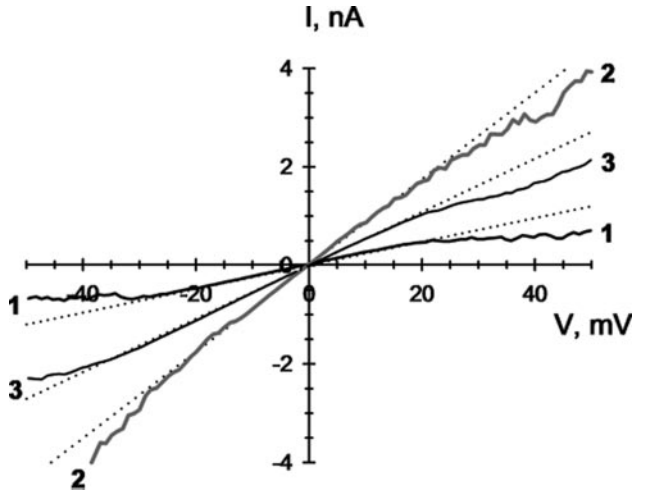

FIG. 7. **Multichannel membrane conductance increases in the presence of Bid.** Shown are current-voltage plots before (*curve 1*) and after (*curve 2*) addition of 20 nM t^{c}Bid to the membrane containing 20 VDAC channels (*N. crassa*). Addition of 100 μM $LaCl_3$ inhibited the conductance induced by Bid alone (*curve 3*). The experimental conditions were the same as described in the legend to Fig. 4.

described previously (55, 56). The insertion and channel-forming activity of membrane proteins greatly depend on the physical properties of the membrane interface and protein-lipid interaction (for review, see Ref. 57). It should be noted that Bax-initiated insertion of the additional VDAC channels was observed only under conditions in which there was an excess of VDAC protein in the surrounding membrane solution and, most likely, on the surface of the membrane. Whenever excess VDAC was removed from the *cis*-chamber by perfusion prior to Bax addition, no additional VDAC channels insertion was observed. Therefore, it seems that the accelerated insertion of channels is a particular feature of protein reconstitution into planar membranes and could be irrelevant to the *in vivo* situation, in which most membrane proteins are delivered to the mitochondrial membranes in the form of precursors (58).

Oligomeric Bax induces channels in lipid membranes. This fact makes the study of the effect of oligomeric Bax on VDAC channel properties rather challenging. There is a practical upper limit to the concentrations of oligomeric Bax that can be used in electrophysiological experiments with VDAC channels because the properties of the combined conductances are difficult to interpret. Therefore, we still cannot rule out the possibility that VDAC might interact with oligomeric Bax at concentrations higher than those at the threshold of Bax channel-forming activity. Although hypothetically possible, this seems to be biologically irrelevant because once oligomeric Bax has formed channels in MOM, it inevitably would lead to MOM permeabilization and cytochrome *c* release independent on whether VDAC is involved or not. One of the purposes of our experiments with channel-forming oligomeric Bax was to investigate whether VDAC can accelerate the ability of Bax to form pores and thus work upstream of the formation of Bax channels.

It has been calculated that the concentration of monomeric Bax in neural cells ranges from 2.2 to 3.7 nM (52). An oligomeric Bax concentration of 2 nM was the lowest used in our experiments (Fig. 3 and Table I). Therefore, most of our experiments were performed at Bax concentrations that were well above those expected *in vivo*.

VDAC and Bid—We have shown here that cut Bid induces closure of VDAC channels both on single and multichannel membranes in a dose-dependent manner. Cut Bid and t^{c}Bid formed channels in planar membranes (Fig. 2). Unlike oligomeric Bax and t^{c}Bid, cut Bid induced channels at higher concentrations (>200 nM) (Fig. 3). Therefore, 100 nM cut Bid used in multichannel experiments (Fig. 6) is still below the threshold at which it increases the membrane conductance. However, it is unclear why in multichannel membranes cut Bid at concentrations >20 nM did not close the entire population of VDAC channels, as expected from its effect on single VDAC channels (Fig. 5). One of the tentative explanations is that VDAC channels could be organized into clusters in the lipid matrix, and this would restrict the access of cut Bid to channels inside the cluster. This could take place if cut Bid interacted with VDAC following cut Bid translocation into the membrane plane.

The mechanism by which Bid alters the gating properties of VDAC remains to be understood. There are indirect indications that König polyanion inhibits tBid-induced cytochrome *c* release by acting through VDAC (59). However, a physical interaction of VDAC with tBid (as well as with Bax) has never been demonstrated. Based on our observations, we suggest that cut Bid most likely affects VDAC following cut Bid insertion into the lipid membrane. It is unclear whether these two proteins physically associate within the membrane or whether cut Bid affects VDAC indirectly through the lipid environment surrounding the proteins (57). It has been shown that tBid promotes negative membrane curvature and, as a result, destabilizes bilayer membranes (60). Therefore, cut Bid might insert into the membrane, alter the local lipid environment in close proximity to VDAC, and induce VDAC closure indirectly. Indirect protein-protein interactions involving membrane lipids were recently demonstrated for a model system of gramicidin channels (61). Whether cut Bid alters VDAC gating properties by direct or indirect interactions, this most likely occurs within

the lipid membrane, and its lipid composition may influence these interaction.

As demonstrated previously, in the closed state, the ability of VDAC to pass small negative charged metabolites such as phosphocreatine, succinate, and HPO_4^{2-} is compromised (21, 27), and VDAC in its closed state is essentially impermeable to ATP (19, 20). Therefore, by decreasing the probability of VDAC opening, cut Bid would reduce the flux of adenine nucleotides and other negatively charged metabolites across MOM, which may affect mitochondrial functions in different ways. One of the possible consequences of VDAC channel closure is the mechanism proposed by Vander Heiden *et al.* (27, 28), according to which the disruption of metabolite exchange across MOM could result in the accumulation of small metabolites in the intermembrane space, followed by mitochondria swelling and consequent loss of MOM integrity. It has also been shown that the anti-apoptotic protein Bcl-x_L favors the open state of VDAC and can maintain the permeability of MOM to ADP and ATP (27). Our data support this model, in which Bcl-2 family proteins control MOM permeabilization by regulating the opening or closure of VDAC channel. However, a number of recent studies show the absence of mitochondrial swelling and intactness of MOM during apoptosis (8). The permeabilization of MOM is a very intricate process, in which several proteins and many factors play a substantial role (for review, see Ref. 62). We propose that the regulation of VDAC gating by Bcl-2 family proteins is part of the process.

Cardiolipin has been shown to play a crucial role in Bid targeting of phospholipid membranes (47, 63) and in promoting formation of large pores by cut Bid and monomeric Bax (64). Cardiolipin has a defined distribution pattern within mitochondria. It is found in high concentrations throughout the inner membrane and at contact sites (17–20%) (65, 66). In the outer membrane, cardiolipin is present at much lower concentrations (0.3–4%) (65, 67). A recent study confirmed the preferential association of tBid with mitochondrial contact sites, which might be enriched with cardiolipin (63). VDAC and the adenine nucleotide transporter are the major components of the permeability transition pore (PTP) thought to be involved in cytochrome *c* release. It has been suggested that PTP components, including VDAC, reside at the contact points between inner and outer mitochondrial membranes (68). Therefore, VDAC, tBid, and cardiolipin are likely to be co-localized in the contact points. Based on this, we may alternatively suggest that tBid could regulate the PTP complex by regulating fluxes of ADP and ATP, the inhibitors of PTP, through VDAC. The relationships between VDAC and the adenine nucleotide transporter within the PTP complex are unknown so far and are very speculative. Therefore, it is hard to predict how VDAC closure would affect PTP functioning. An alternative model in which VDAC and cut Bid interaction might be involved is related to the recently reported reorganization of the cristae induced by tBid (69). It has been proposed that the pro-apoptotic activity of tBid has two parallel pathways: one mediates release of cytochrome *c* across MOM and requires homo-oligomerization of Bax or Bak, and the another initiates mitochondrial cristae remodeling and cytochrome *c* mobilization in the intermembrane space. This structural reorganization of the junctions between the intercristae and the intermembrane space was shown to be PTP-dependent. Thus, the second pathway involves physical or functional interactions of tBid with PTP proteins.

On the other hand, the role of cardiolipin could be simply to affect the structure of the lipid bilayer in a way that makes it more accessible for cut Bid. Similarly, VDAC channels preexisting in the membrane could also influence the efficiency of cut Bid insertion. In other words, each of the channel-forming proteins could affect the other and be influenced by the physical properties of the lipid bilayer (57, 61). This could explain the mutual interactions observed on multichannel membranes when addition of cut Bid stimulated insertion of additional VDAC channels, and vice versa, VDAC channels incorporated into the membrane promoted insertion of Bid channels.

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