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Bax increases the pore size of rat brain mitochondrial voltage-dependent anion channel in the presence of tBid

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Abstract

Voltage-dependent anion channel (VDAC), Bax, and tBid play a central role in apoptosis regulation but their functioning is still very controversial. VDAC forms voltage gated pore in planar lipid bilayers, and acts as the pathway for the movement of substances in and out of the mitochondria by passive diffusion. Here we report that there is increase in the pore size of VDAC in the presence of Bax and tBid through bilayer electrophysiological experiments. We hereby hypothesize that this increase in pore size might cause swelling in the mitochondria, leading to the rupture of mitochondrial outer membrane and release of cytochrome *c* causing brain cell death.

Keywords: Brain VDAC; Bax; tBid; BLM; Electrophysiology; Apoptosis

Apoptosis is critical for normal nervous system development and is tightly regulated by an evolutionary conserved molecular program [1]. It is also essential for tissue homeostasis and elimination of harmful animals in metazoan animals [2,3]. There are several hypotheses regarding the mechanism of apoptosis. Some of these say that, voltage-dependent anion channel (VDAC) is involved in apoptosis. VDAC is an abundant protein in the outer mitochondrial membrane, which forms large voltage gated pore (2.5–3 nm) in planar lipid bilayers, and acts as the pathway for the movement of substances in and out of the mitochondria by passive diffusion [4]. There are many reports, which say that Bax and Bid proteins are the key molecules involved in cell death. Bax and Bid are the members of the Bcl-2 family of proteins, which are well-characterized regulators of apoptosis [5,6].

One of the models proposes that the Bax interacts with VDAC resulting in cytochrome *c* permeation through the outer mitochondrial membrane [7,8]. Cytochrome *c*, which normally resides exclusively in the intermembrane space of mitochondria, is released into cytosol during apoptosis [9]. Release of cytochrome *c* from mitochondria inactivates the electron transfer chain and triggers caspase activation through Apaf 1 [10,11]. Caspases, a group of cysteine proteases that cleave protein substrates after the aspartic acid residues, play a central role in regulation and execution of apoptosis [12]. Caspases that function in the apoptotic pathway exist as inactive zymogens in the cytosol of living cells and become activated through proteolysis when cells receive apoptotic signals.

Another model proposes that closure of VDAC channel prevents the efficient exchange of ATP and ADP between the cytosol and mitochondrial matrix [13]. Loss of the 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 by promoting the open configuration of the VDAC channel, shown in planar bilayer experiments [14].

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Also, activated or truncated Bid (tBid) is a key regulatory component of the apoptotic machinery [15]. Bid usually exists in an inactive form in the cytosolic fraction of living cells. Activation of cell surface death receptors (Fas) leads to rapid activation of caspase-8 that cleaves and activates the Bid protein, which is known to interact with both Bcl2 and Bax through its BH3 domain [16]. One of the models states that tBid interacts with full-length monomeric Bax, but it is not enough for the release of cytochrome *c* [17]. In another model it has been reported that tBid/Bax oligomers may themselves form channels in the outer membrane or they could interact with other outer membrane proteins like VDAC to induce cytochrome *c* release [18].

On the contrary, it has been recently reported that properties of VDAC channels isolated from mammalian mitochondria are unaffected by addition of Bax in the planar lipid bilayer and that Bax does not induce cytochrome *c* release by acting on VDAC [19]. It was also shown that tBid induces closure of VDAC channels and reducing the permeability of VDAC channels it might interfere with metabolite exchange between mitochondria and the cytosol with the subsequent loss of outer membrane integrity.

The role of VDAC, Bax, and tBid in the above-mentioned models is still very controversial. In order to resolve this controversy, we have explored the role of tBid and Bax in the gating of VDAC through electrophysiological experiments. In the present work we have shown that there is an increase in the channel conductance (VDAC) after addition of Bax and tBid through bilayer electrophysiological experiments.

Materials and methods

Purification of VDAC. VDAC was purified from rat brain mitochondria using the method of De Pinto et al. [20]. Recombinant full-length monomeric Bax and tBid were obtained from Professor Jean-Claude Martinou, Department de Biologie Cellulaire Sciences, Ernest-Ansermet, Geneva, Switzerland, as a gift.

Reconstitution of VDAC in planar lipid bilayers and electrophysiological recording. VDAC was reconstituted into the planar lipid bilayers according to the method of Roos et al. [21]. Briefly, the apparatus consisted of a polystyrene cuvette (Warner Instrument, USA) with a thin wall separating two aqueous compartments containing 500 mM KCl, 5 mM $MgCl_2$, and 10 mM Hepes (pH 7.4). The polystyrene divider had a circular aperture with a diameter of 150 μm. Aqueous compartments were connected to an integrating patch amplifier (Axopatch 200 A, Axon Instruments) through a matched pair of Ag/AgCl electrodes. The *cis* chamber was connected to headstage (CV-201) of the amplifier and the *trans* chamber was held at virtual ground. A 1% solution of DPhPC in *n*-decane was painted over the aperture to form the membrane. Reconstitution of VDAC in BLM was initiated by adding 2 μl of VDAC (2–5 ng protein dissolved in 1% Triton X-100, 10 mM Hepes, pH 7.4) to the *cis* chamber. Channel current was recorded at full bandwidth on a videocassette recorder after being digitized through an analog-to-digital converter (VR-10B, Instrutech, New York). Data were filtered through an 8-pole Bessel filter (Frequency Devices, USA) and sampled at a rate greater than the corner frequency, using an ITC-16 interface (Instrutech, USA) connected to a Macintosh computer (Apple Computer, USA). Single channel recording of VDAC was performed in a symmetric bath solution.

Addition of Bax and tBid. After getting the single channel recording of VDAC the solution in the *cis* chamber is removed and fresh buffer is added. Now full-length monomeric Bax protein is added to the *cis* chamber and stirred for 5 min. The concentration of Bax was varied from 10 to 100 nM. Current traces were recorded after an interval of 30 min for 3 h. Then tBid was added to the same chamber in the ratio of 1:20 for tBid:Bax as per the earlier report about the amount of tBid required for activation of Bax [17] under constant stirring (for 5 min) and similar recording was performed after incubation of 30 min.

Analysis of electrophysiological data. Steady state conductance (current/voltage) of VDAC was calculated from the single channel current data using the software Axograph (Axon Instruments, USA). Similarly conductance was measured after addition of Bax and tBid. Finally, a comparison was made for the conductance values of native VDAC, VDAC + Bax, and VDAC + Bax + tBid.

Results

Purified rat brain mitochondrial VDAC, when reconstituted in a planar lipid membrane, showed voltage-dependent gating. Fig. 1A shows the single channel current traces of native VDAC. After getting the single channel recording of VDAC, the solution in the *cis* chamber was perfused out and fresh solution was added to avoid any further insertion of VDAC molecule. Now full-length monomeric Bax was added to the chamber, but no change in the single channel current was observed even after 3 h of addition of Bax (Fig. 1B). Even after the concentration of Bax was gradually varied (from 10 to 100 nM) there was no change in the channel conductance. The experiment was repeated at various potentials but the observations were the same. Our data are consistent with the results observed by Rostovtseva et al. [19] who showed that VDAC incorporated in planar lipid membrane is unaffected by Bax. After addition of tBid to the same chamber a significant increase in the current was observed (Fig. 1C).

Here we have not used cardiolipin in the formation of bilayer to avoid the insertion of tBid into the membrane and formation of channel as reported previously [19]. It has also been reported that addition of tBid to the membrane containing cardiolipin caused membrane rupture [22]. In our experiments membrane rupture was not observed supposedly due to absence of cardiolipin. So the increase in the channel current after addition of tBid is because of the increase in the pore size of the VDAC, and not due to the channel forming activity of tBid.

Further, analysis of the current recordings has revealed that there is no significant difference in the conductance values of VDAC before and after addition of Bax. But after addition of tBid there is a large increase in the channel conductance as shown in Fig. 2. As an example, conductance increased from 4.024 nS (native VDAC) to 19.12 nS (after addition of tBid) at +25 mV applied potential.

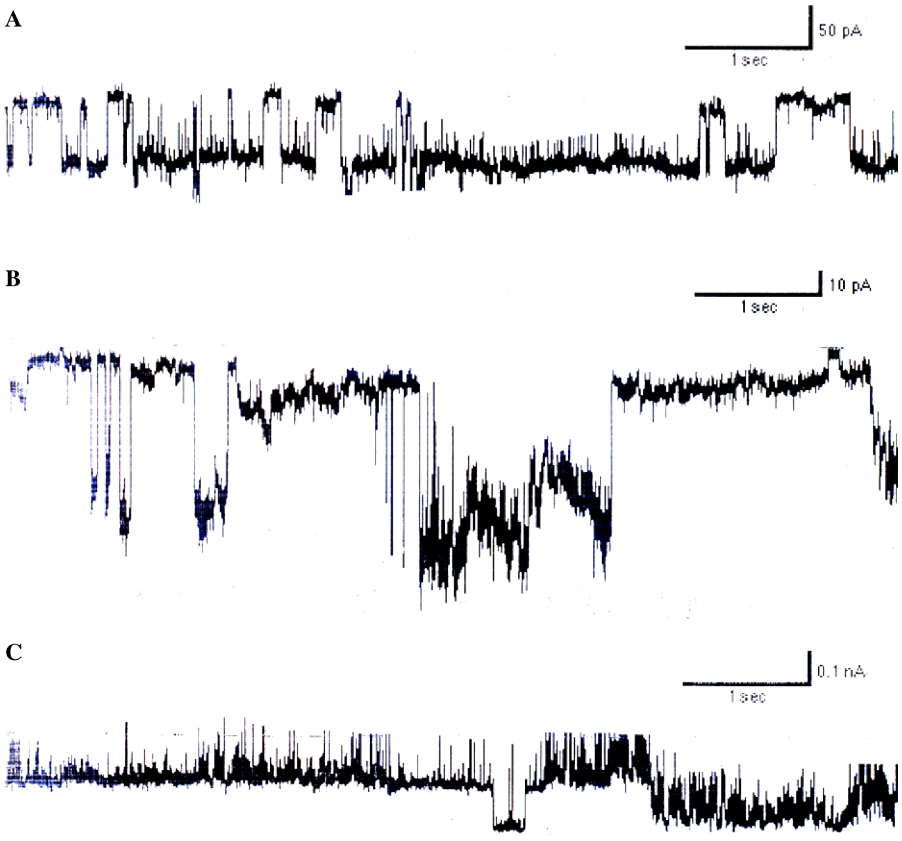

Fig. 1. Continuous current traces at +25 mV of (A) native VDAC, (B) VDAC + Bax, and (C) VDAC + Bax + tBid. The medium consisted of 500 mM KCl, 10 mM Hepes, and 5 mM $MgCl_2$ (pH 7.4).

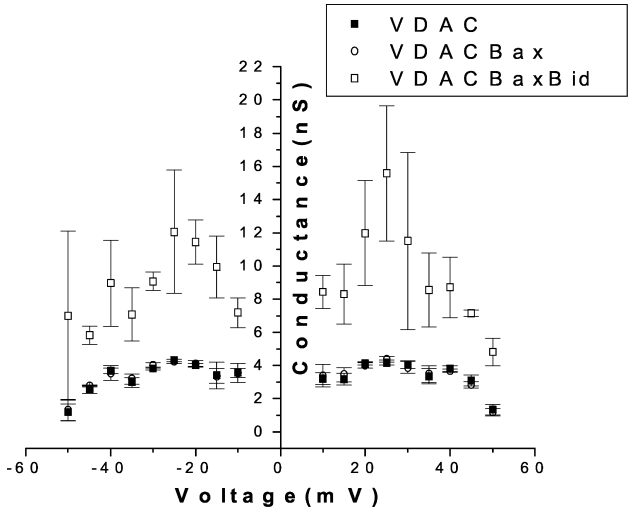

Fig. 2. Conductance profile of native VDAC, VDAC + Bax, and VDAC + Bax + tBid. As shown conductance vs. voltage pattern in each case is double Gaussian. In case of VDAC and VDAC + Bax the plots are coinciding and the variation of conductance in different experiments is 2–3%. Conductance of VDAC + Bax + tBid has large fluctuations in all voltages (up to 28%).

Discussion

Our results on both current and conductance as described in results show that rat brain mitochondrial VDAC forms a large voltage gated pore in the presence of full-length monomeric Bax and tBid. Just after addition of Bax there was no change in the single channel conductance. It is well established that Bax interacts with VDAC and the protein–protein binding site for this interaction is also known [23]. As per our results interaction of VDAC and Bax takes place without changing the gating of the channel. This is also supported by our finding that there is a change in the noise profile of VDAC after addition of Bax. The power spectrum of the native VDAC is following power law and shows $1/f$ noise pattern that fails after addition of Bax indicating interaction (data not shown).

The increase in the conductance value at a particular voltage, after addition of tBid, is not consistent in all the experiments; the fluctuation in conductance is very high (10–28%) after addition of tBid as obvious from Fig. 2. However, single channel conductance of tBid treated VDAC-Bax vs. voltage plot still follows a double Gaussian pattern as in the cases of native VDAC and Bax treated VDAC (Fig. 2). Here after addition of Bax and tBid there must be some kind of structural modification occurring in VDAC that leads to an increase in the pore size. Structural modifications are likely to affect the pattern of gating, hence it is expected to throw some light on the gating mechanism of VDAC. We have demon-

strated earlier that VDAC can be phosphorylated in vitro by protein kinase A, leading to partial closer of the negative gate of the channel [24]. Recently, we have shown that plasminogen protein interacts with VDAC from rat brain causing partial closure of the channel, which might be a mechanism of brain cell death [25]. However, structural mechanism by which the tBid is inducing Bax to increase the pore size of VDAC is still unclear and needs to be studied in detail.

There is evidence that cardiolipin is important in apoptosis [26]. The major fraction of cardiolipin in cell is present on the inner leaflet of the inner membrane of mitochondria, although some cardiolipin has been reported to be present in the outer mitochondrial membrane and at contact sites where inner and outer membranes appear to join [27]. It is well known that cardiolipin is essential for the binding of tBid to the mitochondrial membrane [28]. As in our experiments cardiolipin is not used, the increase in the current is not due to the channel formation by tBid. The only alternative is increase in the pore diameter of VDAC induced by tBid.

The present study shows that the interplay of VDAC, Bax, and tBid is simultaneously required for the change in the gating of VDAC molecule, which might be a possible mechanism of cell death. As reported earlier, activation of Fas leads to rapid activation of caspase-8, which then cleaves and activates Bid. The COOH-terminal part of Bid (tBid) then translocates onto mitochondria and triggers cytochrome *c* release to the cytosol [15]. Based on our findings in the bilayer membrane experiments we hereby propose that tBid along with Bax interacts with VDAC and forms a large pore. This will cause swelling in the mitochondria and finally rupture the outer mitochondrial membrane, thereby releasing cytochrome *c* and other apoptogenic molecules into the cytosol leading to brain cell death.

Our finding will throw light on the ongoing controversy surrounding the role of VDAC, Bax, and tBid in cell death and their interdependence on each other. The change in the gating of VDAC in the presence of Bax and tBid is an important phenomenon for studying the problems related to disorders caused by cell death. Regulation of this tBid and Bax induced increase in pore size of VDAC will be an important therapeutic target for various neurological dysfunctions caused by brain cell death and needs to be verified in detail.

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