Biochemical and Biophysical Research Communications 463 (2015) 751–755

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Bax and Bif-1 proteins interact on Bilayer Lipid Membrane and form pore

Rajeev Gupta, Subhendu Ghosh*

Department of Biophysics, University of Delhi South Campus, New Delhi, India

ARTICLE INFO

Article history:
Received 5 May 2015
Accepted 2 June 2015
Available online 4 June 2015

Keywords:
Bax
Bif-1
Bilayer electrophysiology
Pore
Mitochondria-mediated apoptosis

ABSTRACT

Bax and Bax interacting factor-1(Bif-1) are cytosolic proteins, which translocate towards mitochondria during mitochondria-mediated apoptosis. Bif-1 has been identified to co-immunoprecipitate with Bax in apoptotic cells. We have studied the interaction of Bax and Bif-1 on Bilayer Lipid Membrane (BLM) through electrophysiological experiments. It has been observed that Bax-Bif-1 equimolar mixture can form a pore. The pore conductance is in the range of 4.96–5.41 nS. It also displays a sub-state with a conductance of 2.6 nS. No pore activity is observed on BLM when monomeric Bax and Bif-1 proteins are tested independently. The above-mentioned pore forming activity could be relevant in mitochondria-mediated apoptosis.

1. Introduction

Bax is a proapoptotic protein which resides in an inactive form in cytosol and after activation it gets translocated to mitochondria and play an important role in mitochondria-mediated apoptosis [1,2]. Activated Bax either in homo-oligomeric form or as complex with other proteins associated with mitochondrial outer membrane, e.g. Voltage-Dependent Anion Channel, truncated Bid (tBid) protein, form pores on the outer mitochondrial membranes [3–7]. These pores promote the leakage of ions, essential metabolites and cytochrome c from mitochondrion to cytosol leading to cell death [8]. Bax has been shown to get activated after its interaction with different cytosolic proteins like tBid, Puma etc. [9–11]. One of the newly identified factors which have been proposed for Bax activation is Bax interacting factor-1 (Bif-1). Bif-1 (also known as Endophilin B1) is a cytosolic protein which has been shown to translocate towards mitochondria and co-immunoprecipitate with Bax protein in cell cultures undergoing mitochondria-mediated apoptosis [12,13]. It contains an N-terminal N-BAR (Bin-Amphiphysin-Rvs) domain and a C-terminal SH3 (Src Homology 3) domain that are important for its roles in apoptosis and autophagy [12,14]. Using atomic force microscopy N-BAR domain of Bif-1 protein has been shown to perturb the structure of lipid bilayers [15]. At a low concentration it has been shown to cause bilayer thinning and at higher concentration it forms thin silvers from bilayer sheet [15]. It also binds to liposomal lipid bilayers and self-oligomerize to deform them into tubules [16,17]. Thus, Bif-1 can interact with lipid bilayers. Reports on the interaction of Bax with Bif-1 are diverse. The N-BAR domain of Bif-1 is required for its ability to interact with Bax and promote mitochondrial outer membrane permeabilization [18]. Bif-1 has been shown to activate Bax by inducing its conformational rearrangement [18]. Some believe Bif-1 activates Bax and causes apoptosis [19], while others believe that Bax activates Bif-1 oligomerization and oligomeric Bif-1 might cause apoptosis by inducing outer mitochondrial membrane vesiculation [20]. Thus, the two proteins play an important role in apoptosis. However, the exact mechanism by which Bax and Bif-1 promote mitochondria-mediated apoptosis is not very clear till date. One of the principal ways could be by forming pores/ion channels on the outer mitochondrial membrane. Keeping this in view we have investigated the possibility of formation of pore by Bax-Bif-1 mixture on Bilayer Lipid Membrane (BLM).

2. Materials and methods

Recombinant full length Monomeric GST-Bax protein MW ≈ 49 kDa (SignalChem Company, USA) and Monomeric GST-Bif-1 full length protein MW ≈ 69 kDa (Novus Biologicals, USA) were purchased and used for BLM experiments.

Abbreviations: Bax protein, Bcl-2-associated X protein; Bif-1, Bax interacting factor-1; BAR, Bin-Amphiphysin-Rvs; BLM, Bilayer Lipid Membrane; GST, glutathione-S-transferase.

* Corresponding author.
E-mail address: profsubhendu@gmail.com (S. Ghosh).

http://dx.doi.org/10.1016/j.bbrc.2015.06.007
0006-291X/

3. Reconstitution of proteins on BLM

Reconstitution was done onto BLM by standardized method [4,21,22]. Briefly, perfusion BLM Cup (Warner Instruments Corp., Hamden, CT, USA) made up of polystyrene with a circular aperture of 150 μm diameter was used to separate two compartments (cis and trans) of the BLM chamber. The two compartments were filled with BLM buffer [1 M KCl, 10 mM $MgCl_2$, 10 mM HEPES (pH 7.4)] and connected to an integrating patch clamp amplifier (Axopatch 200B, Axon Instruments, Sunnyvale, CA, USA) using a matched pair of Ag/AgCl electrodes. The cis compartment was connected to the headstage (CV203BU) of the amplifier and the trans compartment was held at virtual ground. BLM was prepared by applying DPhPC lipid (1,2-DiPhytanoyl-*sn*-glycero-3-Phosphocholine) (Avanti Polar Lipids, Alabaster, AL, USA) in *n*-decane to the BLM cup aperture (final lipid concentration is l2.5 mg/ml). After preparation of BLM, Bax and Bif-1 proteins were tested one by one for pore formation. Then, an equimolar mixture of Bif-1 and Bax was prepared and incubated at 4 °C for ≥1 h. The BLM buffer was replaced with fresh one and the mixture was added to the cis chamber and the solution was slowly stirred to check its insertion onto BLM at +10 mV applied potential. The mixture got inserted onto BLM, formed pore and showed current activity. Currents were filtered using an external low pass RC filter (Single pole) at 200 Hz and then acquired and digitized at 1 kHz sampling frequency using data acquisition software Clampex (pClamp 10.2, Axon Instruments, Sunnyvale, CA, USA) through an analog to digital converter (Digidata 1440A, Axon Instruments, Sunnyvale, CA, USA).

4. Analysis of bilayer electrophysiological data

(i) Current values were obtained from the current-time traces and the current-amplitude histograms at different voltages. I–V plot (showing mean of the current values of the full open state of pore from three independent sets of experiments) was made using pClamp 10.2 (Axon Instruments, Sunnyvale, CA, USA) and Origin 5.0 (Origin Lab Corp., MA, USA) software. Best fit to the I–V data was analyzed using Origin 5.0. Histogram analysis was done using pClamp 10.2 and AxoGraph X (Version 1.5.4, AxoGraph Inc., CA, USA).

(ii). The opening probability of a particular pore state at a particular voltage was determined by calculating the fraction of total time pore remained open in that state out of the total time of recording at that particular voltage. It was calculated using pClamp 10.2 software and plotted using Origin 5.0.

5. Results and discussion

We tested monomeric Bax and Bif-1 proteins independently on BLM clamped at different voltages for pore formation at varying concentrations ranging from 1 to 10 μg/ml of BLM buffer. Fig. 1(A, B, C, D) shows the representative current-time traces of the Bax and Bif-1 proteins when tested on BLM independently. As indicated in Fig. 1 no pore formation could be detected from any of the proteins. Then, equimolar mixture of the two proteins was added to the BLM chamber. Pore activity by the mixture was observed. Fig. 2(A, B, C, D) shows the representative current-time traces and their all point

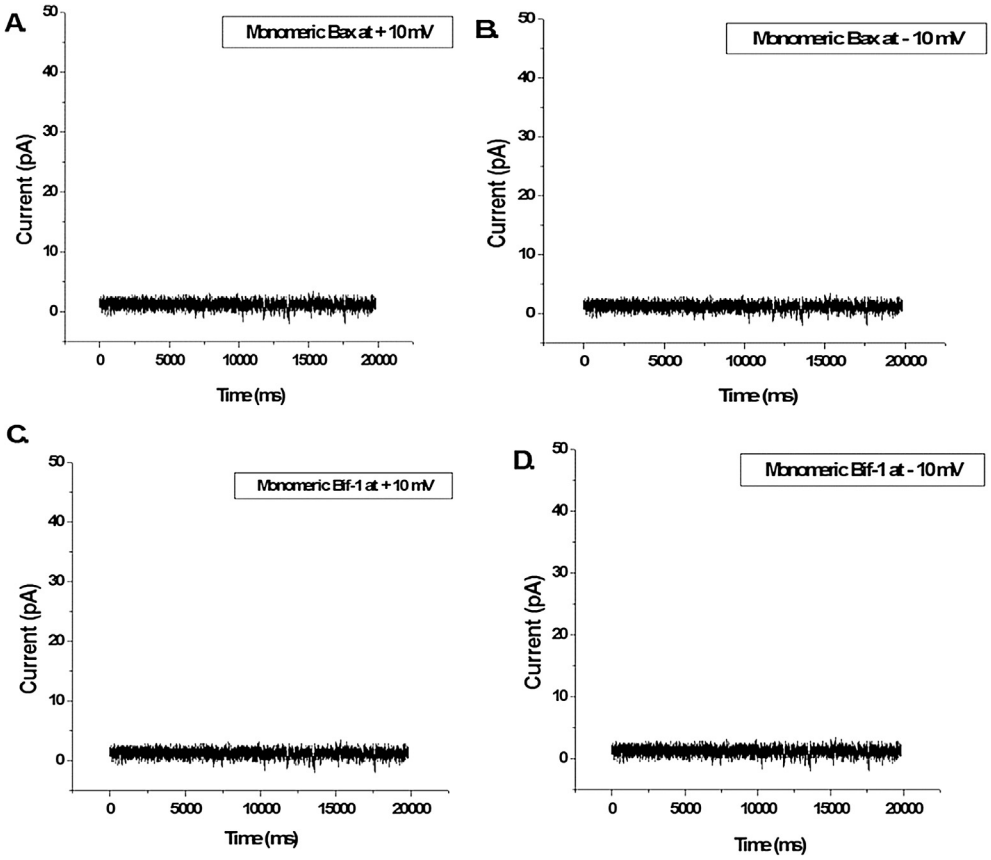

Fig. 1. Representative current–time traces A. Bax at +10 mV; B. Bax at −10 mV; C. Bif-1 at +10 mV; D. Bif-1 at −10 mV on DPhPC BLM with a symmetrical bath solution of 1 M KCl, 10 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 25 °C at clamping potential with respect to the ground. Data shown is filtered at 200 Hz and sampled at 1 kHz.

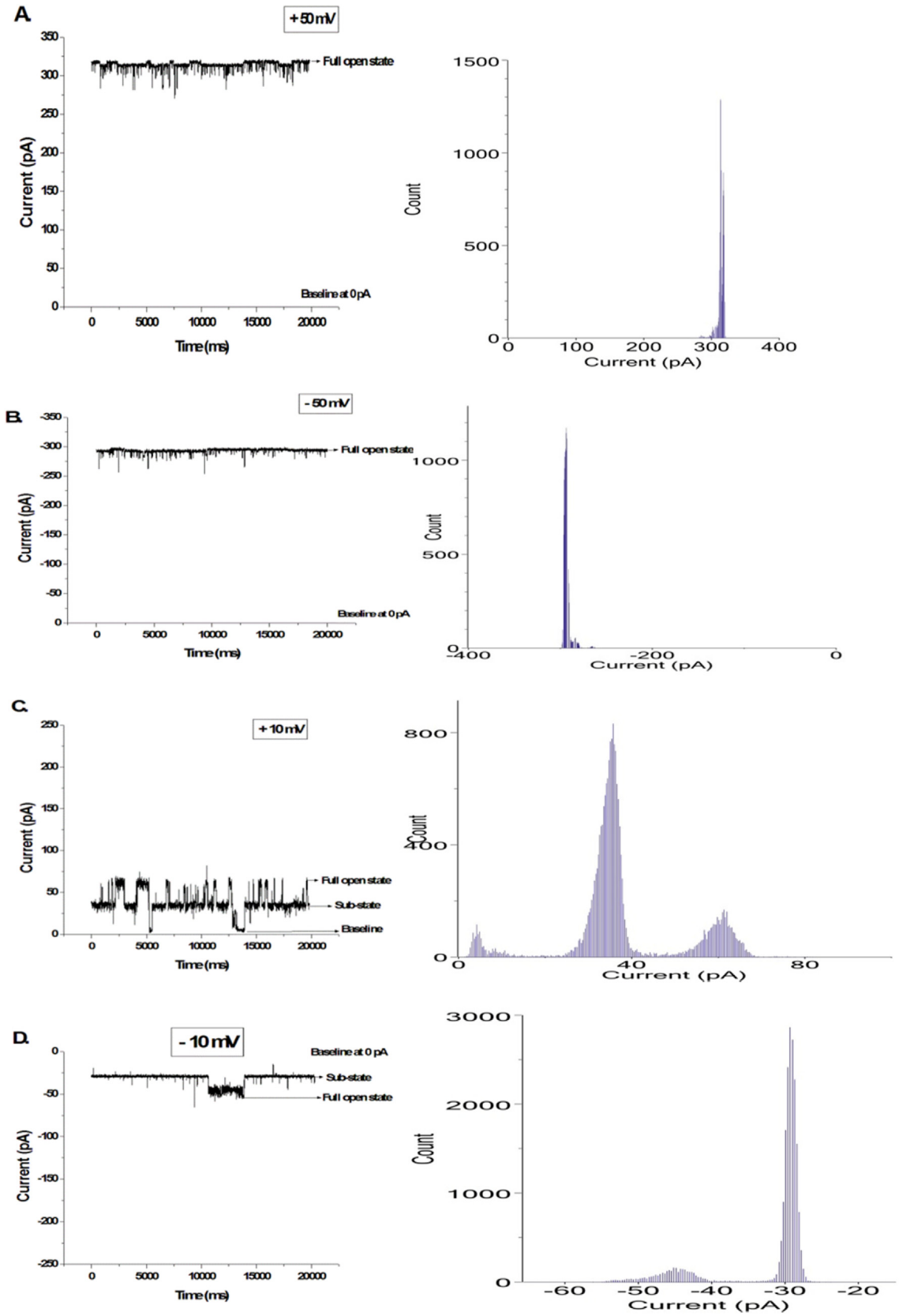

Fig. 2. Representative current–time traces and their current amplitude histograms of pore formed by equimolar mixture of Bax and Bif-1 onto DPhPC BLM with a symmetrical bath solution of 1 M KCl, 10 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 25 °C at A. +50 mV; B. −50 mV; C. +10 mV; D. −10 mV clamping potential with respect to the ground. Data shown is filtered at 200 Hz and sampled at 1 kHz.

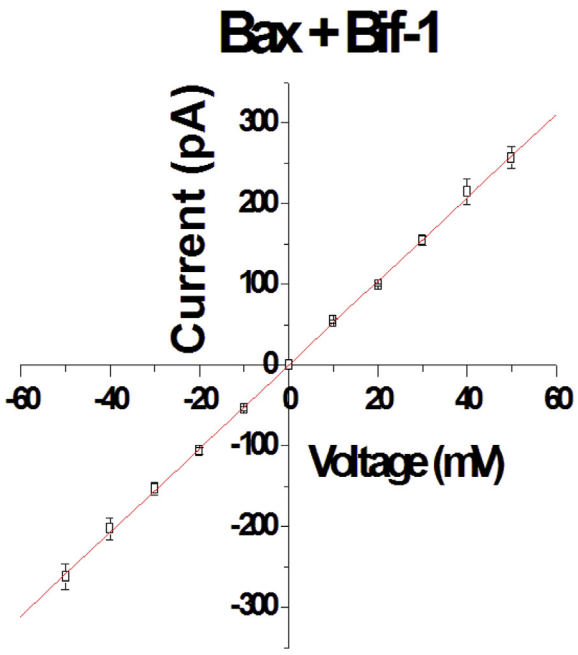

Fig. 3. Current–voltage relationship (I–V plot) of full open state of pore formed by Bax-Bif-1 mixture. The best fit line is drawn using Origin 5.0 software. Values are mean ± S.E. of three independent set of experiments.

current amplitude histograms of the pore activity seen with Bax-Bif-1 mixture. As indicated in the figure the pore has a full open state and a sub-state. The opening probability of the full open state of the pore varied drastically from 0.98 to 0.18 in different sets of experiments because in a given experiment either the pore remained continuously open or it switched between the full open and the sub-state at all the applied membrane potentials (data not shown). Fig. 2A & B show pore activity with high opening probability of the full open state and Fig. 2C & D show the same with low opening probability. Current changes with respect to voltage (I–V plot) of the full open state shows linearity, which indicates the pore is voltage-independent in the range ±50 mV (Fig. 3). The conductance of the full open state is in the range 4.96 nS–5.41 nS and that of the sub-state is 2.6 nS.

As stated above, the pore activity was seen only when the mixture of the two proteins was used for the experiments. Incidentally, when the equimolar mixture was run on Native or Sodium Dodecyl Sulphate polyacrylamide gels, complex formation between Bax and Bif-1 proteins or oligomerization of any of the two proteins was not observed (data not shown). The above-mentioned pore activity on BLM can arise due to several reasons. The first possibility is the formation of a complex between Bax and Bif-1 and we expect this complex formation is only possible on BLM. The structure of inactive cytosolic Bax consists of three Bcl-2 homology domains BH1, BH2, BH3 and nine α helices clustered around helix α5 that constitute the hydrophobic core of the protein [23]. Interestingly, two important domains of Bax, the putative transmembrane domain (helix α9) and the BH3 domain are masked inside the hydrophobic core of the protein [23]. It is believed that in order to insert into the mitochondrial membrane, helix α9 would have to come out from the hydrophobic pocket [23,24]. The above mentioned conformational changes in Bax protein can be brought about by its binding to the membrane but Bax binding to the membrane is sensitive to intrinsic membrane curvature [7,25]. N–terminal region (1–27) amino acids of Bif-1 protein are required for its binding to Bax [19]. This region of Bif-1 protein can form a dimer with other Bif-1 molecule and upon dimerization can induce membrane curvature [17]. Thus, binding of Bif-1 dimer to the Bilayer Lipid Membrane can change the membrane curvature in such a way that the changed membrane induces major conformational change in Bax protein that exposes the hidden hydrophobic domains in Bax protein. The conformationally altered Bax protein can interact with the BLM and Bif-1 protein and they together form a complex which might show the observed pore activity on BLM seen in our experiments. Second possibility is that Bif-1 interacts with Bax protein on BLM and activates it. Activation of Bax can lead to its oligomerization and formation of pores on BLM. BH3 domain is necessary for the formation of Bax/Bax homodimers and Bax/Bcl-2 heterodimers [23]. This possibility is also likely because the reported mean conductance of recombinant oligomeric Bax channel (activated by tBid or 1% Triton X-100) is of the order of 5.0 ± 3.0 nS [26], which is quite close to our results of 4.96 nS–5.41 nS conductance shown by the full open state of Bax-Bif-1 mixture. Third and the last possibility is that Bax activates Bif-1 protein leading to its self-oligomerization and formation of pores by Bif-1 oligomers. This possibility is less likely because Bif-1 has not so far been reported to form pores anywhere in literature. Out of the above-mentioned possibilities we think the first and the second one are most feasible. However it is not possible at this juncture to confirm out of the aforesaid possibilities which one is true. Further studies in this direction are under progress. In conclusion, we report for the first time that it is neither Bax nor Bif-1 can independently form membrane pore, rather their equimolar mixture forms pore on BLM. We believe such pore formation by the Bax-Bif-1 mixture on lipid membrane has relevance in mitochondrion-mediated apoptosis *in vivo*.

Conflict of interest

Both the authors i.e.; Dr. Rajeev Gupta (Ph.D.) and Dr. Subhendu Ghosh (Associate Professor) Dept. of Biophysics, University of Delhi South Campus, India declare no conflict of interest. Also, we both acknowledge Department of Atomic Energy Board of Research in Nuclear Sciences (DAE-BRNS), Indian Council of Medical Research (ICMR), Government of India and University of Delhi for financial assistance.

Acknowledgments

We acknowledge Department of Atomic Energy, Board of Research in Nuclear Sciences (DAE-BRNS) (Grant No. 2007/37/4/BRNS/2629), Indian Council of Medical Research (ICMR) (Grant No. 45/3/2011-BMS/CMB), Government of India and University of Delhi (Grant No. DRCH/R&D/2013-14/4155) for financial assistance.

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