Original Article

J Babol Univ Med Sci
Vol 18, Issu 1; Jan 2016. P:26-31

Electrophysiological Characteristics of Cationic Single-Channel formed by Incorporation of Amphotericin B in Bilayer Lipid Membrane

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J Babol Univ Med Sci; 18(2); Feb 2016; PP:26-31

Received: Apr 15th 2015, Revised: Jul 29th 2015, Accepted: Jan 6th 2016.

ABSTRACT

BACKGROUND AND OBJECTIVE: Amphotericin B (AmB) is the main antibiotic of polyene type, which is widely used in the treatment of systemic fungal infections. One of the key mechanisms of this drug is the formation of ion channels and increasing permeability of the host cell membranes. This study was conducted to evaluate some of the electrophysiological characteristics of cationic single-channel formed by the incorporation of AmB in bilayer lipid membrane (BLM).

METHODS: In this experimental study, phosphatidyl choline was extracted from fresh egg yolk. After the formation of BLM and the incorporation of AmB in the membrane, the channel activity was recorded through +40 to -40 millivolt (mV) voltages. Data analysis was conducted using PClamp10 software to determine the electrophysiological characteristics of the channel.

FINDINGS: Reverse potential of Amphotericin B ion Channel was-36 mV. The Channel activity was more in such positive voltages. This means that in voltage -40 mV the current amplitude was 2 Pico Ampère but in voltage +40 mV reach to 30 Pico Ampère. In addition, the channel open probability at voltage -30 mV voltage was low (less than 0.15) but increased to 0.75 in voltage +40 mV. The channel conductance was also 157±4.9 Picoseimence.

CONCLUSION: AmB forms cationic channels in BLMs with phosphatidylcholine and without cholesterol. This channel has voltage-dependent electrophysiological characteristics and behaviors.

KEY WORDS: *Electrophysiological, Phosphatidylcholine, Amphotericin B, Cationic channel*.

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Please cite this article as follows:

Shahmoradi T, Ashrafpour T, Sepehri H. Electrophysiological Characteristics of Cationic Single-Channel formed by Incorporation of Amphotericin B in Bilayer Lipid Membrane. J Babol Univ Med Sci. 2016;18(2):26-31.

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[DOR: 20.1001.1.15614107.1394.18.2.4.6]
[DOI: 10.22088/jbums.18.2.26]

Introduction

Amphotericin B (AmB) is the main antibiotic of polyene antibiotic family, and after six decades, it is still used as the gold standard in the treatment of systemic fungal infections. This compound is widely used to cure systemic fungal infections after organ transplant, acquired immune deficiency syndrome, chemotherapy, and viral, parasitic, and prion diseases (1, 2). Each molecule of AmB has a linear structure with a molecular weight of 200 KDa. This compound is an almost flat lactone ring with seven pairs of double bonds and a chain of hydrophilic, carboxyl, and amine groups, which is usually combined with the sugar part of the adjacent molecules (1, 3).

The mechanism of action and factors affecting the functioning of AmB are not still clear. The important suggested cellular mechanism for this drug is changing permeability of the host cell membrane to various substances (1, 4, 5).

A number of AmB monomers accumulate in the membrane and form an ion channel. Non-aqueous channels with selective permeability to cations are formed in low AmB concentrations, which not only promotes the transmission of sodium, but also increases pH of the intracellular fluid; these channels may also induce phosphorylation of tyrosine kinases (5). The channels formed in high doses of AmB are comprised of two halves containing eight antibiotic and eight sterol molecules in one layer of the membrane. Electrostatic interaction between an amine group from one molecule and carboxyl group of an adjacent molecule creates a hydrogen bond, which results in the binding of molecules (6-10). In the absence of sterol, ion channels can be formed in high concentrations of AmB in the synthetic membrane (9, 11). Sterol does not have a direct role in the formation of ion channels; rather it facilitates the formation of channels by affecting the structure of the membrane (12-15). Former studies demonstrated that the incorporation of AmB with other drugs reduces side effects and improves therapeutic effects of chemotherapy medications (16-19). Behaviors of ion channels formed by AmB are affected by various factors. AmB is capable of inducing apoptosis through the activation of driving factors of apoptosis, and nowadays, it is used as a complementary medicine in chemotherapy (18, 19). Moreover, voltage variations can modify the time and way of opening and closing of channels. Therefore, this study was conducted to evaluate some of the electrophysiological characteristics of cationic single-channels formed by incorporation of AmB in bilayer lipid membrane (BLM).

Method

To extract phosphatidylcholine chloroform, methanol, ethanol, acetone, petroleum ether, aluminum, acetone, and silica were bought from Merck Co. (USA). Moreover, KCl, KOH, HEPES, and Tris-HCI were bought from Sigma Co. (USA) for single channel recording. Phosphatidylcholine was employed for BLM formation; phosphatidylcholine was extracted from fresh egg yolk using Singleton et al. method (20), for this purpose, lipid organic solvents were applied to separate the yolk from proteins and pigments. Afterwards, phosphatidylcholine was separated from other phosphatides using chromatography column.

The stationary phase of chromatography column was aluminum oxide and its mobile phase was methanol-chloroform solution in a 9:1 ratio. The purity of the obtained phosphatidylcholine was estimated by thin-layer chromatography. The extracted phosphatidylcholine was collected in microtubes, and until being used, it was preserved under nitrogen gas and away from light at -20°C. The method of Muller et al. was used for BLM formation (21); for this purpose, 25 mg/ml concentration of phosphatidylcholine was obtained by dissolving phosphatidylcholine in n-decane. Thereafter, using a dental needle with diameter of 150 µm, BLM was formed on a 250 µm aperture in the Teflon film between cis and trans chambers. The cis and trans chambers of BLM system have potassium chloride concentrations of 200 and 50 mM, respectively. Using a dental needle, AmB solution with 50 mg/dl concentration was put in contact with the membrane from the cis chamber's side until the

[DOR: 20.1001.1.15614107.1394.18.2.4.6]
[DOI: 10.22088/jbums.18.2.26]

channel was accidentally incorporated in the membrane and ion current was produced. We recorded electrophysiological activity of the cationic channel formed by AmB through the single channel recording method. The ion currents were transferred to a computer after being amplified. Silver and silver chloride electrodes (Ag/AgCl) were used to record activities of the channel. The electrode connected to the trans chamber was used as the source electrode, and the other electrode connected to the cis chamber was regulated for voltage generation. The current flowing through the channel was recorded and quantified using bilayer clamp amplifier (BC-535 model, Warner Co.).

All the recordings were performed after 1 kHz low-pass filter with 10 kHz rate was sampled, and then the data were saved into a computer. The recordings were analyzed using Pclamp10 software (Axon Co.). To analyze the data, after determination of a base line for the open and close states of the channel, the range of the current flowing through the channel (based on pico-ampere [pA]) was used. In addition, the possibility of openness of the channel (Po) was identified using records with sufficient time and with duration of one minute for each voltage. Then, for each voltage, amplitude histogram of the ion current channel was drawn by Pclamp10.

To determine the AmB cationic channel conductance and activity, the current-voltage curve was drawn using Excel. Thereafter, using the relevant formula, the slope of the aforementioned curve was estimated as an indicator of the channel conductance.

Results

After the formation of BLM and incorporation of AmB in the membrane, ion channel activity was recorded at various voltages (from +40 to -40 millivolts [mV]). Some examples of channel activity recordings (made at +40, +20, 0, -20, -40 mV voltages) and their histograms are shown in figure 1. The reverse potential of the channel activity was estimated to be -36 mV. There was no current in reverse potential since electrochemical gradient of potassium ions neutralize each other (fig 1). In potentials more positive than the reverse potential of the outward current (to the cis chamber) and positive in potentials more negative than the reverse potential, a negative inward (to the trans chamber) current was produced. Since the reverse potential of channel activity was close to equilibrium potential of potassium ion (EK^+=-36mV), the channel was cationic, which shows a higher level of permeability compared to potassium ion.

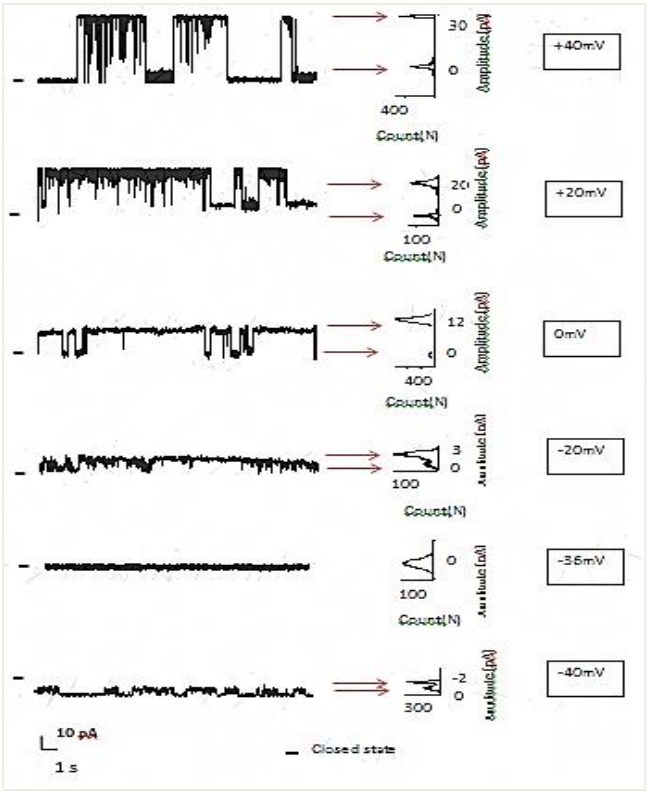

Figure 1. A record of Amphotericin B channel activity and amplitude histogram of the current flowing through the channel in bilayer lipid membrane at various voltages; the medium contained 200 and 500 mM potassium chloride solutions for cis and trans chambers, respectively (n=7 for each voltage); shows the closed state of the channel

Using Goldman–Hodgkin–Katz equation, the potassium-chlorine permeability ratio was estimated to be higher than 25 (22); this means that the ion channel formed by AmB in BLM, comprising of phosphatidylcholine, demonstrated more than 25 times higher selective permeability compared to potassium

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ion, while it had slight permeability to chlorine ion. The range of these currents gradually increases at voltages more positive than -30 mV, that is, the mean range of the current flowing through the channel was -3±1 pA at -20 mV voltage, while it raised to 30±3.5 pA at +40 mV voltage.

The cationic channel formed by AmB in BLM is voltage-dependent and it shows ohmic behavior with no convergence properties. The electrophysiological behavior and voltage-dependent functioning of the channel were in a way that by increasing the applied potential to the membrane and at more positive voltages, the channel got more active and range of the flowing ion current increased (fig 2). The slope of current-voltage curve of channel activity demonstrates a conductance channel, which was estimated to be 470±4.90 picosiemens using slope equation. The average possibility of channel's existence raised as the potential applied to the membrane increased from -30 mV (fig 3). The mean level of channel Po was not significant at -30 mV voltage, but the possibility of an open channel was increased to 0.8 by elevating the potential to 0 mV. The mean level of channel activity Po did not vary as the voltage was increased to +40 mV (from 0 mV), and it was kept at 0.75 level.

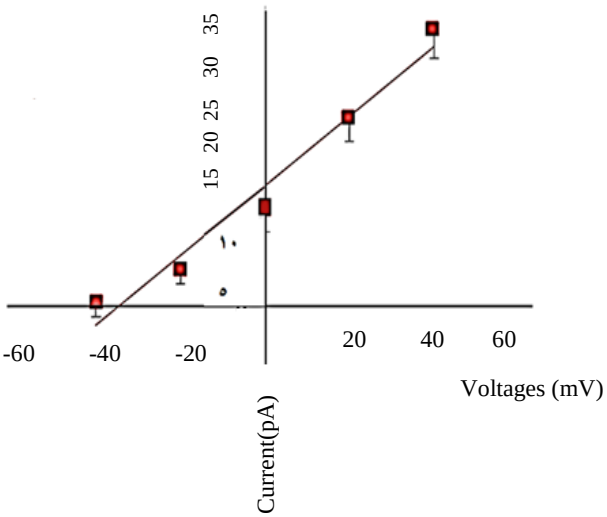

Figure 2. The current-voltage relationship in Amphotericin B channel of lipid bilayer membrane consisting of phosphatidylcholine, under 50 millimole potassium chloride of cis and 200 millimole of potassium chloride of cis conditions; the points are shown as mean±standard error (n=7)

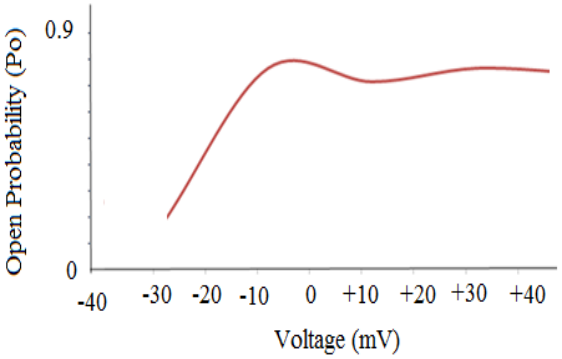

Figure 3. The relationship between the possibility of an open channel and Amphotericin B channel voltage at different voltages; mean±standard error and n=7 for each voltage

Discussion

The results of the present study indicated that a kind of cationic channel with selective permeability to potassium ion was formed as a result of AmB incorporation in BLM consisting of phosphatidylcholine. This channel exhibits voltage-dependent behaviors in terms of electrophysiological properties.

The range of ion current of the channel activity and the possibility of an open channel were higher in more positive voltages, and the channel had a linear current-voltage relationship curve. The results of other studies were in agreement with those of the present one regarding the fact that AmB is capable of forming channels in the membrane even in the absence of sterols (11, 23).

In addition, in the study of Brutyan et al., it was found that adding AmB to one side of the phosphatidylcholine membrane leads to the formation of selective monovalent cationic channels, while by adding antibiotics to both sides of the membrane, the formed channels convert to anionic types (16).

In another study, the channel formed by AmB in BLM was of cationic type (7). Huang et al. demonstrated that with monomeric and accumulated forms of AmB ion channels can be formed in membranes containing ergosterol. However, in the absence of estrols, ion channels can be formed in the membrane only in relatively high concentrations of

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AmB (11). Their results showed that the form of AmB has a key role in the formation of ion channels in membranes without estrol.

In addition, the results of a study by Venegas et al. suggested that a considerable amount of AmB is required to form channels in this membrane (23). As was observed, the mean conductance in cationic channels formed by AmB in BLMs containing phosphatidylcholine was 470±4.90 picosiemens. Nevertheless, in a study by Cohen et al., the mean channel conductance in the membranes with phosphatidylcholine and without cholesterol was 35.4±3.9 picosiemens (24). These findings were not consistent with the results of our study. This discrepancy might be attributed to differences in pH of the study settings and recorded voltages, since the study by Cohen was conducted in pH=8 and at 200 mV voltage. In a study by Brutyan et al., AmB channel conductance in pH=7 equaled to 6.5 picosiemens, and adding AmB to one side of cholesterol-phospholipid containing membrane formed a channel with the maximum conductance of 15 picosiemens and another channel in the ergosterol-phospholipid containing membrane with 6-7.5 picosiemens conductance. According to the results of the Cohen study, in dipalmitoyl phosphatidylcholine membrane without cholesterol, conductance of less than 11 picosiemens in AmB channels is related to their short lifespan (16). In general, our results indicated that AmB can form cationic channels with selective permeability to potassium even in phosphatidylcholine membranes without cholesterol. The electrophysiological activity of this channel is highly voltage-dependent, that is, in more positive potentials, the amount of conductance, current range, and Po of channel activity significantly increase toward channel cis.

Acknowledgments

We would like to thank Deputy of Research and Technology of Golestan University of Medical Sciences for financial support. We also appreciate the efforts of Deputy of Research of School of Medicine and cooperation of Department of Physiology.

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[DOR: 20.1001.1.15614107.1394.18.2.4.6]
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