Original Article

pH regulation of amphotericin B channels activity in the bilayer lipid membrane

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Abstract

Background: Amphotericin B (AmB) is a polyene antibiotic frequently applied in the treatment of systemic fungal infections in spite of its secondary effects. The pH plays a crucial role in modulating biophysical features of ion channels in the bilayer lipid membranes. **Aim:** In this study, the role of pH in the regulation of AmB channel was assessed by single channel recording of ion channel incorporated in the artificial membrane. **Materials and Methods:** Bilayer lipid membrane was formed by phosphatidylcholine in a 350 μm diameter aperture between two chambers, cis and trans contained 200/50 mMKCl solutions, respectively; then AmB was incorporated into the bilayer lipid membrane. Single channel recordings were used to indicate the effects of pH changes on AmB channels activity. The records were analyzed by Clamp fit 10 software. **Results:** A kinetic analysis of single channel currents indicated a cation ion channel with 500 pS conductance and voltage-dependence of the open probability of the AmB channel (P_o). A reduction of *cis* pH to 6 decreased P_o and conductance. This effect was also voltage-dependent, being greater at a more positive above −40. The pH changes in the range of 6-8 had no effect on the reversal potential and ion selectivity. **Conclusion:** Our data indicated that extracellular acidity can reduce AmB activity.

Key words: Amphotericin B, bilayer lipid membrane, phosphatidylcholine, single channel recording

INTRODUCTION

Amphotericin B (AmB) is a polyene belonging to the macrolide antibiotic family used to treat serious, life-threatening fungal infections in spite of its secondary effects.[1,2] AmB is also known to be active against a variety of viruses including HIV virus,[3,4] and it has been recently shown that this drug is able to potentiate the cytotoxicity of several different anticancer agents.[5,6] Despite more than half a century of intensive study, the mechanism of biological action of AmB at the molecular level has not been totally elucidated. Various interaction models with membrane have been proposed. However, the most recent models are based on the ability of the drug to form pores in the membrane, where it increases the permeability of the cells to ions, and small molecules that eventually lead to cell death.[7,8] The accepted channel model assumes that the pore can be made with a different number of monomers[9,10] and is confirmed by the multiple conductivities found in single channel recordings.[11,12] AmB possesses a hydrophobic part (hydrocarbon chain) and a hydrophilic part (polyhydroxyl chain); its amphipathic properties allow interactions with

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the membrane.[8] Membrane sterols seemingly play an important role in the formation of these pores. For this reason, organisms sensitive to these drug contain sterols, whereas those insensitive to them such as bacteria do not have sterols. These molecules assist the assembly of the channel by linking the AmB monomers through a consequence of hydrogen bond between hydroxyl groups of sterol as a hydrogen acceptor and amino group of AmB as hydrogen donor.[13,14] As an evidence against the proposal that sterols are necessary for channel formation, the presence of AmB channels has demonstrated in sterol-free membranes.[10,15] These researchers have proposed that AmB's NH_3^+ group forms specific hydrogen bonds with PO_4^- groups from dimyristoylphosphatidylcholine, and such interactions are responsible for the creation of the AmB-Phospholipid complexes.[16,17] Further knowledge of the factors that may affect the function of AmB channel may help to eliminate or reduce drug toxicity. The solution pH affects many membrane-mediated biologicals, such as membrane fusion,[18] drug-membrane interactions,[19] electric features of membranes, and ion channel activity. Normally, extracellular fluids have a pH at –7.4, and cells regulate their internal pH at –7.0. However, in some situations biological membranes are exposed to environments with various pH values. Thus characterizing and understanding the effect of extracellular pH changes on the AmB may be useful for increasing or reducing AmB toxicity. Searching in the literature showed that few research works are done on pH effect on the AmB activity reporting controversial results. It has been reported that acidic pH has diminished the antifungal effect of AmB on *Candida albicans* cells.[20] While the acidic pH increased the Amb channel activity in the ergosterol-containing zwitterionic lipid membranes.[21] Black lipid membranes are useful model systems for studying the structure and function of ion channels. The simple composition of an artificial bilayer in contrast to the complex mixture of lipids and proteins in biological membranes facilitates a detailed examination of ion channels.[22] In this work, we investigated at the single channel level the regulatory role of pH in control of AmB channels inserted in phosphatidylcholin lipid membranes, in an attempt to further clarify some of the core biophysical properties of this antibiotic.

MATERIALS AND METHODS

Lipid bilayer membranes were formed as described by Mueller across a round aperture (0.1-0.2 mm) in a thin Teflon partition clamped between two symmetrical cell compartments designed to minimize any unstirred areas in the membrane bathing solution.[23] The cis and trans chambers hold 5 ml of KCl (Sigma-Aldrich), 200 and 50 mM, respectively. The pH on both sides was adjusted to 7.4 with Tris-Hepes (Sigma-Aldrich). Planar lipid membranes were formed without cholesterol and ergesrol by using only L-α-Phosphatidylcholine. L-α-lecithin dissolved in decane at a concentration of 25 mg/ml and painted to the hole. Bilayer formation was monitored by applying a 2.5 mV peak-to-peak 20 Hz triangular wave with a typical membrane capacitance of 100-200 pF. L-α-Phosphatidylcholine (L-α-lecithin) was extracted from fresh egg yolk according to the protocol described by Singleton and Gray.[24] Changes in pH in the cis chamber were conducted by addition of small volumes of concentrated solutions of either HCl or KOH (Sigma-Aldrich), ranging between 0.5 and 2.0 N. The final pH was calibrated with a pH mini-electrode. Fusion of the AmB was initiated mechanically by gently touching the bilayer from the cis face using a small stainless steel wire of 150 diameter, on the tip of which was deposited a small drop of solution. the stock AmB solution was obtained by dissolving AmB in dimethyl sulphoxide (Sigma-Aldrich), and the final concentration attained during single channel recording was kept at 2×10^{-5} M. All experiments were performed at a room temperature of –23°C. Single channel currents were measured with a BC-525A amplifier (Warner Instrument). Ag-AgCl electrodes were connected to the bilayer chamber via salt bridges made of agarose (–1%, w/v) dissolved in 3 M KCl. To minimize liquid junction potentials, the cis chamber was voltage-clamped relative to the trans chamber, which was grounded. All recordings were filtered at 1 kHz using an 8pole Bessel filter, digitized at a sampling rate of 5 kHz and stored on a personal computer for offline analysis by PClamp10 (Axon Instruments Inc.). Data for figures were filtered at 200-400 Hz. The results are expressed as means ± standard error of the mean. Statistical significance was obtained by paired *t*-test comparison of sample groups of similar size.

RESULTS

Kinetic Analysis of Single Channel Currents of AmB were reconstituted in the presence of asymmetrical KCl (200 mM) in the cis side and 50 mM K in the trans side in the pH (7). AmB single channel currents were obtained at different holding potentials [Figure 1], and current amplitudes and open probabilities were calculated from all-point histograms. The mean open probability (area under open state) depended on the holding potential.

Effect of cis pH on AmB Channel function: To assess the regulatory effect(s) of pH on AmB channel activity, the pH of the cis chamber was reduced to 6 by the addition of a small volume of HCl (2.0 N) and increased to 8 by the addition of KOH (2N). Single channel current amplitude were reduced after lowering pH cis in the voltages of 40

and 20 ($P < 0.05$). However, the single channel current did not change by increasing pH cis from 7 to 8 [Figure 1]. Theoretical current-to-voltage (I/V) relationships were obtained for currents at three different pH, single channel conductance for each pH was calculated from the slope of the I/V curves [Figure 2]. The conductance for pH 7 and 8 were 500 and 560 pS, respectively. By reduction of cis pH to 6 the single channel conductance decreased to 250 pS. As seen in this figure the reversal potential in the pH of 6, 7, and 8 were 35, 36, and 40 mV, respectively. The calculation of ionic selectivity based on the average values of corresponding reversal potentials indicated highly cation selective channel and by changing the pH among 6-8 the ion selectivity did not significantly change. The effect of voltage on the channel activity was investigated by measuring the channel open probability (P_o) as a function of voltage in asymmetrical K^+ conditions (200 mM K cis/50 mM K trans [Figure 3]. As seen the channel open probability increased at potentials above −40 mV in the pH of 7 and 8. By reduction of cis pH to 6, the open probability significantly decreased in all voltages ($P < 0.005$).

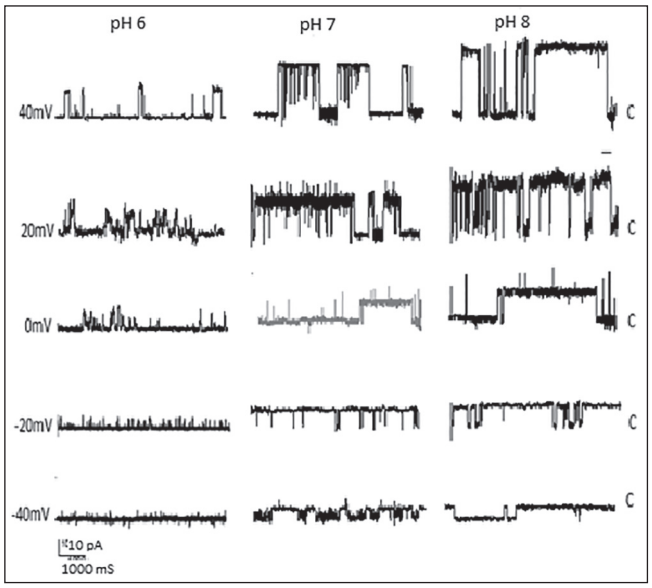

Figure 1: Typical current recordings of single ion channels formed by amphotericin B in phosphorylcholine bilayer membranes measured at different potential when the membrane-cis solutions were buffered to various pH values (at column width)

DISCUSSION

In this work, we investigated at the single channel level the effects of pH changes on biophysical properties of AmB channels inserted in bilayer lipid membranes. Our data indicated the pH-induced modulation of AmB channels open probability, and conductance. Although the ion selectivity did not change. The acidity largely decreased the open probability of the channel. This effect was also voltage-dependent, being greater at a more positive voltages above −40 mV. This data suggest that the net positive charge present on the head group of AmB molecules at low pHs may be a possible cause for the observed effect. Also these data suggest that probably an H^+ on the regulatory site is accessible from the extracellular (cis) side of the channel where pH changes. The pH changes also altered the single-channel conductance of AmB oligomers. Our data show that the single channel conductance of AmB channels value is larger at pH 7 and pH 8, showing a drop at acid pH values [Figure 2]. This finding is in contrast to Asandei and Luchian who reported increased conductance in acidic pH.[21] An intriguing possibility is that the hydroxyl backbone of AmB, perhaps combined with its associated H molecules, produces hydrogen-bonded chains in the pore which by preventing K current decreases AmB channel conductance. Another reasonable explanation is that protons and hydroxyl ions can alter the lipid membrane dipole potential,[25] Ohki observed the effect of variations in pH on the capacitance of pure bilayers. The capacitance of the bilayers was smaller in a solution of lower pH than in a solution of medium pH.[26,27] Since phosphorylcholine

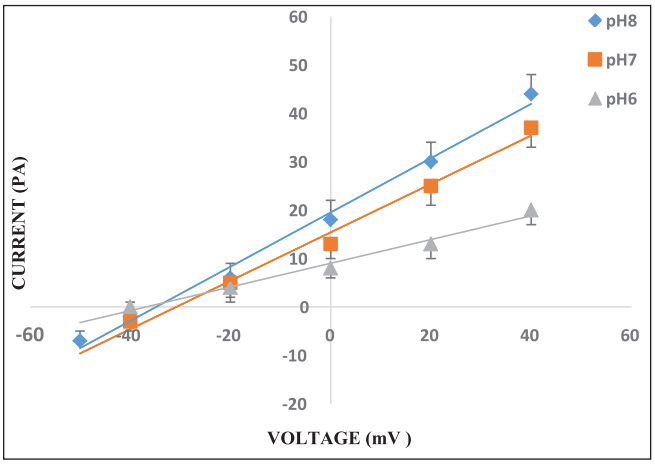

Figure 2: Current-voltage relationships for single channel recordings, in asymmetrical KCl (200 mM) in the cis side and 50 mM KCl in the trans side when the membrane-cis solutions were buffered to pH 6, 7, and 8). Each point represents the means ± standard error of the mean of six different experiments (at column width)

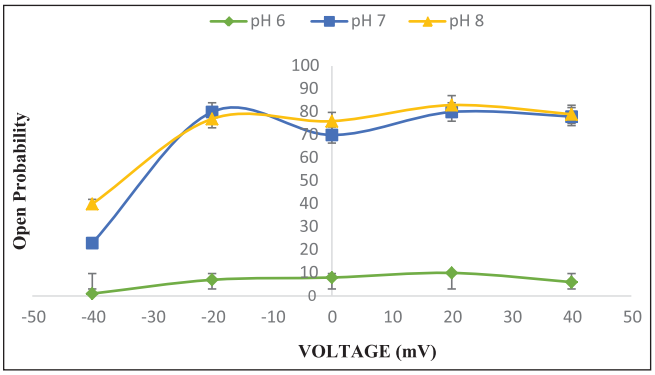

Figure 3: Channel open probability (P_o) at different voltages. When the membrane cis solutions were buffered to pH 6, 7, and 8 (at column width)

has a zwitterionic group, the polar groups would be positively charged at lower pH values, and this would lead to a mechanical constriction of the AmB pore manifested by a drop in its conductance at pH = 6.[28] In contrary to open probability and conductance the reversal potential and ion selectivity did not change by pH. The reversal potential analysis indicated that Amb in these pH was cation channel. At pH range from 6 to 8 the carboxyl group of AmB is deprotonated, so that the selectivity of channels is influenced mostly by the electric profile within the conductive pore, leading to a cation-selective behavior. Luchian found that AmB channels become even more cationic selective in the acidic pH and can even change to anionic in the alkaline pH.[21] In conclusion, our data demonstrates that when pH changes from basic values (pH = 8) to acidic (pH = 6), the visible decrease occurred in the conductance and open probability. These data may be useful to better understand the mechanism of action of AmB and the toxic side effects manifestations.

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Conflicts of interest

There are no conflicts of interest.

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