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The interaction of dipole modifiers with amphotericin-ergosterol complexes. Effects of phospholipid and sphingolipid membrane composition

Olga S. Ostroumova · Svetlana S. Efimova · Ekaterina V. Mikhailova · Ludmila V. Schagina

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Abstract The influence of agents, known to affect the membrane dipole potential, phloretin and RH 421, on the multi channel activity of amphotericin B in lipid bilayers of various compositions, was studied. It was shown that the effects were dependent on the membrane's phospholipid and sphingolipid type. Phloretin enhanced amphotericin B induced steady-state transmembrane current through bilayers made from binary mixtures of POPC (DOPC) and ergosterol and ternary mixture of DPhPC, ergosterol and stearoylphytosphingosine. RH 421 increased steady-state polyene induced transmembrane current through membranes made from binary mixtures of DPhPC (DPhPS) and ergosterol and ternary mixture of DPhPS, ergosterol and stearoylphytosphingosine. It was proposed that the observed effects reflect the fine balance of the interactions between the various components present: amphotericin B, ergosterol, phospholipid, sphingolipid and dipole modifier. The shape of lipid molecules seems to be an important factor impacting the responses of amphotericin B modified bilayers to dipole modifiers. The influence of different phospholipids and sphingolipids on the physical and structural properties of ordered lipid microdomains, enriched in AmB, was also discussed. It was also shown that RH 421 enhanced the antifungal activity of amphotericin B in vitro.

Keywords Planar lipid bilayers · Ion channels · Amphotericin · Dipole modifiers · Phospholipids · Sphingolipids

Introduction

Amphotericin B (AmB) is a polyene antibiotic that is frequently used for the treatment of systemic fungal infections (Hartsel and Bolard 1996). It is widely accepted that macrolide polyenes exert their antifungal effects by binding to membrane sterols (de Kruijff et al. 1974; Baginski et al. 2002). Although the role of sterols in polyenes' mechanisms of action is known to be important, the influence of other membrane components, such as phospholipids and sphingolipids, on the activity of polyenes is still unknown. Understanding the effects of other membrane components may be of fundamental importance for understanding the mechanisms of pore formation by polyenes and for the clinical use of antibiotics. There is some evidence that phospholipids influence polyene activity. It has been reported that polyenes are able to form pores in membranes devoid of sterols (Hsuchen and Feingold 1973; Vertut-Croquin et al. 1983; Hartsel et al. 1988; Cohen 1992; Cotero et al. 1998; Ruckwardt et al. 1998). Fujii et al. (1997) showed that AmB can interact specifically with phospholipids. Based on their 2H-NMR studies of dimyristoylphosphatidylcholine liposomes containing AmB, Dufourc et al. (1984) noted an ordering effect of the acyl chains of dimyristoylphosphatidylcholine molecules in contact with AmB. In addition, based on an analysis of the CD spectra of AmB in sterol-free vesicles, Balakrishnan and Easwaran (1993) proposed the existence of an organized multimolecular structure within the bilayer in which AmB interacts with the acyl chains of dipalmitoylphosphatidylcholine,

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forming a 1:1 complex. The results of the differential scanning calorimetric studies performed by Fournier et al. (1998) showed that AmB induces phase separation within the membrane and that three phases coexist when AmB is dispersed in pure dipalmitoylphosphatidylcholine. One phase corresponds to the phospholipid alone.

The two other phases are characterized by a broad transition at temperatures higher than the main transition temperature of the pure phospholipid and correspond to the drug interacting with the aliphatic chains of the lipid due to enhanced Van der Waals interactions. Paquet et al. (2002) showed that pure dipalmitoylphosphatidylcholine bilayers undergo an AmB dose-dependent restructurization of the lipids and an increase of the gel-to-lamellar fluid phase transition temperature. Milhaud et al. (2002) suggested that the established AmB-induced gel-to-subgel transformation of dipalmitoylphosphatidylcholine bilayers is due to interactions between AmB and polymolecular phospholipid assemblies at the bilayer-water interface. A molecular dynamics study performed by Sternal et al. (2004) supports the hypothesis that AmB's polar head interacts with the polar head of dimyristoylphosphatidylcholine. Such interactions have been noted, in particular between the carboxyl group of AmB and the amino group of the lipid. Herec et al. (2005) proposed that hydrogen bonding between the horizontally oriented AmB and the polar groups of the lipids makes the membrane more compact and less permeable to ions.

The results of HsuChen and Feingold (1973) demonstrated that the responses to polyenes in a liposome membrane are affected by the composition of the fatty acyl chains. In the absence of sterols, AmB induces appreciable glucose leakage from saturated dipalmitoylphosphatidylcholine liposomes. In contrast, sterol-free unsaturated dioleoylphosphatidylcholine liposomes are unaffected by AmB. The response to AmB of the sterol-free egg lecithin liposome system, which contains saturated and unsaturated fatty acid residues, is intermediate. The authors hypothesized that these effects were not related to the role of the fatty acyl composition of phospholipids in determining the fluidity of the membrane.

There is only limited and indirect evidence of a possible interaction between polyenes and sphingolipids. For instance, Zager (2000) showed that polyene antibiotics alter the phospholipid and ceramide levels in the plasma membrane. Nagiec et al. (2003) demonstrated that the *Saccharomyces cerevisiae* sphingolipid bypass mutant strain, which is able to grow without producing sphingolipids, is more susceptible to amphotericin B than wild-type cells.

Incorporating into the membrane certain compounds possessing large dipole moments, dipole modifiers can alter membrane dipole potential, which originates from specific orientation of lipid and water dipoles at the interface. The number of studies clearly demonstrates that membrane dipole modifiers, such as plant flavonoids and styrylpyridinium dyes, are convenient and sensitive instruments for the investigation of the formation and function of ion channels in model membranes (Sun and Garlid 1992; Antonenko et al. 1999; Rokitskaya et al. 2002; Hwang et al. 2008; Luchian and Mereuta 2006; Ostroumova et al. 2007, 2008, 2010, 2011; Asandei et al. 2008; Mereuta et al. 2008; Ostroumova and Schagina 2009; Apetrei et al. 2009; Lundbaek et al. 2010; Mereuta et al. 2011; Efimova et al. 2011).

In particular, recently we showed that the effect of dipole modifiers on the conductance of single AmB channels in sterol-containing lipid bilayers primarily resulted from changes in the membrane dipole potential (Ostroumova et al. 2012a). While the effects of dipole modifiers on the channel forming activity of macrolide polyenes in sterol-containing bilayers can be attributed to the influence of these modifiers on the stability of the different polyene-sterol complexes that form the ion-permeable pores (Ostroumova et al. 2012b). We showed that the addition of phloretin to cholesterol-containing membranes from diphytanoylphosphocholine (DPhPC) leads to a significant increase in the steady-state AmB-induced transmembrane current. Furthermore, RH 421 increases the current through ergosterol-containing DPhPC-bilayers. Using different sterols, polyenes and dipole modifiers, it was shown that the presence of double bonds in the 7 and 22 positions of sterol molecules, the number of conjugated double bonds and amino sugar residues in polyene molecules, and the conformation and adsorption plane of dipole modifiers are important factors impacting this interaction. For further study of the mechanisms of polyenes' membrane activity, including the interactions between polyenes and both phospholipids and sphingolipids in the membrane, here we examined the effects of phloretin and RH 421 on the AmB induced steady-state transmembrane current through planar lipid bilayers composed of various phospholipids and sphingolipids.

Materials and methods

All chemicals were of reagent grade. Synthetic 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), 1,2-diphytanoyl-*sn*-glycero-3-phospho-L-serine (DPhPS), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-ioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), N-stearoyl-phytosphingosine (*Saccharomyces cerevisiae*) (PhSG), sphingomyelin (Brain, Porcine) (SM), N-stearoyl-D-*erythro*-sphinganine (SG), and ergosterol (Erg) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Phloretin

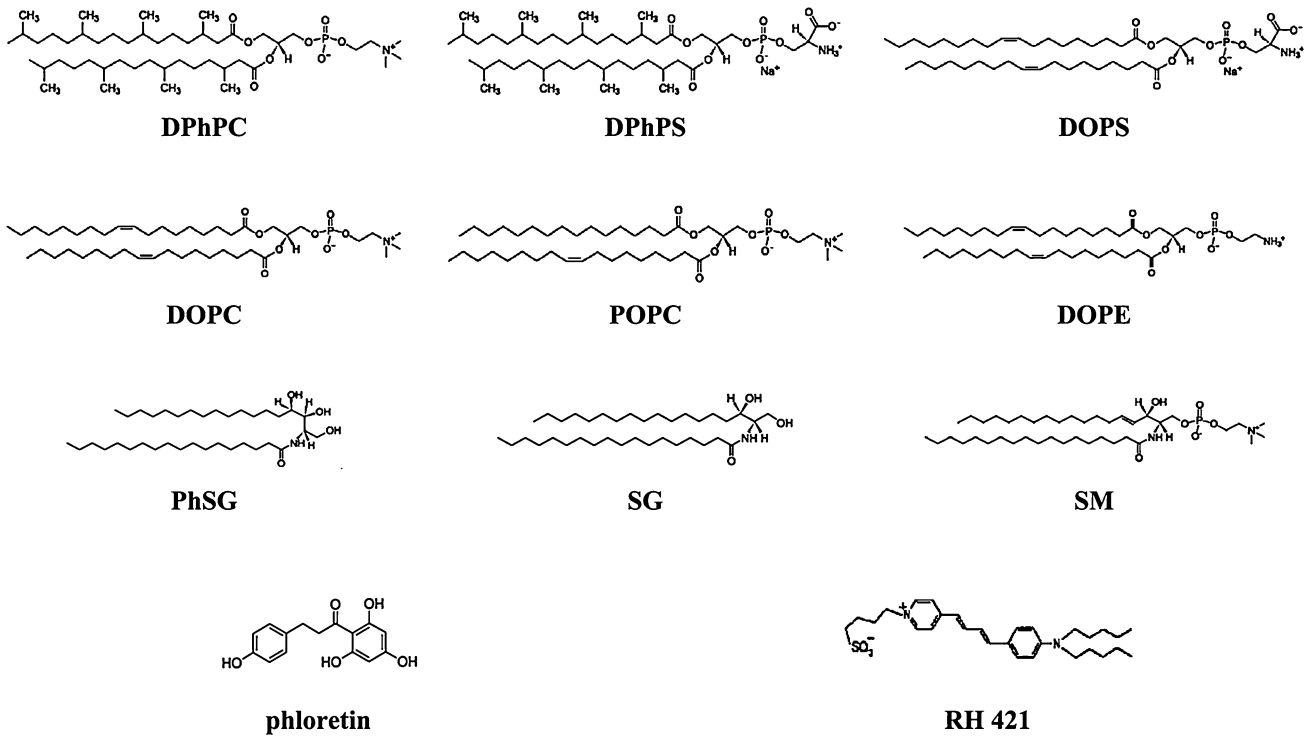

Fig. 1 The chemical structures of the phospholipids (DPhPC, DPhPS, DOPS, DOPE, DOPC, and POPC) and sphingolipids (PhSG, SG, and SM)

(3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone) and phloxine B (2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein disodium salt) were purchased from Sigma (St. Louis, MO), and RH 421 (N-(4-sulfobutyl)-4-(4-(4-(dipentylamino) phenyl)butadienyl) pyridinium, inner salt) was purchased from Molecular Probes (Eugene, OR). Yeast nitrogen base (YNB) and other components of the yeast media were obtained from Difco (BD). The water used in this study was double distilled and deionized. The 2 M KCl solutions used were buffered with 5 mM HEPES, pH 7.0. AmB was purchased from Sigma St. Louis, MO). The structures of tested phospholipids, sphingolipids, and dipole modifiers are shown in Fig. 1. The major criterion of phospholipid selection was a low gel-fluid transition temperature which allows to form bilayer lipid membrane at room temperature. The structural features of lipid molecules and their distribution in the membranes of target cells were the criteria for the selection of sphingolipids.

Virtually solvent-free planar lipid bilayers were formed using a monolayer-opposition technique (Montal and Muller 1972) with a 50-μm-diameter aperture in a 10-μm-thick Teflon film separating the two (*cis* and *trans*) compartments of a Teflon chamber. The aperture was pretreated with hexadecane. Lipid bilayers were made from 67 mol % phospholipid (DPhPS, DOPS, DOPE, DOPC or POPC) and 33 mol % Erg, or from 53 mol % phospholipid (DPhPC, DPhPS, DOPS, DOPE, DOPC or POPC), 27 mol % Erg, and 20 mol % sphingolipid (PhSG, SG or SM). After the membrane was completely formed and stabilized, an aliquot of AmB stock solution (0.1 mg/ml in DMSO) was added to each compartment to a final concentration that ranged from 10^{-7} to 10^{-6} M. Ag/AgCl electrodes with agarose/2 M KCl bridges were used to apply the transmembrane voltage (*V*) and measure the transmembrane current (*I*). "Positive voltage" refers to the situation in which the *cis*-side compartment was positive with respect to the *trans*-side. All experiments were performed at room temperature.

The two-sided addition of phloretin or RH 421 from stock solutions (mM) in ethanol to the membrane-bathing solution, yielding a final concentration of 20 μM for the flavonoid and 5 μM for the RH dye, was used to modulate the channel-forming activity of AmB after the polyene induced transmembrane current had reached its steady-state level. The final concentration of ethanol in the chamber did not exceed 0.1 %. These concentrations of solvent did not affect the integrity of the lipid bilayers and did not increase their conductance.

The current measurements were taken using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were digitized with Digidata 1440A digitizer and analyzed using pClamp 10 (Axon Instruments) and Origin 7.0 (OriginLab). The current tracks were processed with an 8-pole Bessel 100-kHz filter. The channel-forming activity of AmB in the absence and presence of the modifier (flavonoid or styryl dye) was characterized by the

steady-state transmembrane current (I_∞) under the given experimental conditions ($V = 50$ mV and the given polyene concentration). It should be mentioned that the dipole modifier's effects did not depend on initial value of the steady-state transmembrane current before their addition (the coefficient of correlation is about zero). Therefore, the mean ratios (I_∞/I^0_∞) of the steady-state transmembrane current induced by AmB in the presence (I_∞) and the absence of modifiers (I^0_∞) were calculated from the data for three to seven bilayers of each lipid composition type as the mean (average) score and standard error of mean (mean ± se).

Minimal inhibitory concentrations (MIC) of AmB and its composition with RH 421 were measured under steady-state conditions in liquid media. The *Saccharomyces cerevisiae* strain 168t (*MAT* a *his* 5) was used. Yeast cells were grown on YEPD at 30 °C for 48 h. The cells were harvested with 1 ml of sterile saline, and 250 μl of this suspension was inoculated into flasks containing 1.25 ml of YNB media supplemented with 1 % glucose. The yeast concentration was 2.0–3.0 × 10^3 cells/ml. The flasks were incubated at 30 °C for 24 h on a gyratory shaker with a speed of 100 rpm.

The MIC was defined as the lowest drug concentration that caused the complete inhibition of yeast growth. The MIC values were determined at least three times with triplicate measurements. Growth inhibition was assessed by measuring the absorbance at 600 nm. The optical density of media at MIC was equal to 0.01 ± 0.01. Initial optical density of cell suspension at zero concentration of AmB was 0.3 ± 0.1. Cell viability was determined using phloxine B.

Results

Figure 2 illustrates the time course of the AmB-induced transmembrane currents after the addition of 20 μM phloretin to both sides of the lipid bilayers containing 33 mol % Erg and 67 mol % phospholipid (POPC, DOPC, DOPS, or DPhPS). The effect of phloretin was dependent on the type of phospholipid in the bilayer. Table 1 summarizes the data obtained. The addition of phloretin to the bilayer bathing solution led to an increase in the channel-forming activity of AmB in POPC-containing and DOPC-containing membranes, whereas this modifier did not influence the AmB-induced steady-state transmembrane current through DOPE-containing, DOPS-containing, DPhPC-containing, and DPhPS-containing bilayers. These results most likely indicate that the effect of phloretin is related to both the unsaturation in the phospholipid acyl chains (e.g., the presence of one and two double bonds in the acyl chains of POPC and DOPC molecule, respectively) and the choline moiety in the phospholipid's polar head.

The observed changes in the AmB-induced transmembrane current resulting from the introduction of 5 μM RH 421 into the aqueous bilayer bathing solutions are presented in Fig. 3. The effect of RH 421 was also dependent on the membrane's phospholipid content. From the data in Table 1, one can see that RH 421 increased I_∞ through DPhPC- and DPhPS-bilayers, but it did not alter steady-state current through bilayers made from a mixture of ergosterol and POPC, DOPC, DOPE, or DOPS. Table 1

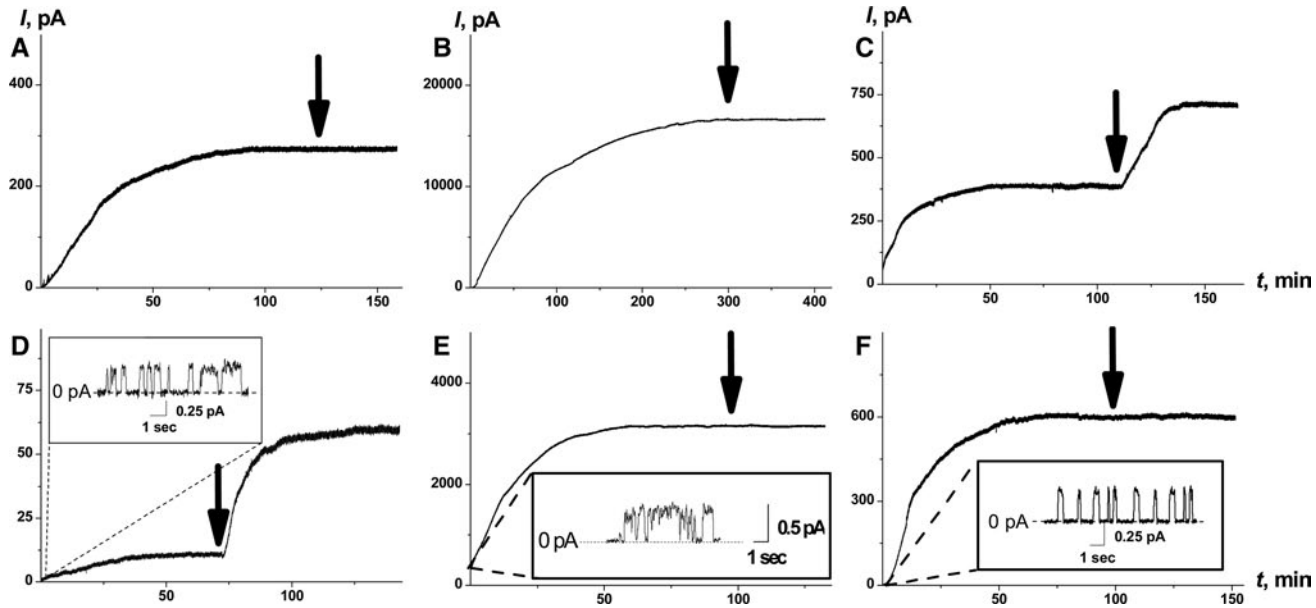

Fig. 2 The effect of phloretin on the steady-state transmembrane current induced by AmB. The membranes were composed of DPhPC/Erg (67:33 mol %) (**a**), DPhPS/Erg (67:33 mol %) (**b**), POPC/Erg (67:33 mol %) (**c**), DOPC/Erg (67:33 mol %) (**d**), DOPE/Erg (67:33 mol %) (**e**), or DOPS/Erg (67:33 mol %) (**f**), and were bathed in 2.0 M KCl, pH 7.0. The moment at which 20 μM phloretin was added to the bilayer bathing solution is indicated by an *arrow*. $V = 50$ mV

Table 1 The mean ratios (I_∞/I_∞^0) of the steady-state transmembrane currents induced by AmB in the presence (I_∞) and absence (I_∞^0) of dipole modifiers (phloretin and RH 421) in phospholipid/Erg (67:33 mol %) bilayers bathed in 2.0 M KCl, pH 7.0

Dipole modifier	Membrane lipid composition					
	DPhPC/Erg*	DPhPS/Erg	POPC/Erg	DOPC/Erg	DOPE/Erg	DOPS/Erg
Phloretin	0.8 ± 0.1*	1.0 ± 0.1	12.2 ± 9.6	3.9 ± 2.5	1.0 ± 0.1	1.0 ± 0.1
RH 421	15.2 ± 6.1*	42 ± 23	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1

* The results for the DPhPC/Erg bilayers are from Ostroumova et al. (2012b)

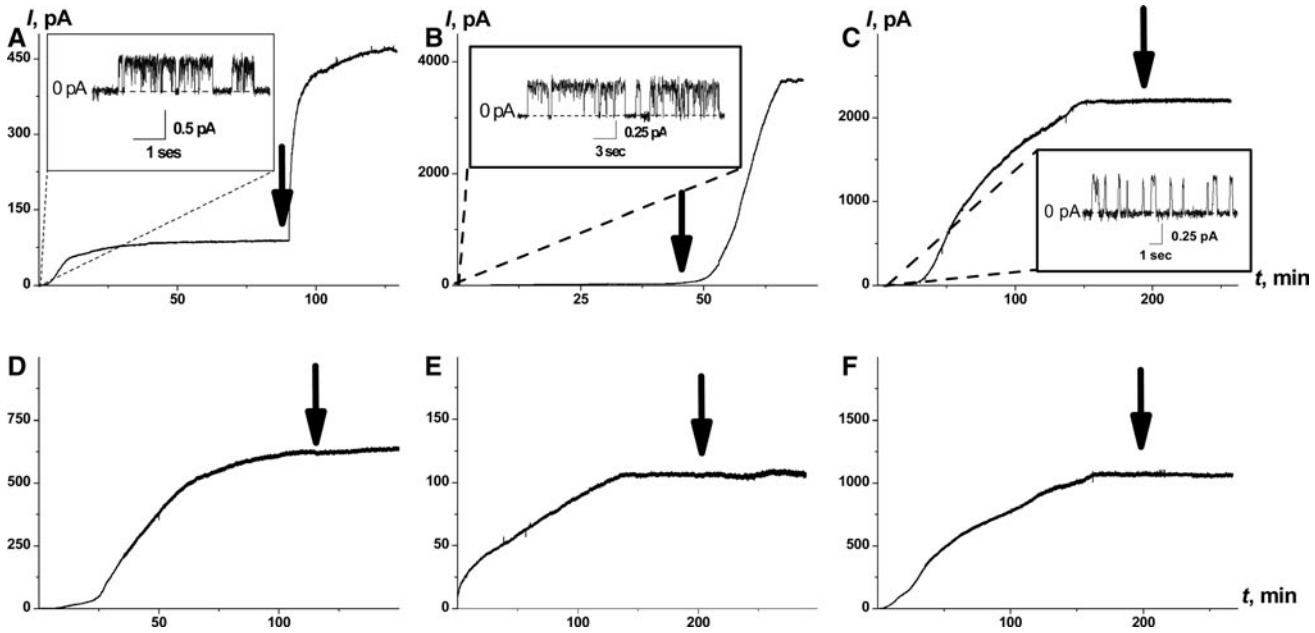

Fig. 3 The effect of RH 421 on the steady-state transmembrane current induced by AmB. The membranes were composed of DPhPC/Erg (67:33 mol %) (**a**), DPhPS/Erg (67:33 mol %) (**b**), POPC/Erg (67:33 mol %) (**c**), DOPC/Erg (67:33 mol %) (**d**), DOPE/Erg (67:33 mol %) (**e**), or DOPS/Erg (67:33 mol %) (**f**), and were bathed in 2.0 M KCl, pH 7.0. The moment at which 5 μM RH 421 was added to the bilayer bathing solution is indicated by an *arrow*. $V = 50$ mV

demonstrates that the effect of RH 421 is related to the phytanoyl chains and is independent of the type of amino alcohol in the polar head (choline or serine).

To investigate the possible role of other membrane components, such as sphingolipids, the AmB-induced steady-state transmembrane currents through bilayers containing different sphingolipids were compared. Figure 4a shows that phloretin increased I_∞ through the DPhPC/Erg/PhSG membrane. It should be mentioned that phloretin did not affect the AmB induced steady-state current through DPhPC/Erg membranes in the absence of phytosphingosine (Table 1). Herewith phloretin did not affect the AmB modified POPC/Erg-bilayers and DOPC/Erg-bilayers in the presence of phytosphingosine (Table 2) despite the fact that phloretin increased I_∞ through these membranes in the absence of this sphingolipid (Fig. 2; Table 1). Figure 4b shows that the introduction of RH 421 led to an increase in I_∞ through DPhPS/Erg/PhSG bilayers. Surprisingly, RH 421 did not alter AmB induced steady-state current through DPhPC/Erg/PhSG bilayers (Table 2) even though it affected I_∞ through both DPhPS/Erg and DPhPC/Erg membranes in the absence of a sphingolipid (Fig. 3; Table 1). One can assume that the charge of the amino alcohol moiety and the phytanoyl chains are important factors affecting the response to dipole modifiers of AmB-modified phytosphingosine-containing bilayers. Substitution of phytosphingosine for sphinganine or sphingomyelin caused the loss of RH 421 effect on AmB modified DPhPS/Erg-bilayers (Table 3). Phloretin did not influence I_∞ through DPhPS/Erg/SG and DPhPS/Erg/SM bilayers (Table 3).

Phytosphingosine and ergosterol are ubiquitous constituents of the plasma membranes of fungi cells (Rest et al. 1995). The observed increase in AmB's channel-forming activity in DPhPC/Erg/PhSG membranes and DPhPS/

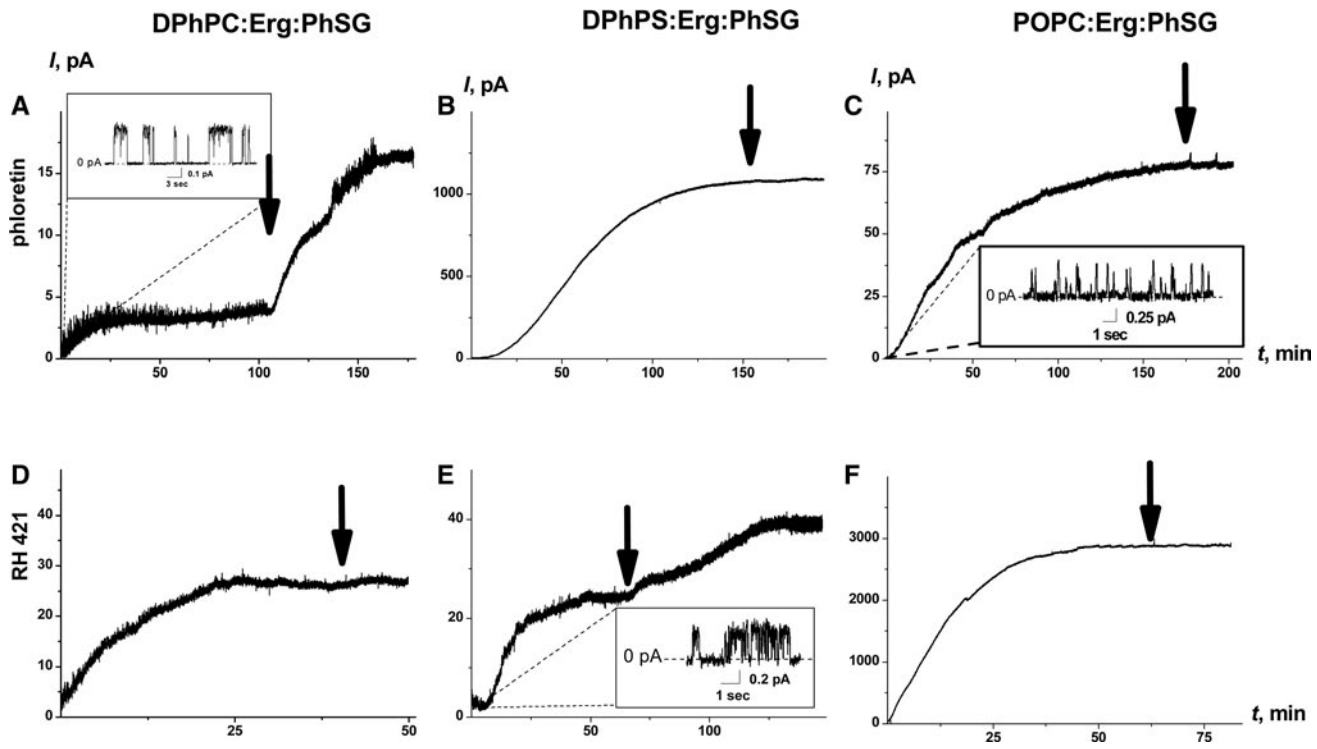

Fig. 4 The effects of dipole modifiers on the steady-state transmembrane current induced by AmB in sphingolipid containing bilayers. The membranes were composed of DPhPC/Erg/PhSG (53:27:20 mol %) (**a, d**), DPhPS/Erg/PhSG (53:27:20 mol %) (**b, e**), POPC/Erg/PhSG (53:27:20 mol %) (**c, f**) and were bathed in 2.0 M KCl, pH 7.0. The moment at which 20 μM phloretin (*upper panel*) or 5 μM RH 421 (*low panel*) was added to the bilayer bathing solution is indicated by an *arrow*. $V = 50$ mV

Table 2 The mean ratios (I_∞/I_∞^0) of the steady-state transmembrane currents induced by AmB in the presence (I_∞) and absence (I_∞^0) of dipole modifiers (phloretin and RH 421) in phospholipid/Erg/PhSG (53:27:20 mol %) bilayers bathed in 2.0 M KCl, pH 7.0

Dipole modifier	Membrane lipid composition					
	DPhPC/Erg/PhSG	DPhPS/Erg/PhSG	POPC/Erg/PhSG	DOPC/Erg/PhSG	DOPE/Erg/PhSG	DOPS/Erg/PhSG
Phloretin	2.3 ± 1.4	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
RH 421	1.0 ± 0.1	1.7 ± 0.4	1.3 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

Table 3 The mean ratios (I_∞/I_∞^0) of the steady-state transmembrane currents induced by AmB in the presence (I_∞) and absence (I_∞^0) of dipole modifiers (phloretin and RH 421) in DPhPS/Erg/sphingolipid (53:27:20 mol %) bilayers bathed in 2.0 M KCl, pH 7.0

Dipole modifier	Membrane lipid composition	
	DPhPS/Erg/SG	DPhPS/Erg/SM
Phloretin	0.9 ± 0.2	1.0 ± 0.1
RH 421	1.1 ± 0.2	1.0 ± 0.1

Erg/PhSG bilayers after phloretin and RH 421 addition, respectively (Table 2), prompted us to compare the susceptibilities of *Saccharomyces cerevisiae* to AmB alone and in combination with dipole modifiers. According to Werner and Morgan (2009), phloretin is metabolized by yeast cells. For this reason, the influence of phloretin on yeast cells has not been studied. Using the paper disk agar diffusion method, we have recently demonstrated that RH 421 enhanced the antimicrobial effect of AmB leading to an increase in the zone of growth inhibition of *S. cerevisiae* strain by 1.5 times (Mikhailova et al. 2013). Here, we compared the minimal inhibitory concentrations of AmB and its combination with RH 421. RH 421 was included in the medium at a constant concentration of 1.5 μM. The presence of RH 421 in combination with AmB resulted in a decrease in the MIC compared to AmB alone from 0.075 to 0.06 μg/ml. In addition, we determined the concentration of viable cells after 24 h of growth in 0.05 μg/ml AmB with and without RH 421 in the media. The number of viable cells in the media containing only AmB was 68 % higher than the number of viable cells in the media containing the

combination of AmB and RH 421 ($p < 0.01$). These tests demonstrate that RH 421 can modify the antifungal activity of AmB in vitro.

Discussion

This study aimed to probe the interactions between AmB and various membrane components such as sterols, phospholipids and sphingolipids by examining the polyene channel forming activity in the presence of dipole modifiers. In this regard, it should be pointed out that these interactions (AmB-sterol, AmB-phospholipid, and AmB-sphingolipid) are most likely not independent but interdependent; i.e., one interaction could promote and/or prevent the others. Moreover, different membrane components may affect AmB–AmB-interaction. Thus, Neumann et al. (2013) showed that AmB self-aggregation is less favorable in ergosterol-containing membranes compared to cholesterol-containing ones. This leads to increase of the concentration of active AmB monomers which are able to form specific pore-forming AmB-ergosterol complexes or to bind with membrane lipids.

We found that the structure of phospholipid greatly influence the effects of dipole modifiers on the AmB induced steady-state transmembrane current (Table 1). We varied both the acyl chain and head group structure of phospholipids and tested DPhPC, DPhPS, POPC, DOPC, DOPE, and DOPS. As DPhPC differs from DOPC and POPC by not only the absence of double bounds but also by the branched structure of its acyl chains, so one can assume that bilayers from unbranched fully saturated phospholipids, for example, dipalmytoylphosphocholine (DPPC), should be compared with membranes made from unbranched and unsaturated DOPC and POPC to make a conclusion about the effects of acyl chain saturation. However, it is impossible due to different packing density of DPPC and DOPC molecules in the membranes. According to literature data, the area per DPPC molecule is about 50 A^2 while this parameter is about 70 A^2 for both DOPC and DPhPC molecules (Pownal et al. 1987; Tristram-Nagle et al. 1993; Reddy et al. 2012). It is known that the phloretin effect significantly depends on the density of lipid molecules in the membranes (Lairion and Disalvo 2004). This indicates that the comparison of bilayers characterized by the close areas per lipid molecules, i.e., composed of DOPC and DPhPC should be done.

Matsumori et al. (2009) demonstrated a reverse correlation between AmB-phospholipid and AmB-sterol interactions: stronger binding between phospholipid and AmB relatively weakens the AmB-sterol interaction, while weaker interaction of phospholipid with AmB allows the AmB-sterol interaction. DPhPC and DPhPS have bulky tails, DOPE and DOPS have a relatively small and large head groups, respectively, such that these lipids take the shape of cones/inverse cones, whereas DOPC and POPC take the shape of cylinders (Sakuma et al. 2010; Bezrukov 2000). One can assume that DOPC and POPC better complement the rigid AmB molecule than other tested phospholipids. Stronger AmB-POPC(DOPC) interaction may impede AmB-sterol in the vicinity. We speculate that phloretin due to its high flexibility and four hydroxyls can play the role of a mediator for the formation of hydrogen bonds between the OH-group of ergosterol and the AmB polar head, which stabilizes the complex similar to the AmB-cholesterol-complex (Ostroumova et al. 2012b). This assumption was made basing on the data by Neumann et al. (2010). The authors demonstrated that AmB-cholesterol- and AmB-ergosterol-complexes had different molecular geometries. Due to their rigid form and parallel orientation, ergosterol and AmB in the AmB-ergosterol-complex are held together by stronger van der Waals forces than are observed between cholesterol and AmB in the AmB-cholesterol-complex. We speculated that phloretin can stabilize the AmB-cholesterol-complex by leading to the formation of additional mediated point-to-point interactions that maximize van der Waals forces. Following this logic, one can think that a very poor ordering ability of branched phospholipids prevents the strong interaction with the rigid AmB molecule and does not significantly change AmB-ergosterol interaction. We previously proposed that RH 421 induced an increase in channel forming activity of AmB in DPhPC-ergosterol bilayers that is a result of the dipole modifier contribution to a network of hydrogen bonds and π-π electronic interactions between the ergosterol and AmB molecules stabilizing the complex (Ostroumova et al. 2012b).

From Table 2 one can see that the addition of sphingolipids into the membrane significantly affects the AmB-phospholipid interactions. We found that phloretin slightly increased AmB-induced steady-state transmembrane current through DPhPC/Erg/PhSG bilayers while RH 421 insignificantly enhanced polyene induced steady-state transmembrane current only through DPhPS/Erg/PhSG bilayers. Substitution of phytosphingosine for sphinganine or sphingomyelin caused the loss of the RH 421 effect (Table 3). One possibility to explain the obtained results is a stronger interaction between AmB and saturated phytosphingosine that promote AmB-Erg complex formation. It is known that AmB molecules have a higher affinity for ordered lipid domains (Czub and Baginski 2006) that are enriched in sterols and sphingolipids. So that another possible reason could be the dependence of properties of such liquid-ordered domains on sphingolipid and phospholipid membrane content. Bakht et al. (2007) showed that phospholipids with low melting temperature may induce the formation of domains in which they do not locate. This

ability is lipid structure dependent, and it is decreased in the following order: DPhPC > DPhPS > POPC = DOPC. Phytosphingosine differs from sphinganine with one hydroxyl group in the C4-position. Work by Idkowiak-Baldys et al. (2004) showed that C4-hydroxylation influences the physical and structural properties of lipid microdomains. This is likely as C4-hydroxylation promotes condensation of lipid lateral packing due to increased hydrogen bonding (Lofgren and Pascher 1977). The ability to affect lipid ordering and to promote rafts is also sterol type dependent (Hsueh et al. 2007; Cournia et al. 2007; Róg et al. 2009). The plasma membranes of fungi cells contain phytosphingosine and ergosterol (Rest et al. 1995), while plasma membranes of mammalian cells have sphingomyelin and cholesterol (Simons and Toomre 2000). Evolutionary preference to a given combination may be associated with the different properties of liquid-ordered domains. Sterol-dependent membrane activity of AmB suggests that the observed therapeutic efficacy of AmB might be related to a differential preference between sterols found in cell membranes. Our data might also suggest that AmB selectivity relates to both sterol and sphingolipid composition of target cell membranes.

Conclusions

In our recent work (Ostroumova et al. 2012b), we showed that the effects of dipole modifiers on the channel-forming activity of macrolide polyenes in sterol-containing bilayers were dependent on sterol, polyene and modifier types and may be attributed to the influence of the dipole modifiers on the stability of the different polyene/sterol complexes that form the ion-permeable pores. Taken together, the above findings and the results obtained in this study clearly indicate that phospholipids and sphingolipids greatly modulated the polyene channel forming activity as well as sterols do. This gave grounds to speculate that phospholipid and sphingolipid molecules may be also involved in the formation of pores, together with polyene and sterol molecules. These results might have fundamental importance in understanding the molecular mechanisms of the effects of polyenes on membranes.

Here we compared the susceptibilities of *Saccharomyces cerevisiae* to AmB alone and in combination with RH 421 and demonstrated that RH 421 can increase the antifungal activity of AmB in vitro. These results may have implications for the clinical use of polyenes, including their lipid-associated formulations.

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