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Membrane dipole modifiers modulate single-length nystatin channels via reducing elastic stress in the vicinity of the lipid mouth of a pore

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ABSTRACT

The polyene antifungal antibiotic nystatin confers its biological activity by forming pores in the membranes of target cells. Exposure of only one side of the membrane to nystatin is more relevant than two-side exposure because in vivo antibiotic molecules initially interact with cell membrane from the exterior side.

The effect of flavonoids and styryl dyes on the steady-state conductance induced by a *cis*-side addition of nystatin was investigated by using electrophysiological measurements on artificial membranes. The assessment of changes in membrane dipole potential by dipole modifiers was carried out by their influence on K^+-nonactin (K^+-valinomycin) current. The alterations of the phase segregation scenario induced by nystatin and flavonoids were observed via confocal fluorescence microscopy.

The introduction of phloretin, phlorizin, biochanin A, myricetin, quercetin, taxifolin, genistin, genistein, and RH 421 leads to a significant increase in the nystatin-induced steady-state transmembrane current through membranes composed of a mixture of DOPC, cholesterol and sphingomyelin (57:33:10 mol%). Conversely, daidzein, catechin, trihydroxyacetophenone, and RH 237 do not affect the transmembrane current. Three possible mechanisms that explain the observed results are discussed: changes in the membrane dipole potential, alterations of the phase separation within the lipid bilayer, and influences of the dipole modifiers on the formation of the lipid mouth of the polyene pore.

Most likely, changes in the monolayer curvature in the vicinity of *trans*-mouth of a nystatin single-length channel prevail over alterations of dipole potential of membrane and the phase segregation scenarios induced by dipole modifiers.

1. Introduction

Nystatin is one of the most commonly used antibiotics to treat human fungal infections [1–3]. Polyene antibiotics predominantly impart their biological activity via pore formation in the membranes of target pathogenic cells [2,4]. One-side addition of polyenes to the membrane is considered to be biologically relevant because in vivo nystatin molecules initially interact with the cell membrane from the exterior surface of the membrane. Kleinberg and Finkelstein [5] hypothesized that nystatin forms two distinct types of channels in sterol-containing lipid membranes. That is, when nystatin is added to one side of planar lipid membranes, single-length channels form; when added to both sides, double-length channels form. The steady-state transmembrane current induced by one-side addition of nystatin is achieved in tens of minutes or faster; in contrast, addition to both sides requires hours to reach a steady-state [6]. In addition, different concentrations of nystatin in membrane bathing solutions are required to induce measurable transmembrane currents. The sidedness of nystatin addition leads to the formation of channels with different selectivities, which suggests that they are significantly different species [2,5,7].

Despite the high therapeutic efficiency of polyenes, serious side effects limit their pharmaceutical applications [3,8,9]. One possible method to improve their therapeutic efficiency is to use a combination of polyenes and other biologically active agents that may enhance the activity of polyenes. To this end, membrane dipole modifiers, such as flavonoids and styryl dyes, are especially attractive because their influence on the channel forming activity of several different antimicrobial agents has been established [10–18]. Flavonoids are a class of polyphenols that are found ubiquitously in plants. Their biological activity is related to antioxidant, anti-allergic, anti-inflammatory, antimicrobial, and anticancer properties [19–21]. In vitro studies indicate that some flavonoids alter lipid packing [22–24] and decrease the membrane dipole potential [25,26]. Synthetic styryl dyes are widely used in bio-labeling and in medicinal analysis [27] and may be applied for the modulation of membrane properties [17,18]. As shown by Ostroumova et al. [18] flavonoids and some other dipole modifiers may alter the steady-state transmembrane current induced by the two-sided addition of polyenes.

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In this paper, some possible mechanisms of action that explain the effects of the dipole modifiers on the transmembrane conductance induced by one-sided action of nystatin are considered. First, changes in the dipole potential may potentiate channel formation via electrostatic interactions. Second, the influences of amphiphilic compounds on the lateral phase separation of lipids within the membrane may expand the area containing the phase that polyene antibiotic prefers. Third, interactions of the dipole modifiers with the channel may lead to the stabilization of the lipid mouth of a pore.

2. Materials and methods

All of the chemicals were reagent grade. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol (Chol), ergosterol (Erg), brain bovine sphingomyelin (SM), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DPPE) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Dimethylsulfoxide (DMSO), phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone), phlorizin (1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one), taxifolin ((2R,3R)-3,3′,4′,5,7-pentahydroxyflavanone, (2R,3R)-dihydroquercetin), genistin (genistein-7-O-β-D-glucopyranoside), genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), daidzein (4′,7-dihydroxyisoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 7-hydroxy-3-(4-hydroxyphenyl)chromone), catechin ((2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol), 2′,4′,6′-trihydroxy-acetophenone monohydrate (THAP), triton X-100 (TX-100), and nystatin A were purchased from Sigma Chemical (St. Louis, MO). RH 237 (N-(4-sulfobutyl)-4-(6-(4-(dibutylamino)phenyl)hexatrienyl)pyridinium, inner salt) and RH 421 (N-(4-sulfobutyl)-4-(4-(4-(dipentylamino)phenyl)butadienyl) pyridinium, inner salt) were purchased from Molecular Probes (Eugene, OR). The water used in this study was double distilled and deionized. The KCl solutions were buffered with 5 mM HEPES, pH 7.0. The chemical structures of the flavonoids, RH dyes, and nystatin are shown in Fig. 1.

Planar lipid bilayers were formed using a monolayer-opposition technique [28] on a 50-μm-diameter aperture in a 10-μm-thick Teflon film that separated the two (*cis*- and *trans*-) compartments of the Teflon chamber. The volume of chamber each was 1.5 ml. The aperture was pretreated with hexadecane. Lipid bilayers were made from 57 mol% DOPC or DOPE, 33 mol% sterol (Chol or Erg), and 10 mol% SM. After the membrane was completely formed, nystatin, from a stock solution (20 mM in DMSO), was added to the *cis*-compartment to a final concentration that ranged from 20 to 40 μM. Ag/AgCl electrodes with agarose/2 M KCl bridges were used to apply the transmembrane voltage (*V*) and

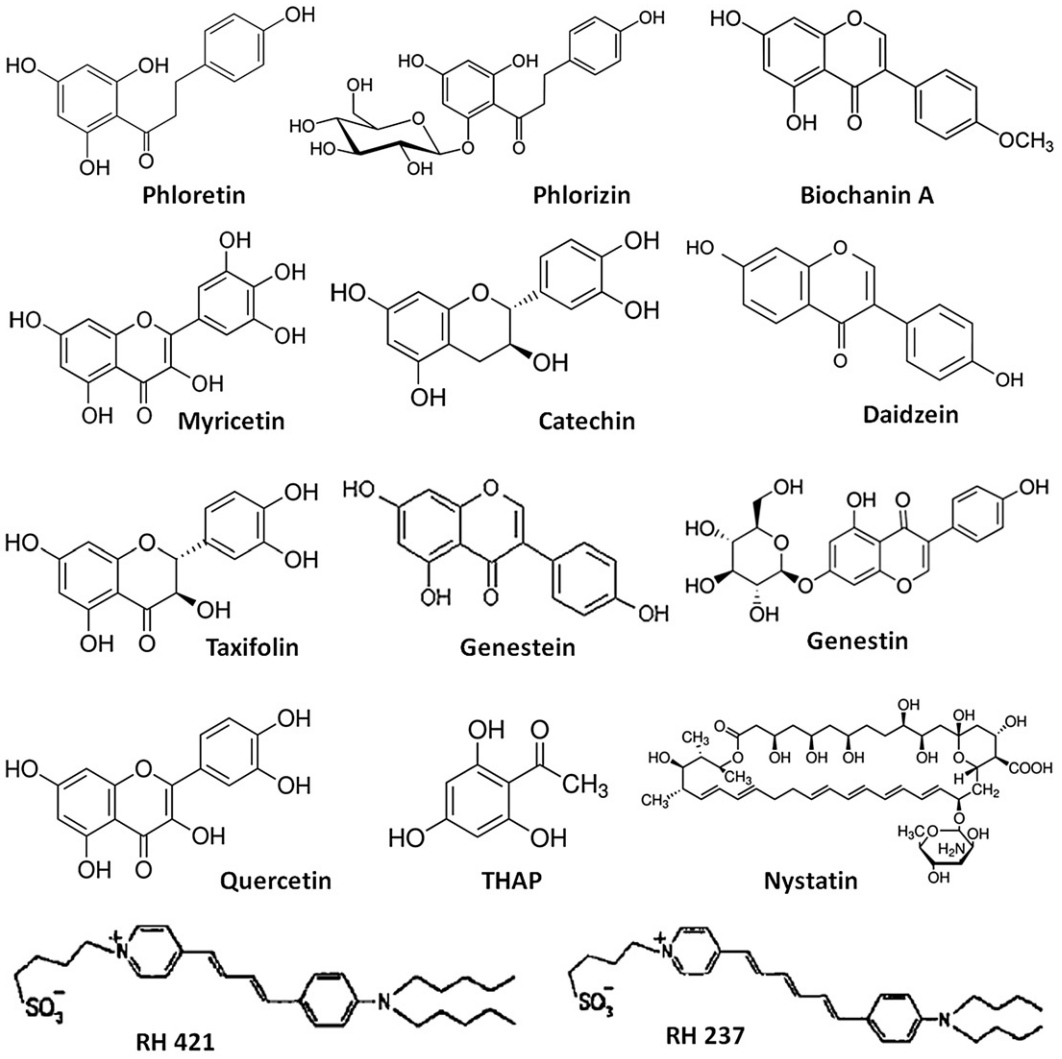

Fig. 1. Chemical structures of tested dipole modifiers and nystatin.

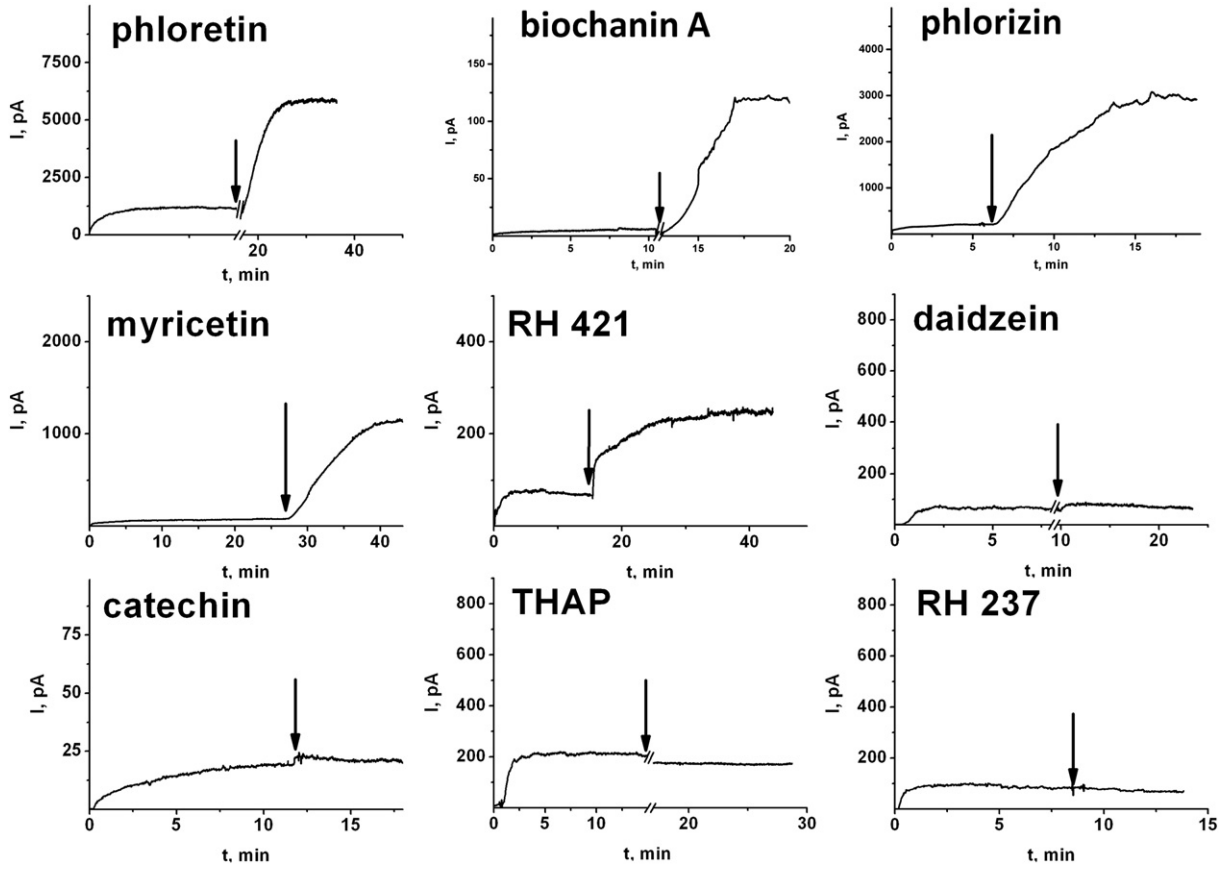

Fig. 2. The effect of both-side introduction of different dipole modifiers on the steady-state transmembrane current induced by one-side addition of nystatin. Arrows indicate the moment of addition of: 20 μM phloretin, phlorizin, biochanin A, myricetin, daidzein, catechin, THAP, or 5 μM RH 421 and RH 237. The membranes were composed of DOPC:Chol:SM (57:33:10 mol%) and bathed in 2.0 M KCl, pH 7.0. The transmembrane voltage was 50 mV.

measure the transmembrane current (I). "Positive voltage" refers to the condition where the *cis*-side compartment was positive with respect to the *trans*-compartment. The nystatin channel-forming activity was modulated by a one-side or both-side addition of flavonoids or styryl dyes using stock solutions in ethanol (or DMSO for genistin and daidzein) to the membrane-bathing solution, yielding final concentrations of 20 or 5 μM for the flavonoids or styryl dyes, respectively. The final concentration of ethanol (or DMSO) in the chamber did not exceed 0.2%. These concentrations of solvents did not affect the integrity of the lipid bilayers and did not increase their conductance. Added alone dipole modifiers did not affect membrane conductance at tested concentrations.

The current measurements were conducted using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. The data were digitized with Digidata 1440A and analyzed using pClamp 10 (Axon Instruments) and Origin 8.0 (OriginLab). The sampling frequency was 5 kHz. The current tracks were processed through an 8-pole Bessel 100-kHz filter. The channel-forming activity of nystatin in the absence and after the introduction of the modifier (flavonoids or styryl dyes) was characterized by a logarithm of steady-state transmembrane current I_∞ under the given experimental conditions (V = 50 mV and the given polyene concentration). Mean logarithm of a ratios $\ln(I_\infty/I_\infty^0)$ of steady-state transmembrane current induced by nystatin in the presence (I_∞^0) and in the absence of modifiers (I_∞^0) were averaged from 4 to 9 bilayers (mean ± standard error).

The alterations in K^+-nonactin (or K^+-valinomycin in cases of the RH dyes) induced steady-state conductance were measured to assess changes in the membrane dipole potential that were induced by each dipole modifier. Nonactin and valinomycin were added over a range of 1–4 μM to both bathing solutions from ethanol stock solutions after the formation of a bilayer. Estimation of the magnitude of these changes was based on the Boltzmann distribution [29]: $G_m/G_m^o = \exp(-q_e\Delta\varphi_d/kT)$, where G_m and G_m^o are the steady-state membrane conductance after and before the addition of dipole modifiers, respectively; $\Delta\varphi_d$ represents the change in the membrane dipole potential after the addition of modifier to both sides of membrane; q_e, k, and T have standard definitions. The membrane bathing solution contained 0.1 M KCl, 5 mM HEPES–KOH, pH 7.0. At least 3 independent experiments were performed for each tested system. The transmembrane voltage was 50 mV.

The influence of the flavonoids on the lateral membrane heterogeneity in the presence of nystatin was investigated via confocal fluorescence microscopy on giant unilamellar vesicles (GUVs) as described by Ostroumova et al. [24]. GUVs were formed by the electroformation method on a pair of indium tin oxide (ITO) slides using a commercial Nanion vesicle prep pro (Munich, Germany). Labeling was carried out by the addition of 1 mol% Rh-DPPE to the chloroform stock solution of lipids. Approximately 20 μl of a lipid stock was placed on the ITO slide in the center of the O-ring. After solvent evaporation, a 0.5 M aqueous sorbitol solution was added to the dry lipid film before being covered with another ITO slide. Alternating voltage with an amplitude of 3 V and a frequency of 10 Hz was applied across the ITO slides for 1 h. Afterwards, the upper ITO slide was removed and the liposome suspension was vigorously taken away. Nystatin, from a 20 mM stock solution in DMSO, was added to the liposome suspension to a concentration of 200 μM and was incubated for 30 min. Then, biochanin A, phloretin, or myricetin from 40 mM stock solutions in ethanol was added to the GUV suspension. The liposome suspension with flavonoid was allowed to equilibrate for 15 min at room temperature. The final flavonoid concentration in the sample was 400 μM, and the ethanol concentration was 1% v/v. The addition of ethanol and DMSO up to 1% v/v did not have an effect on the character of the lipid phase separation. These samples,

Table 1
The changes in the logarithm of nystatin induced steady-state transmembrane current $\ln(I_\infty/I_\infty^0)$ and changes in the membrane dipole potential ($\Delta\varphi_d$) after both-side addition of different dipole modifiers. The membranes were composed of DOPC:Chol:SM (57:33:10 mol%). The transmembrane voltage was 50 mV.

	Phloretin	Phlorizin	Biochanin A	Myricetin	Quercetin	Taxifolin	Genistin	Genistein	Daidzein	Catechin	THAP	RH 237	RH 421
$\ln(I_\infty/I_\infty^0)$[a]	2.5 ± 1.2	2.4 ± 1.6	2.9 ± 1.1	2.7 ± 1.4	3.3 ± 0.4	3.4 ± 1.7	1.1 ± 0.5	1.3 ± 0.6	0.1 ± 0.2	0.1 ± 0.1	−0.1 ± 0.0	0.2 ± 0.1	2.7 ± 1.2
$\Delta\varphi_d$[b], mV	−153 ± 18	−104 ± 5	−97 ± 7	−125 ± 10	−152 ± 12	−17 ± 2	−1 ± 1	−48 ± 10	−8 ± 1	−10 ± 7	−19 ± 5	66 ± 14	54 ± 10

[a] The natural logarithm of ratios of the nystatin induced steady-state transmembrane currents in the presence (I_∞) and in the absence (I_∞^0) of dipole modifiers is presented. The membranes were bathed in 2 M KCl, pH 7.0.
[b] The membranes were bathed in 0.1 M KCl, pH 7.0. Concentrations of flavonoids and RH dyes in the membrane bathing solution were equal to 20 and 5 μM, respectively.

as a standard microscopy preparation, were imaged through an oil immersion objective 100×/1.4 HCX PL in a Leica TCS SP5 confocal laser system Apo (Leica Microsystems, Mannheim, Germany). Rh-DPPE was excited at wavelengths of 543 nm (helium–neon laser). All of the experiments were performed at room temperature. Because the fluorescence probe prefers the liquid disordered phase (ld), the solid ordered (gel) phase (so) remained uncolored [30]. The number of GUVs within single field of view with and without visible phase separation was counted. Several neighboring fields of view were analyzed. The percentage of phase separated vesicles (p_g) at each tested system was calculated as the ratio of phase-separated GUVs to the total number of GUVs: $p_g = N_g/N_t \cdot 100$ %, where N_g — number of vesicles with uncolored domains, N_t — total number of counted vesicles in sample (from 50 to 100).

3. Results and discussion

3.1. Altering the steady-state transmembrane current by dipole modifiers

Fig. 2 presents the changes in the transmembrane current induced by the *cis*-side addition of nystatin to DOPC:Chol:SM (57:33:10 mol%) membrane after the introduction of different dipole modifiers to both (*cis*- and *trans*-) compartments. Phloretin, phlorizin, biochanin A, myricetin, and RH 421 increased nystatin induced transmembrane current, while daidzein, catechin, THAP, and RH 237 did not affect it. Table 1 shows the summary of mean logarithms of the ratios ($\ln(I_\infty/I_\infty^0)$) of transmembrane currents before (I_∞^0) and after (I_∞) addition of the modifiers.

3.1.1. Nystatin single-length channels in DOPC:Chol:SM membranes

Step-like fluctuations induced by *cis*-side addition of nystatin to DOPC:Chol:SM membrane are not resolved at given conditions (concentration of electrolyte, pH and etc.). To prove the channel formation induced by one-side addition of nystatin in appropriate lipid composition additional experiments were performed. Fig. 3A presents step-like fluctuations induced by *cis*-side addition of nystatin to DOPC:Chol:SM membrane at acidic bathing solution (pH 2.5). At low pH polyenes added from one side form more stable channels [31] probably due to the protonation of COOH group of antibiotic molecule which becomes less exposed to water that facilitate the formation of more stable pore. From Fig. 3B one can see the spectrum of current noise induced by one-side action of nystatin at neutral pH. The shape of this spectrum indicates that the transmembrane current is induced by ion channels, i.e. it represents Lorentzian spectrum with frequency-independent limit at low frequencies and a decline toward high frequencies: $S(f) \sim 1/(1 + (f/f_c)^2)$, where $S(f)$ — spectral density of noise, f — frequency, and f_c — corner frequency. In contrast to the present spectrum, carrier or hydrophobic ions transport through membrane used to shows "white" or inverse Lorentzian spectra of current noise which increase toward high frequencies [32,33].

Additionally Fig. 3C presents the dependence of steady-state conductance of a DOPC:Chol:SM bilayer treated with *cis*-side nystatin on transmembrane voltage. A positive voltage represents a much higher conductance compared to a negative one. Kleinberg and Finkelstein [5] also observed a similar dependence of the pore opening of single-length nystatin channels on the sign of the transmembrane voltage. Since generally conductance of single-length polyene channels does not depend on the sign of transmembrane voltage [5,31] the increase in steady-state membrane conductance at a positive voltage may be attributed to the increase in the number of open channels.

3.2. Role of dipole potential in the regulation of nystatin activity

It is known that a membrane has a positive dipole potential of about hundreds millivolts inside the lipid tail region [29]. Also flavonoids decrease while RH dyes increase dipole potential [27,29,34]. Actually the dependence of the membrane conductance induced by the *cis*-side

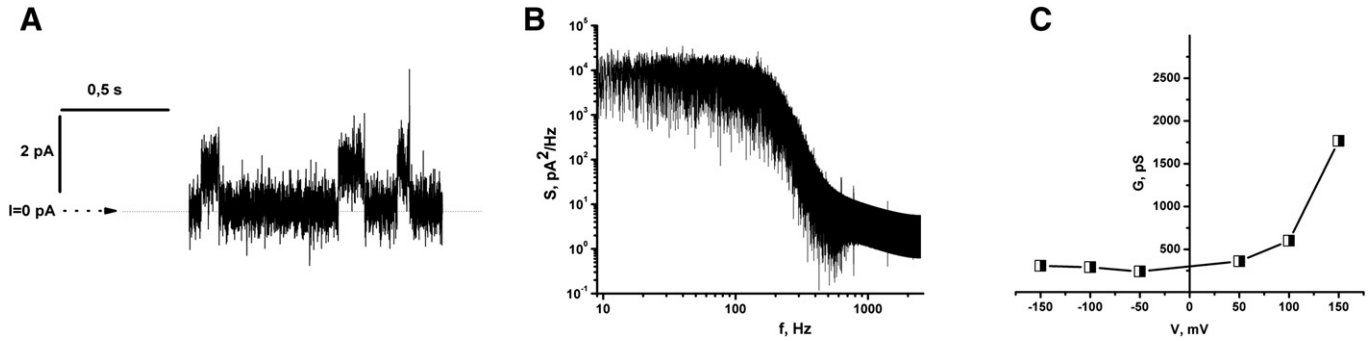

Fig. 3. A — current fluctuations of bilayer treated with nystatin from *cis*-side. The membrane was composed of DOPC:Chol:SM (57:33:10 mol%) and bathed in 3 M KCl, pH 2.5. The transmembrane voltage was 100 mV. B — spectral density (S) of current noise from DOPC:Chol:SM membrane bathed in 2 M KCl, pH 7.0 treated with 20 μM nystatin from *cis*-side. The transmembrane voltage was 100 mV. The sampling frequency was 5 kHz and cutoff frequency 100 kHz. C — the dependence of steady-state conductance of bilayer treated with nystatin from *cis*-side on transmembrane voltage. The membrane was composed of DOPC:Chol:SM (57:33:10 mol%) and bathed in 2 M KCl, pH 7.0.

addition of nystatin on the sign of the applied potential may be attributed to an interaction between the polyene dipole with the electric field (Fig. 3C). Czub and Baginsky [35] showed that the positive charge of the dipole of amphotericin B is embedded more deeply in the hydrophobic core of the membrane than the negative charge. Similar study related to the distribution of charge in single-length amphotericin channel showed its asymmetry [36]. Thus, the positive transmembrane potential may facilitate the formation of amphotericin B and nystatin channels. In addition to transmembrane voltage, the dipole potential also contributes to the intrinsic electric field of the membrane hence the reduction of the membrane dipole potential would therefore promote channel formation. For the sake of testing this assumption we measured the $\Delta\varphi_d$ of DOPC:Chol:SM membrane that was caused by the addition of 20 μM of flavonoids or 5 μM of RH dyes. Phloretin, phlorizin, biochanin A, myricetin, quercetin, and genistein significantly decreased φ_d; RH 421 and RH 237 increased φ_d; and taxifolin, genistin, daidzein, catechin, and THAP insignificantly affected φ_d (Table 1). Assumption that the dipole potential plays a key role in the regulation of the conductance induced by the one-side addition of nystatin is not provided by increasing the transmembrane current in the presence of RH dyes which enlarge φ_d.

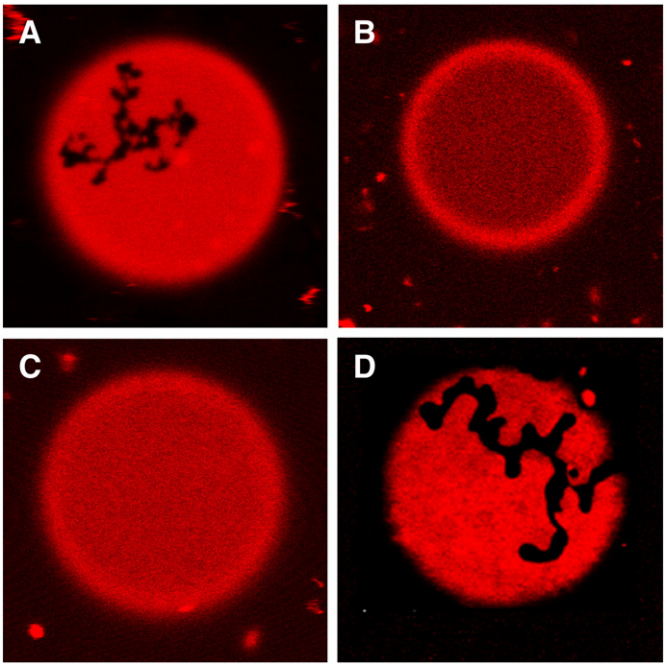

Fig. 4. Micrographs of typically encountered DOPC:Chol:SM GUVs in suspension containing 200 μM nystatin. In the absence of flavonoids (A) and in the presence of 400 μM phloretin (B), biochanin A (C), and myricetin (D). Image size is 26 × 26 μm.

3.3. Does lateral heterogeneity of membrane involve in the regulation of nystatin channel activity?

The existence of the lateral heterogeneity of lipid membranes composed of ternary mixtures of DOPC, Chol, and SM may complicate the interpretation of the results. Many studies have shown that polyenes prefer more rigid bilayers, i.e. their distribution throughout a membrane is dependent on the physical state of the lipids [37–39]. The most widely accepted channel model includes sterols as a component of a barrel-like polyene pore [4]. It is known that sterols have differential orientations in ordered and disordered lipid phases. Particularly, the angle between the long axis of the cholesterol and the bilayer normal is 10° in the ordered phase, while it is approximately 40° in the disordered phase [40]. It allows one to believe that polyene conducting units prefer more ordered lipid phases because the sterols in the ordered phase would support the barrel-like structure and orient it along the bilayer normal. The transmembrane current (I) should be considered as a sum of the independent components: $I = \sum_{(p)} j_p S_p$, where j, S, and p represent the current density, the phase area, and the type of phase (e.g., liquid disordered and solid ordered), respectively. Dipole modifiers may fluidize or condensate the membrane [23,24,41]. As a result, the effect of dipole modifiers on the nystatin-induced current can be mediated by their influence on the phase separation.

We examined the effects of 400 μM phloretin, biochanin A, and myricetin on lipid phase separation in GUVs that were previously treated with 200 μM of nystatin. Confocal fluorescence microscopy of GUVs was used to demand high concentrations of substances because the resolution of light microscopy is limited. Altering of tested compounds on the phase separation becomes more pronounced and easier for detection at high concentration both antibiotic and modifiers. Actually, the action of flavonoids on the phase segregation scenarios is significantly manifested around the concentration of 400 μM [24]. Moreover the ability of flavonoids to penetrate through the lipid bilayer leads to their location on both sides of GUV membrane. 10-fold increased concentration of nystatin was chosen for the sake of observing well-defined solid ordered domains induced by it which poorly appears at 20 μM [42].

Fig. 4 represents the micrographs of typically encountered nystatin treated GUVs formed from DOPC:Chol:SM in the absence and presence of the tested modifiers. One can see that in the presence of nystatin, solid ordered domains were observed (Fig. 4A). The addition of biochanin A or phloretin led to the disruption of these domains (Fig. 4B, C), while myricetin did not affect the phase separation (Fig. 4D). A statistical description of the GUV data is given in Fig. 5.

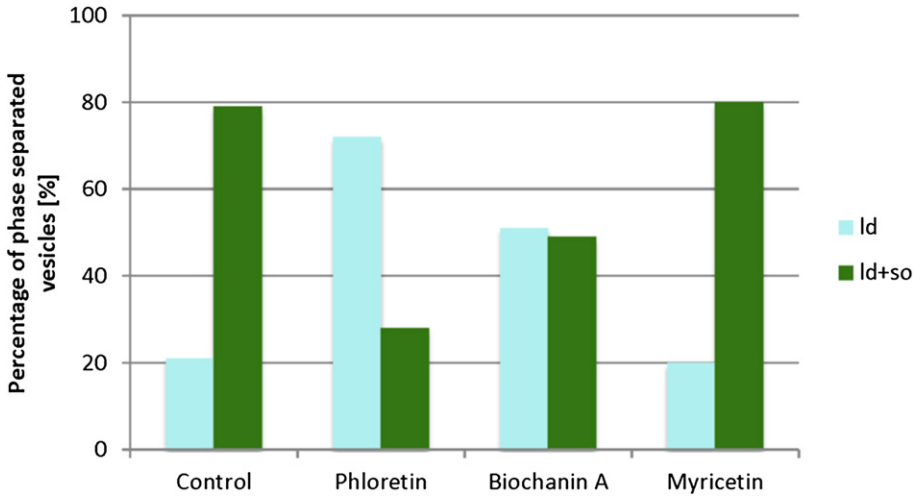

Fig. 5. Diagrams of the percentage distribution of the GUVs made from DOPC:Chol:SM and treated with 200 μM nystatin in the absence (control) and in the presence of 400 μM of phloretin, biochanin A, and myricetin in suspension.

More than 500 GUVs were measured in 9 independent experiments. Fig. 5 (control) shows that approximately 80% of the GUVs treated with nystatin had solid ordered domains (so), while the addition of 400 μM of phloretin reduced this percentage to 30% (Fig. 5, phloretin). Four hundred micromolar biochanin A decreased the fraction of the phase separated vesicles to 50% (Fig. 5, biochanin A). The presence of 400 μM myricetin in suspension did not change the number of GUVs containing solid ordered domains (Fig. 5, myricetin). The data mentioned above indicate that biochanin A and phloretin led to disrupting solid ordered domains induced by nystatin, while myricetin did not. One can see that the increased conductance induced by nystatin added from the *cis*-side in the presence of phloretin, biochanin A, and myricetin did not correlate with their different influences on the phase segregation scenarios.

3.4. May dipole modifiers affect curvature stress in the vicinity of lipid mouth of nystatin single-length channel?

The barrel-stave channel model of polyene action assumes that when an antibiotic is added to both sides of the membrane, the tail-to-tail connection of the two polyene–sterol half-pores from the opposite monolayers of the lipid membrane forms the conducting pore [4]. Furthermore, nystatin has a highly hydrophilic aminosugar residue that is anchored to the aqueous phase. However, if an antibiotic is added only to one side of the bilayer, a channel spanning the membrane would be expected to have a pore mouth composed of lipids because the length along the long axis of a nystatin molecule (~2 nm) is significantly smaller than the thickness of the membrane (~4 nm). In particular, membranes formed from monoglycerides with acyl chains of approximately 2.3 nm in length are insensitive to nystatin one-sided action [5]. This model implies that the spontaneous curvature of membrane lipids should play a crucial role in the formation of channels with a lipid mouth [43–47]. To test this idea, we substituted DOPC with DOPE, which has a smaller hydrophilic head. The spontaneous curvatures of DOPE and DOPC are −0.4 and −0.1 nm^{-1}, respectively [48]. The larger negative spontaneous curvature of DOPE should prevent the formation of the single-length nystatin channel spanning the membrane. We revealed that approximately 400 μM nystatin in the *cis*-chamber was required to induce the measurable transmembrane current in DOPE containing membranes, while in DOPC containing membranes only 20–40 μM nystatin was needed. This increase in the concentration of the channel forming agent that was required to induce measurable currents supports the inference of the presence of a lipid mouth in a single-length nystatin channel. Indirectly, the findings are

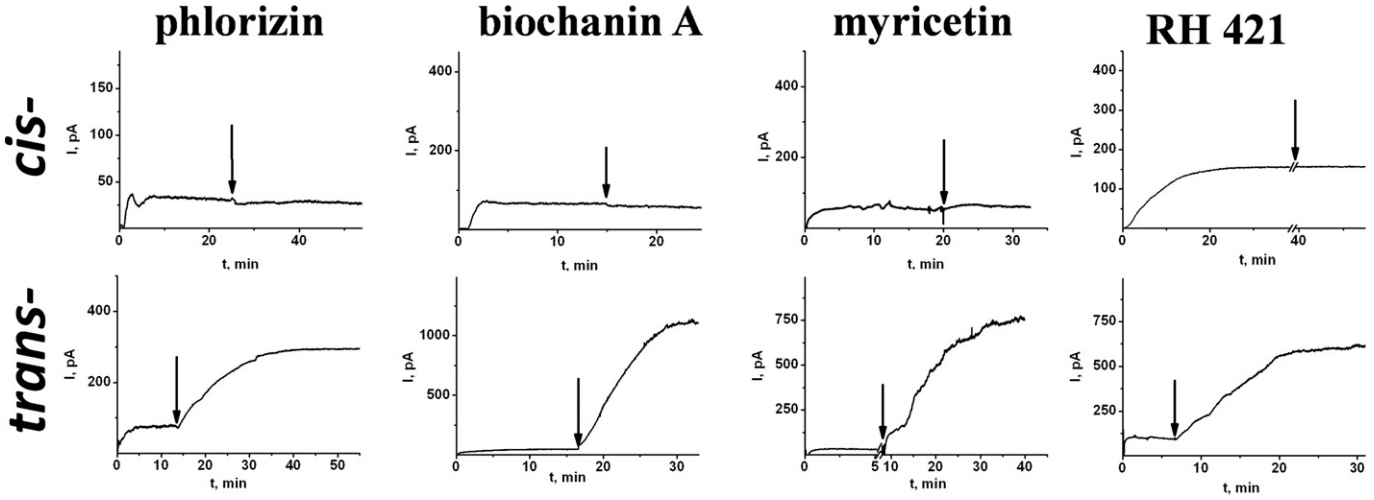

Fig. 6. The effect of *cis*- or *trans*-side addition of the dipole modifiers on the nystatin induced steady-state conductance. Arrows indicate the moment of addition to *cis*- or *trans*-side of 20 μM phlorizin, biochanin A, myricetin or of 5 μM RH 421. The membranes were composed of DOPC:Chol:SM (57:33:10 mol%) and bathed in 2.0 M KCl, pH 7.0. The transmembrane voltage was 50 mV.

Table 2
The logarithm of ratio of the nystatin induced steady-state transmembrane current $\ln(I_\infty/I_\infty^0)$ in the absence (I_∞^0) and in the presence (I_∞) of dipole modifiers from *cis*- and *trans*-side. The membranes were composed of DOPC:Chol:SM (57:33:10 mol%) and bathed in 2 M KCl, pH 7.0. The transmembrane voltage was 50 mV.

	Biochanin A	Myricetin	Phlorizin	RH 421
Cis	−0.1 ± 0.4	−0.1 ± 0.2	−0.1 ± 0.1	0.2 ± 0.2
Trans	2.9 ± 0.6	2.6 ± 1.7	2.7 ± 1.5	2.5 ± 0.8

consistent with the observation that bacteria, with plasma membranes containing predominantly phosphoethanolamines [49], are completely insensitive to polyene antibiotics [1]. Therefore, the energetic cost to form single-length nystatin channels depends on the shape of the membrane lipids and their spontaneous curvature. It may be proposed that the appearance of dipole modifiers reduces elastic stress and increases monolayer curvature in the vicinity of the lipid mouth of the channel that may contribute to a pore formation.

From this point of view, the action of the dipole modifiers on the *cis*- and *trans*-sides of the membrane should be qualitatively different, i.e. a relief of stress in the *trans*-leaflet should promote channel formation, while in the *cis*-leaflet, it should not. Fig. 6 indicates that the addition of the given modifiers to the *cis*-side did not lead to growing conductance, whereas *trans*-addition of phlorizin, biochanin A, myricetin, or RH 421 increased transmembrane current. Moreover, the logarithms of ratios ($\ln(I_\infty/I_\infty^0)$) had the same order of magnitude as the both-side addition of dipole modifiers (Table 2). A proposed model of action is shown in Fig. 7. Most likely, embedding of dipole modifiers into head-group region reduces the free space and modulates geometry of *trans*-leaflet via locally increasing the membrane curvature. These changes reduce the free energy of *trans*-mouth of a channel and facilitate pore formation. Also changes in the free energy of a channel may be estimated on the basis of Boltzmann distribution: $\ln(I_\infty/I_\infty^0)$ gives it in kT units. Additionally well-known membrane active agent TX-100 with high positive spontaneous curvature was tested. It was revealed that both-side addition of 20 μM TX-100 increases the transmembrane current induced by one-side addition of nystatin. Also it is known that flavonoids may induce the transition of lipids into non-bilayer (hexagonal) phase [24] that supports the idea about their role in the orientation of lipid molecules.

Additionally the influence of catechin and taxifolin on *cis*-side nystatin induced transmembrane current was investigated in DOPC:Erg:SM (57:33:10 mol%) and DOPE:Chol:SM (57:33:10 mol%) bilayers. Table 2 presents $\ln(I_\infty/I_\infty^0)$, where I_∞^0 and I_∞ are the transmembrane currents before and after the addition of the dipole modifiers, respectively. Taxifolin significantly increased while catechin did not affect the single-length nystatin channel transmembrane current as in DOPC:Chol:SM membranes. Experiments with varied lipid compositions of membrane (Table 3) allowed us to consider the effects of modifiers on nystatin induced membrane conductance as a non-specific action. With the aim to estimate the number of modifier molecules involved in the interaction with the *trans*-mouth of a single-length nystatin channel we investigated the dependence of nystatin induced transmembrane current on the concentration of phloretin. It was revealed that the slope of growth region of dependence of $lg(I)$ on lg [phloretin] is equal to 2 ± 1. It seems possible that 2 modifier molecules interact with the *trans*-mouth of a pore.

Table 3
The logarithm of ratio of the nystatin induced steady-state transmembrane current $\ln(I_\infty/I_\infty^0)$ after both-side addition of catechin and taxifolin to membranes composed of DOPC:Erg:SM (57:33:10 mol%) and DOPE:Chol:SM (57:33:10 mol%) and bathed in 2 M KCl, pH 7.0. The transmembrane voltage was 50 mV.

	Catechin	Taxifolin
DOPC:Erg:SM	0.0 ± 0.1	3.5 ± 1.6
DOPE:Chol:SM	0.3 ± 0.2	3.3 ± 2.4

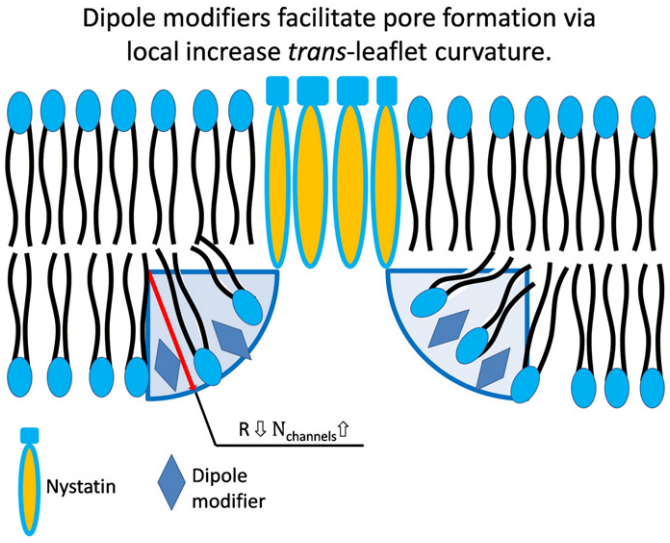

Fig. 7. Schematic illustration of the mechanism of action of dipole modifiers on the *trans*-mouth of the single-length nystatin pore. Dipole modifiers embedded into the region of hydrophilic lipid heads and facilitate the channel formation via a local decrease of radius of curvature near *trans*-mouth of a single-length nystatin channel. Radius of curvature of the *trans*-leaflet in the vicinity of the lipid mouth of a pore is approximately 1 nm.

One point remains unclear: why did some dipole modifiers act on the steady-state transmembrane conductance induced by the one-side addition of nystatin while others did not? Studying the features of the chemical structures of the tested dipole modifiers may reveal possible mechanisms to explain the increase in nystatin-induced conductance caused by the addition of phloretin, phlorizin, biochanin A, myricetin, quercetin, taxifolin, genistin, genistein, and RH 421. Noticeably, all of the tested flavonoids that modulate nystatin activity have a combination of carboxyl and hydroxyl groups (or a carboxyl group and an O-atom in a glycoside bond in a case of phlorizin). Most likely, this moiety participates in an interaction with the membrane components. Of key importance is the formation of two hydrogen bonds between the flavonoid and the O-atoms in the phosphate residue in the phosphocholines, which may localize the flavonoid in the appropriate part of membrane. Phloretin and phlorizin may form hydrogen bonds with the heads of the phospholipids [23]. They compensate locally for the excess of negative membrane curvature in the *trans*-leaflet in the vicinity of the lipid mouth of a pore and decrease the energetic cost of channel formation. The insensitivity of a nystatin current to THAP may be explained by its relatively small size. The discrepancies between the effects of RH 421 and RH 237 on the steady-state transmembrane conductance induced by nystatin may be explained by the difference in how deep they embed into the membrane. RH 237 penetrates more deeply into the membrane than RH 421 [50]. The highly hydrated SO_3^- residue of RH 421 may more effectively compensate the negative curvature of the *trans*-monolayer. Apetrei et al. [51] showed that RH 421-induced membrane elasticity changes alter the activity of pores formed by alamethicin, mellitin, and magainin.

The changes in the monolayer curvature in the vicinity of *trans*-mouth of a nystatin single-length channel probably prevail over alterations of dipole potential of membrane and of the phase segregation scenarios induced by dipole modifiers. The location in the membrane most likely determines the effectiveness of action of dipole modifiers on the steady-state *cis*-side nystatin induced conductance.

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