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Effect of Membrane Structure on the Action of Polyenes: I. Nystatin Action in Cholesterol- and Ergosterol-Containing Membranes

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Abstract A detailed and thorough characterization of nystatin-induced permeability on lipid bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)-containing ergosterol or cholesterol is presented. The results show that the same collection of transmembrane pores appears in membranes containing either sterol. The concentration range for the appearance of these pores is sterol-dependent. Another mechanism of action, membrane disruption, is also observed in ergosterol-POPC membranes. The greater potency of nystatin present in ergosterol-containing membranes cannot be explained simply by the longer opening times of its pores, as has been suggested; it is also due to an increased number of events in these membranes. The present results and those of a companion paper lead us to propose that membrane structure is the determining factor for drug selectivity in membranes with different sterols.

Keywords Nystatin · Permeability model · Membrane structure · Cholesterol · Ergosterol

Introduction

In recent years there has been growing interest in understanding the role that lipid bilayer structure has on the action of transmembrane biomolecules (Phillips et al. 2009; Schmidt et al. 2009; Ursell et al. 2009; Silva et al. 2009; Lundbaek et al. 2004). Of particular interest is the effect that sterols present on the bilayer have on the latter (Eggeling et al. 2009; Pan et al. 2009; Epshtein et al. 2009; Qiu et al. 2009). Polyene antibiotics such as nystatin (Nys) and amphotericin B (AmB) have been studied for more than five decades (Hazen and Brown 1950; Dutcher et al. 1959), precisely because of their known dependence on sterol presence in the membrane. Interest in these molecules is derived mostly from their sustained medical use in systemic fungal infections through induced permeability of the cellular membrane, which has undesirable collateral toxicity (Hartsel and Bolard 1996; Borgos et al. 2006). Over half a century ago, it was proposed that the drug acted via the assembly of pores in the membrane (Andreoli 1974; Finkelstein and Holz 1973; de Kruijff and Demel 1974). These pores facilitated the loss of K^+ from the cell interior, finally leading to cell death (Brown et al. 1953; D'Arcy and Scott 1978). Furthermore, it was thought that the potency of the drug in membranes containing ergosterol (erg) or cholesterol (chol) was due to the influence that sterol had on the molecular structure of the pores (Dennis et al. 1970; Cass et al. 1970). This was supported by the facts that increasing amounts of sterol in the membrane lead to an increase in ion permeability (de Kruijff et al. 1974; Marty and Finkelstein 1975), that drug potency is higher in cells and model membranes when erg is present instead of chol (Dennis et al. 1970; Kinsky 1963), that bacteria are insensitive to these polyenes (D'Arcy and Scott 1978), and that sterols added to the aqueous medium of in vitro experiments compete for the polyene and therefore diminish the effects these drugs have on sensitive organisms (Gottlieb et al. 1958). These observations have contributed to the acceptance of the polyene antibiotics action model based on direct participation of sterol in the polyene

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channel. However, there are experimental observations that do not entirely agree with this proposal, and alternative hypotheses have emerged (Cotero et al. 1998; Venegas et al. 2003; Romine et al. 1977; Vertut-Croquin et al. 1983).

A compendium of the latter experimental results is presented in the introduction to part two of this series, where the role of sterols is further assessed. Experimental results supporting the idea of sterols as an integral part of the channel have also appeared recently. In particular, a series of works of Prieto and coworkers (Coutinho and Prieto 2003; Coutinho et al. 2004; Silva et al. 2006a, b) have led them to propose that the erg/chol selectivity of Nys is due to architectural differences in the pore structure that each of these molecules will produce. They posit that these differences in turn will be conducive to a larger average open time for the channels formed in the presence of erg than those formed in the presence of chol. However, there is no molecular characterization of the Nys channels in these conditions, and therefore, a Nys single-channel study in the presence of both sterols is called for. In this work we present a thorough characterization of the Nys-induced permeability in erg- and chol-containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membranes as a function of Nys concentration. The single-channel behavior is described and analyzed for the concentrations at which channels appear. In part two of this series the characterization of Nys activity along the phase diagrams of chol-POPC and erg-POPC membranes is presented.

The results of this and the companion paper are totally congruent with the Finkelstein and Holz (1973) model for the pore structure, and they also support the idea that sterols, through a modulatory effect on the membrane structure, are responsible for regulating polyene adsorption, insertion or pore formation, rather than just acting as promoters of stave in the barrel. This latter conception is more in accordance with recent observations on the role of sterols (Feigenson 2009; Sasaki and White 2009).

Materials and Methods

Materials

POPC dissolved in chloroform and powdered chol was purchased from Avanti Polar Lipids (Alabaster, AL) and stored in the dark at −18°C. Nys was purchased from Sigma-Aldrich (Toluca, Mexico), stored at 4°C under vacuum and used without further purification. Tetraethylammonium cloride (TEA) was also purchased from Sigma-Aldrich. Sealed vials of erg dissolved in chloroform were purchased from Supelco (Bellefonte, PA) and stored at 4°C in the dark. Dimethyl sulfoxide (DMSO, synthesis grade), potassium chloride (KCl, ACS grade) and calcium chloride ($CaCl_2$, ACS grade) were purchased from Merck (Naucalpan, Mexico). All organic solvents were ACS grade and were purchased from J. T. Baker (Xalostoc, Mexico).

Small Unilamellar Vesicle Preparation

POPC, chol and erg were stored at −20°C and used without further purification. Stock chloroform solutions for every lipid were prepared once a week and stored at −20°C. Chol or erg was mixed with POPC to obtain the mol fraction of 30 mol% of sterol. Solvent was evaporated in a rotative evaporator (B-177; Büchi Labortechnik, Flawil, Switzerland). The suspension was prepared by adding the working solution (2 M KCl, 2 mM $CaCl_2$, 10 mM HEPES [pH 8.0]) to the film deposited in the flask and then treating in an ultrasonic bath to produce dispersion and obtain unilamellar vesicles (Paternostre et al. 1996). The suspension was stored for 2 h under refrigeration (4–6°C) prior to Nys incorporation.

Nys Incorporation

Powdered Nys was stored at −18°C and used without further purification. Nys was dissolved in methanol (1 mM) and used the same day of preparation. In order to homogenize and produce unilamellar vesicles, Nys in the small unilamellar vesicle (SUV) suspension was dispersed via ultrasonic bath. Different volumes of the stock were added to the SUV preparation in order to obtain the desired concentrations.

The concentrations used were 1.0, 2.5, 4.0, 5.0, 6.0 and 7.5 μM for erg-POPC and 1.0, 2.5, 4.0, 5.0, 10.0, 15.0, 20.0 and 25.0 μM for chol-POPC membranes. The amount of Nys present in the membrane will depend not only on the concentration in solution but also on the molar ratio Nys/total lipid. The molar ratios corresponding to the previous concentrations are $(1.33, 3.33, 5.32, 6.65, 7.98, 9.98) \times 10^{-4}$ for the erg-POPC membranes and $(1.33, 3.33, 5.32, 6.65, 13.33, 20.00, 26.66, 33.33) \times 10^{-4}$ for the chol-POPC membranes. The maximal methanol fraction was 2.5% vol/vol. Homogenized suspension was obtained via ultrasonic dispersion in an N_2-enriched atmosphere after adding the polyene. The mixture was stored at 4°C for 24 h before use in an N_2-enriched atmosphere.

Micropipette Fabrication

Glass capillaries with filament (1.00 mm external diameter and 0.58 mm internal diameter) from World Precision Instruments (Sarasota, FL) were pulled using the P2000 instrument from Sutter (Novato, CA). Micropipettes were then filled with the same working solution used for the

preparation of SUVs and used within the following 20 min. The average resistance of micropipettes in the working solution was 100 ± 15 MΩ.

Solvent-Free Tip-Dip Lipid Bilayer Formation

Aliquots from the SUV preparation were placed in a temperature-controlled chamber preheated to 27.0°C with N_2 flux. A TC2Bip temperature controller from Cell Micro-Controls (Norfolk, VA) was used. The chamber was isolated from electrical and mechanical noise with a Faraday cage and an antivibration table, respectively. The sample was allowed to settle for 10 min, and the lipid bilayer was then formed at the tip of the micropipette following the tip-dip technique (Suarez-Isla et al. 1983). Only seals greater than 70 GΩ were used. The rate of success was 0.5 on average. We determined the time average conductance as a function of time and discovered that this observation needs 15 min to attain equilibrium.

Sidedness of Experiments

It could be thought that channel formation by the protocol previously described would include double-sided barrels (Marty and Finkelstein 1975) or different orientation of the pore resulting from the presence of Nys on both sides of the membrane. However, this is not so; the micropipette it is not filled with liposomal solution, and therefore, there is no Nys in it, thus producing a washing out of any residual drug (Cass et al. 1970) that could have appeared in the pipette membrane side. This idea was corroborated by performing two sets of experiments: first, the formation of the membrane in the SUV suspension without antibiotic followed by perfusion of SUVs with the antibiotic; second, the blocking of Nys pores with TEA (Brutyan and McPhie 1996; Borisova et al. 1979). The results are discussed below.

Electrical Measurements

Electrical measurements were done with the Axopatch 200B amplifier from Molecular Devices (Union City, CA) set to voltage-clamp mode, 2-kHz low pass filter and maximal scaling output. The bilayer seal and pipette resistance were measured by applying a square 5-mV step potential. The signal was digitalized using a Digidata 1200A, stored and visualized in a personal computer using the software PClamp 8.2 (both from Molecular Devices). The sampling rate was set to 20 ms. Once the seal was formed, the root mean square basal current (I_{rms}) obtained consistently was 0.15 ± 0.03 pA. Five experiments were performed for every point. Each experiment lasted 5 min, and data were collected when an equilibrium situation had been reached.

Data Analysis

All records were a posteriori baseline-corrected and digitally filtered at 1 kHz. The events were automatically detected using the segmental k-means algorithm (SKM) implemented in the free software QuB (www.qub.buffalo.edu/wiki/index.php/Main_Page) (Qin et al. 1996; Qin 2004). Amplitudes, lifetimes, numbers of events and occupancies were obtained from the resulting statistics. The amplitudes for the starting model were obtained by fitting the amplitude frequency histogram with a multicomponent gaussian function also implemented in the QuB software. All graphics were done with OriginPro 7.5 from OriginLab (Northampton, MA).

For each experiment, the time average conductance change occurring in the membranes upon Nys addition was obtained from the following expression:

$$\langle G \rangle = \sum \langle G_i \rangle = \frac{\sum g_i l_i n_i}{t_{\text{exp}}} \quad (1)$$

where the index i runs over the different types of channels observed; $\langle G_i \rangle$ is the time average conductance of channel type i, g_i is the actual conductance of channel type i, l_i is the lifetime of channel type i, n_i is the number of events of channel type i and t_{exp} is the time duration of the record analyzed.

Results

Figure 1 shows examples of Nys channels in chol- and erg-containing POPC membranes. In both cases the channels are clearly defined, showing that Nys can induce permeability through channel formation. This agrees with previous

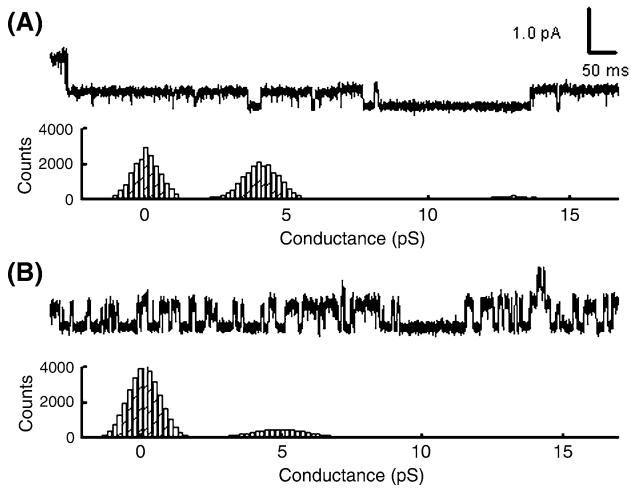

Fig. 1 Examples of a continuous current trace of Nys channels in a POPC membrane with (**a**) 30 mol% ergosterol, [Nys] = 5 μM, and (**b**) 30 mol% cholesterol, [Nys] = 20 μM, both at 27°C. Their respective conductance histograms are also presented

reports (Ermishkin et al. 1976; Kleinberg and Finkelstein 1984), even though reports from noise analysis applied to Nys-induced ion permeability experiments showed no evidence of channel formation, leading to the proposal of a different mode of action (Romine et al. 1977). In this case, the absence of channels could be due to the very low concentration of Nys, but a detergentlike effect was also proposed for AmB in the absence of sterol (Bolard et al. 1980) and a different mechanism of Nys-induced permeability in chol-POPC vesicles (Coutinho et al. 2004).

As in the case of AmB, a set of channels with different conductances appeared. Table 1 shows the different conductances observed in POPC membranes containing chol or erg and compares them to those of equivalent AmB channels in egg yolk lecithin membranes (Cotero et al. 1998; Venegas et al. 2003). There is no significant difference in these unitary conductances for Nys channels in erg-POPC or chol-POPC membranes; furthermore, these conductances are very similar to those formed by AmB. A greater similarity can be expected if we take into account that improved experimental control (which is reflected in the reduced standard error) enabled the distinction of two channels at around 7 and 11 pS, which had been mixed in previous works. As will be shown later, the absence of the large Nys conductance channels can be explained by the different potentials used in the two sets of experiments, 200 mV for AmB vs. 100 mV for Nys. The previously reported single-channel conductances for Nys are 1.8 ± 0.2 pS (Ermishkin et al. 1976) and 4.5 pS (Kleinberg and Finkelstein 1984). The second one corresponds to the smaller channel reported here, which is the more common. A possible channel in the range of 2 pS was also observed in the present work, but it was considered that there was not enough resolution to perform a reliable analysis.

Neither conductances nor average lifetimes are dependent on polyene concentration. On the other hand, lifetimes are dependent on the type of sterol, mainly in the case of the smaller conductance channels. Table 2 shows a summary of the average lifetimes for all polyene concentrations and compares them to that of their AmB counterparts. The discrepancy between the lifetimes reported here and those reported by Ermishkin et al. (1976) and Kleinberg and

Table 1 Conductance of the different Nys channel types observed in POPC membranes in the presence of 30% chol or erg at 27°C and comparison with equivalent channels of AmB in egg yolk lecithin bilayers

Channel type	Channel conductance (pS)			
	15 μM Nys, POPC, 30 mol% Chol, 27°C, 100 mV	6 μM Nys, POPC, 30 mol% Erg, 27°C, 100 mV	10 μM AmB, egg yolk lecithin, 30 mol% Chol, 25°C, 200 mV	10 μM AmB, egg yolk lecithin, 30 mol% Erg, 25°C, 100 mV
I	4.8 ± 0.1	4.4 ± 0.4	3.9 ± 0.5	5.2 ± 0.6
II	7.8 ± 0.2	8.5 ± 0.8	9.7 ± 1.8	11.9 ± 0.6
III	12.1 ± 0.6	12.0 ± 0.9	21.4 ± 6.1	19.7 ± 0.4
IV	17.9 ± 1.2	N.O.	37.1 ± 4.3	31.8 ± 1.5
V	N.O.	N.O.	51.2 ± 7.1	41.4 ± 0.8
VI	N.O.	N.O.	70.3 ± 9.0	62.9 ± 1.6

N.O. not observed

Table 2 Lifetimes of the different Nys channel types observed in POPC membranes in the presence of 30% chol or erg at 27°C and comparison with equivalent channels of AmB in egg yolk lecithin bilayers

Channel type	Channel lifetime (ms)			
	15 μM Nys, POPC, 30 mol% Chol, 27°C, 100 mV	6 μM Nys, POPC, 30 mol% Erg, 27°C, 100 mV	10 μM AmB, egg yolk lecithin, 30 mol% Chol, 25°C, 200 mV	10 μM AmB, egg yolk lecithin, 30 mol% Erg, 25°C, 100 mV
I	7.3	21.9	~40	~60
II	4.1	10.7	~1	~390
III	2.8	4.4	~1	~350
IV	2.1	N.O.	~20	~50
V	N.O.	N.O.	~10	~20
VI	N.O.	N.O.	~10	~10

N.O. not observed

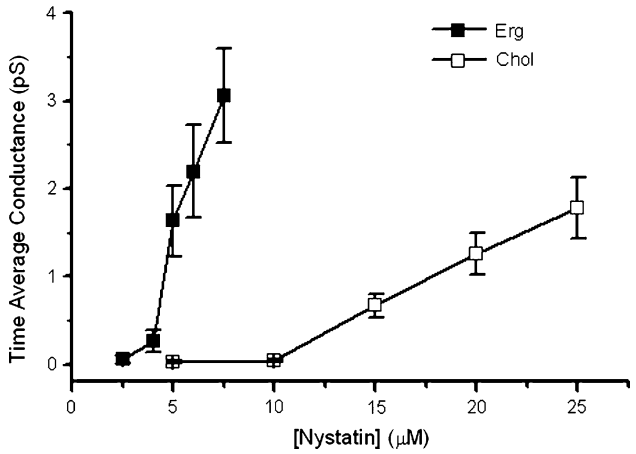

Fig. 2 Time average conductance, Eq. 1, as a function of Nys concentration in POPC membranes at 27°C and with cholesterol or ergosterol presence

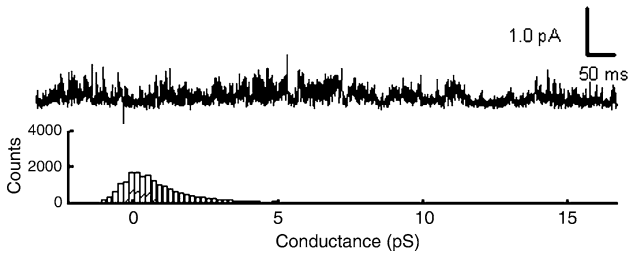

Fig. 3 Nonquantized current surges observed in a POPC membrane with 30% ergosterol at 27°C. These surges were observed only at lower concentrations. The example shown here was obtained with 2.5 μM of Nys. The corresponding conductance histogram is also presented

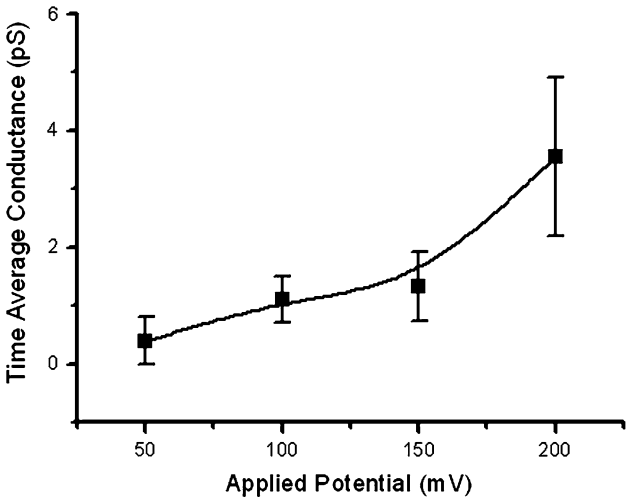

Fig. 4 Time average conductance produced by 15 μM of Nys in POPC membranes with 30 mol% cholesterol at 27°C under different applied potentials ($n = 4$)

Finkelstein (1984) (220 and 2.3 s, respectively) for the Nys channels could be due to the difference in time resolution. As will be shown, the number of channels that appear in membranes containing erg is larger than in those containing chol. If there is not enough time resolution, as was the case in early experiments, short closing or opening events will be missed and longer-lasting events will be recorded (Qin 2004; Chung et al. 1991).

Figure 2 presents the effect of Nys concentration on the time average conductance change of the membrane, as defined by Eq. 1, for erg- or chol-containing POPC bilayers. It is clear that the threshold for channel appearance is quite different for the two sterols. The threshold for channel detection is lower for erg-POPC than for chol-POPC, and the time average conductance attained by the former at 5 μM (6.65×10^{-4} Nys/total lipid molar ratio) is attained by the latter at 25 μM (3.33×10^{-3} Nys/total lipid molar ratio), resulting in a ratio quite close to the known relative drug potency between initial action on fungi vs. hemolysis for several polyenes (Preobrazhenskaya et al. 2009). As a matter of fact, there are so many openings at larger concentrations that single-channel analysis cannot be used any longer.

In addition to the differences observed between erg-POPC and chol-POPC in regard to channel characteristics, another phenomenon was noted. Small irregular surges of current appeared consistently at low concentrations of Nys in erg-POPC bilayers. In some cases, these could be seen as small noisy channels, but in most cases, they lacked onset definition and stable conductance (Fig. 3). These events did not appear in the chol-POPC membrane, either at the same concentrations that appeared in erg-POPC or at the threshold for drug action in chol-POPC membranes. Furthermore, these irregular events presented an optimal concentration $\sim$2.5 μM (3.34×10^{-3} Nys lipid molar ratio): They rise around 1 μM (1.34×10^{-4} Nys lipid molar ratio) and fade out as a result of bona fide channels starting to appear at 5 μM, which suggests a different mechanism of ion permeability.

Figure 4 shows the dependence of the time average conductance as a function of the applied potential. It is clear that channel appearance is induced by the applied potential, but more importantly, the formation of larger channels is facilitated by the electric field: This indicates that the supramolecular structure is stabilized by a large field, possibly by giving a preferential orientation to the monomers. Here, it is important to note that this is due to an increased number of events since there is no dependence on the applied potential of the average open time or the unitary conductance for the different types of channels.

In order to determine clearly if the pores observed corresponded to one- or two-side formation, a set of experiments was performed. First, the antibiotic was added after the formation of the membrane in the tip of the micropipette. It was found that all observations (conductance of the pores, occupancy, spectra of pore types and

average open time) fall within the error of the experiments. This is explained because the micropipette is not filled with liposomal solution, and therefore, there is no Nys in it. Furthermore, the absence of Nys will conduce to a washing out of any residual drug (Cass et al. 1970) that could have appeared in the pipette membrane side. However, it could be argued that this type of experiment does not detect the possible fusion of liposomes to the bilayer that could produce the formation of pores on the *trans* side. Because of this an additional set of experiments with TEA blocking was done. TEA was added to the side where the liposomes with antibiotic were present. It has been shown that TEA blocks the polyene pores when added to the same side as the antibiotic in black lipid membranes (Brutyan and McPhie 1996; Borisova et al. 1979); therefore, this is a very stringent test of sidedness. We obtained results very similar to those of Brutyan and McPhie (1996); when 15 μM TEA were added, the time average current diminished 50%. We went further and added 40 μM of TEA, obtaining a residual activity of 0.4%, indicating that the membrane patch was still intact but that the amount of channel oriented toward the pipette is negligible.

Discussion

The results presented here show that Nys is able to aggregate in membrane-spanning pores (as in the case of those assembled by AmB) and that certain characteristics are different depending on whether the POPC membranes contain erg or chol. The Nys concentration threshold needed for the appearance of channels is greater for chol-POPC than for erg-POPC. This could be explained by a difference in the partition coefficient so that the threshold for adsorption and insertion of Nys is attained at larger concentrations for the chol-POPC membranes. However, as Prieto and coworkers (Silva et al. 2006b) posit, it can also be explained by a substantial difference in channel lifetimes for erg-POPC vis-à-vis chol-POPC membranes. They argue that, at low concentrations, the channel lifetimes in chol-POPC are so small they cannot be detected; therefore, larger concentrations should be used in order to observe a detectable channel signal. Brutyan and McPhie (1996) observed a 100-fold decrease in the average lifetime of AmB channels in DOPC membranes containing 30 mol% chol compared to those appearing in DOPC membranes containing the same amount of erg. Venegas et al. (2003) reported a similar difference for channel types II and III of AmB (Table 1) on egg yolk lecithin membranes with a chol or erg content of 30 mol%. However, in the present work, the observed increase in lifetime was only 4.6-fold for Nys type I channel at sterol concentrations of 30 mol%. This difference could be explained by the difference in temperature and membrane potential used; the activity's dependence on these two parameters will be shown here and in the companion paper. Also, due to the distinct interaction that AmB and Nys have with phospholipids and sterols (Hac-Wydro and Dynarowicz-Latka 2006), Nys interacts more markedly with phospholipids than AmB. Therefore, a relatively reduced interaction with sterols can be expected.

At 5 μM Nys, the difference in the time average conductance appearing in erg-containing membranes relative to chol-containing ones is 54.4 times, which cannot be explained merely by the increase in lifetime. The number of events for the most frequent channel (type I) in erg-POPC is 10.3 times larger than that in chol-POPC. It is possible that the increase in the number of events is produced by the increase in lifetime because events shorter than 1 ms are not considered. We can estimate this correction by integrating the event distribution as a function of the binned lifetime from 0 to 1 ms (with a decay constant equal to the average lifetime) and correct the observed number of events by this proportion. The corrected values are 360 for the chol-POPC and 3,227 for the erg-POPC membranes (instead of 303 and 3,110, respectively). Hence, the more substantial drug effectiveness brought about by the presence of erg is also (and to a large extent) produced by an increased frequency of channel appearance (as can be seen in Fig. 5). If sterols only affected pore architecture, then one would have to assume that the greater stability produced by erg leads to longer opening lifetimes as well as a higher frequency of opening. This could also be due to a larger amount of drug in the erg-POPC membrane or to an effect of membrane structure on channel stability, an effect that is reported in the companion paper. However, the recent results of Prieto and coworkers (Coutinho et al. 2004) go

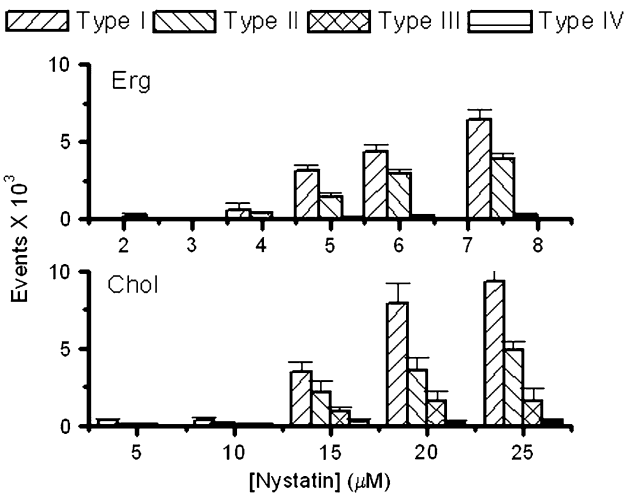

Fig. 5 Number of events for different Nys channel types as a function of antibiotic concentration in POPC membranes with 30 mol% cholesterol (*top*) and 30 mol% ergosterol (*bottom*) at 27°C

against the idea that the partition of the polyene into the membrane is sterol-dependent. Having undertaken a series of spectroscopic studies, they report that the partition of Nys into POPC membranes is sterol-independent ($K_p = 1.36 \pm 0.38 \times 10^4$). It could be that partition into the membrane is, on the whole, sterol-independent, whereas the ratio of adsorption to insertion is sterol-dependent. Milhaud et al. (2002) have provided evidence for different types of interaction between polyenes and lipid bilayers depending on the presence or absence of sterol. These studies showed that the aggregates produced after the lengthy deposition of AmB in 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC)–supported bilayers are quite different when the membrane contains erg or has no sterol. The evidence of a different adsorption of polyenes to dipalmitoylphosphatidylcholine (DPPC) monolayers and bilayers containing either sterol (Barwicz and Tancrede 1997; Gagos et al. 2005) also supports this idea. The results presented here show that low Nys concentrations in erg-POPC bilayers produce ion permeation in a form suggesting a carpet model mechanism (Shai 2002). In a recent work (Rzepiela et al. 2010) few peptide molecules produced disordered pores in the membrane. This phenomenon is not present in chol-POPC membranes at any of the tested concentrations of Nys. The idea of different Nys action mechanisms in erg-POPC vs. chol-POPC membranes was proposed by Coutinho and Prieto (2003), who even suggested the absence of pores in chol-POPC membranes. However, this hypothesis had the difficulty of explaining the action of the drug in K^+-release experiments for chol-POPC membranes (Coutinho et al. 2004). Here, it is shown that pore formation prevails in POPC membranes with either chol or erg at polyene concentrations greater than 5 μM. At lower concentrations, only the erg-POPC membrane shows induced ion permeability. This can also explain the sensitivity of AmB to membrane structure reported by Zumbuehl et al. (2004), who observed a large sensitivity toward erg-containing membranes around 0.1 M, a concentration where membrane disruption should be present if the Nys results are extrapolated to AmB. It has already been reported that channel conductance for AmB or Nys is independent of the type of sterol, its absence or the type of phospholipid. Unitary conductance is, in fact, quite similar in DMPC (Cotero et al. 1998), DOPC, DPhPC (Brutyan and McPhie 1996), monoglycerides (Kleinberg and Finkelstein 1984), chicken lecithin (Venegas et al. 2003), asolecthin (Cotero et al. 1998), *Escherichia coli* membrane (Venegas et al. 2003) and brain phospholipids (Kasumov et al. 1979). On the other hand, the thresholds of concentration for channel appearance, the average open times and the spectral distribution of the different types of channels vary considerably with lipid composition (Cotero et al. 1998; Venegas et al. 2003; Kleinberg and Finkelstein 1984), sterol content (Cotero et al. 1998; Venegas et al. 2003; Kleinberg and Finkelstein 1984; Kasumov et al. 1979), transmembrane potential (addressed here) and temperature (Venegas et al. 2003). These results can be taken as evidence of the fact that channel formation and stability are environmentally dependent but that the supramolecular structure is determined by the polyene monomer itself. The present results show a remarkable similarity in the conductances for Nys and AmB channels. The structural similarity between the two molecules leads us to suppose that pore structure is determined by the self-aggregation of the polyenes in the membrane. Furthermore, the observed increase in channel open times can also be seen as a result of differences in membrane structure. The increment in open times is quite large for the smaller channels but decreases until no change is observed for the larger channels. If the effect was architectural, we would expect that the larger channels would be the most favored. This unequal effect on channel open time was also observed for AmB channels (Venegas et al. 2003). Further explanation of this fact is presented in the companion paper, where lifetime is shown to be correlated to the membrane phase diagram. The fact that transmembrane voltage, which will orient molecules with such a large dipole as Nys and AmB and is conducive to increased open times, is also relevant to the assumption that membrane structure affects polyene action.

The observed alternative mode of action at low polyene concentrations, the disruption of the membrane, has an antecedent in the work by Bolard et al. (1980). They found, using circular dichroism, that a particular aggregation of AmB at 10^{-5} M occurred in the gel phase of egg yolk phosphatidylcholine vesicles with or without low cholesterol presence (<10%). This aggregation was associated to glucose permeability in the gel phase. The fact that aggregation occurred in the gel phase and that it vanished with high cholesterol presence suggests its relation to a structured membrane. This agrees with the presence of membrane disruptions in erg-POPC membranes, which possess strong induced structure, whereas the less structured chol-POPC membrane do not show this. This mode could also be related to the strong sensitivity displayed by 10^{-7} M AmB to lipid phase transition (Zumbuehl et al. 2004) and the work of Silva et al. (2006b), who have proposed different forms of action for membranes containing either erg or chol. In a recent work Romero et al. (2009) clear evidence of two mechanisms of ion permeability in chol-egg-phosphatidylcholine membranes and human erythrocytes appearing at low (<1 μM) and high (>1 μM) AmB concentrations is presented. This is therefore a mode of action that highly depends on the type of sterol and membrane phase. Since there are no proper pores, disruption must depend on the different interaction of the polyenes with membrane structure, thus supporting

the idea that membrane structure is involved in drug selectivity for membranes with different or no sterols.

The results presented here, in addition to those of the companion paper (which reports that Nys activity clearly follows the phase diagram for chol- and erg-containing POPC membranes) and research on behavior of antimicrobial peptides (Bechinger and Lohner 2006; Huang 2006), lead us to a molecular model for the action of polyenes. At low concentrations, the polyene monomers become deposited on the bilayer surface; according to Prieto et al. (Silva et al. 2006b; Coutinho and Prieto 1995), this happens at equal rates in erg- and chol-containing membranes. As proposed by Milhaud et al. (2002), a distinct form of adsorption occurs in ordered membranes, such as erg-containing ones or those in the gel phase. This adsorption compromises the membrane integrity, producing membrane disruptions that give rise to transmembrane currents that could be related to the nonaqueous pores proposed by Cohen et al. (Ramos et al. 1996; Cohen 1998). Further concentration of the polyene leads to its insertion into the membrane, with the ensuing disappearance of membrane disruptions and the appearance of transmembrane pores. Both mechanisms have been observed in the action of antimicrobial peptides; it has been proposed they could be the result of distinct environmental conditions (Bechinger and Lohner 2006; Oren and Shai 2000). The pore's stability and, concomitantly, open times are also affected by environmental conditions, leading to a strong polyene action in ordered membranes. In less ordered membranes (those containing chol or no sterol), the deposition does not provoke membrane disruption. Increasing the concentration of the polyenes in these system leads to larger aggregation in solution and its insertion into the membrane, in agreement with the proposal of Huang et al. (2002). As a matter of fact, there is evidence that Nys aggregation starts at 1 μM (Mazerski et al. 1982). This value is close to the maximal presence of membrane disruptions and to the onset of transmembrane pore formation. This would suggest that the disruption is produced by a monomeric form of Nys, whereas dimerization leads to transmembrane pores and is required for insertion into the chol-POPC membrane. The possibility that aggregation in aqueous solution is behind target cell selectivity has also been proposed for antimicrobial peptides (Oren and Shai 2000). The larger aggregates in solution going into chol-containing membranes can even lead to larger pores. Still, the overall insertion is less than the one occurring in a more structured membrane; a relatively minor activity is therefore observed. In conclusion, the selectivity of polyenes for sterols present in the membrane is determined mainly by their modulatory effect on the membrane phase. Matsumori et al. (2009) have recently argued against this idea by presenting evidence of direct interaction between AmB and erg and not between AmB and chol in POPC membranes with sterol presence. This important finding might suggest that membrane structure and direct interaction could both be involved. This subject is discussed in detail in the companion paper.

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