Amphotericin B Channels in the Bacterial Membrane: Role of Sterol and Temperature

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ABSTRACT Amphotericin B is an antibiotic that forms ion channels in the membrane of a host cell. The change in permeability produced by these channels is greatly improved by sterols; nevertheless, the single channel conductivity remains invariant. Hence, it is proposed that sterols do not act directly, but rather through the modulation of the membrane phase. We look at the formation of these channels in the bacterial membrane to determine the mechanism of its known antibiotic resistance.We found that channels can indeed be formed in this membrane, but a substantial amount of amphotericin B is required. We also study the effects of the antibiotic concentration needed for channel expression as well as the dynamics of channels affected by both sterol and temperature in phosphatidylcholine membranes. The results support the idea that membrane structure is a determining factor in the action of the antibiotic.

INTRODUCTION

Amphotericin B (AmB) is an antibiotic widely used for the treatment of systemic fungal infections despite the severe side effects that it produces (Hartsel and Bolard, 1996; Brajtburg et al., 1990). Even though it has been used clinically for a long time, the knowledge of its action mechanism at a molecular level is very poor. This molecule produces a permeability change in the plasmatic membrane of the host cell, presumably caused by pores where several monomers of AmB interact to form a barrel-like structure whose interior has a hydrophilic environment that allows for the passage of ions and other small molecules like urea and glucose (Hartsel et al., 1994, 1991; Holz and Finkelstein, 1970). This unregulated leakage of metabolites damages the cell and eventually provokes its death.

The accepted channel model assumes that the pore can be made with a different number of monomers ranging from 4 to 12 (Cass et al., 1970; Gruszecki et al., 2003) and is confirmed by the multiple conductivities found in single channel recordings (Brutyan and McPhie, 1996; Cotero et al., 1998). In this model it has also been proposed that AmB interacts with the sterols that are present in the membrane previous to channel formation and is an integral part of the pore structure (De Kruijff and Demel, 1974). The need for sterol has been substantiated by the observation that the antibiotic action generally increases with sterol concentration (Cohen, 1998, 1992; Brutyan and McPhie, 1996; De Kruijff et al., 1974). This idea was used to explain the known AmB resistance of bacteria, as well as the dissimilar action on mammalian and fungal cell, which is proposed to be due to the differences in interaction of the AmB molecule with the distinct sterols in the membrane of each cell.

As evidence against the proposal that sterols are necessary for channel formation, recent works have demonstrated the presence of AmB channels in sterol-free membranes (Gruszecki et al., 2003; Huang et al., 2002; Cotero et al., 1998; Ruckwardt et al., 1998; Wolf and Hartsel, 1995). Different authors have proposed that these particular channels are not responsible for the antibiotic activity, but can evolve to the pharmacologically active channel when sterols are present in the membrane, thus referring to them as protochannels (Cohen, 1998, 1992; Ramos et al., 1996). We found in a previous work, however, that single channel conductivities are independent of cholesterol (Cotero et al., 1998), thus suggesting that channels in the absence of sterol are not different from those in its presence. This proposes that the role of sterols in promoting the antibiotic action happens through the modulation of the membrane structure, which in turn affects the expression of a given channel rather than being an integral part of it. Of course if sterols are not needed for AmB activity, the bacterial resistance to the antibiotic should be explained.

In this work we explore the bacterial resistance to AmB, using single channel recording techniques. To evaluate our proposal that sterol modulation of the membrane phase, rather than direct action on the channels, is the reason for the promotion of drug activity, we induced changes in the bacterial membrane structure using two known modifier agents: sterols and temperature. To make sure that the results are not due to the singularity of the bacterial membrane, we also present results obtained for egg yoke lecithin and 1,2-dimiristoyl-*sn*-glycerol-3-phosphatidyl choline (DMPC) membranes. Additionally, we determined the ionic selectivity of the different channels to further understand their role. A comparison between channels present in cholesterol- or ergosterol-containing membranes is also presented.

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MATERIAL AND METHODS

Bacterial membrane extraction

The *Escherichia coli* wild-type NM522 strain was used in all experiments. LB medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter, pH 7.5) was used to grow the cells, in both agar plates and liquid medium. Single colonies were transferred from agar plates to LB liquid medium and incubated overnight at 37°C under agitation. Cells were harvested by low-speed centrifugation (11,000 $\times$ g_{max} for 20 min at 4°C), washed once, and finally suspended in a buffer.

Two types of extractions were performed. One was a total extraction of the lipids (IOM: inner-outer membrane) made after lysing the cells by sonication and recovering the membranes by centrifugation. The membrane proteins were removed by phase separation in chloroform-methanol (Okorov et al., 1996). The other type of extraction was of the internal membrane lipids (IM) that were separated from those of the outer membrane (OM) by a Percoll gradient as described by Morein et al. (1994). The fatty acids were not removed, and tocopherol was added to avoid lipid oxidation. The extracted lipids were concentrated in chloroform-methanol 2:1 mixture and stored at -18°C.

Amphotericin B was purchased from Sigma (Toluca, Mexico) and stored as a dry powder in the dark at 4°C. An AmB stock solution (1 mM) in dimethyl sulfoxide (DMSO) was prepared each week and also stored at 4°C in the dark. Egg yolk lecithin and ergosterol were purchased from Sigma. Cholesterol was from Avanti Polar Lipids, Inc. (Alabaster, AL). Both were stored in chloroform solution at -18°C.

Liposome formation and single channel recordings

The liposomes were prepared by evaporating the residual solvent off of the lipids in a Rotavapor B-177 (Büchi Labortechnik AG, Flawil, Switzerland) at 60°C for 30 min, and then adding the solution (2 M KCl, 1 mM $CaCl_2$, 10 mM Hepes, pH 8.0) and stirring vigorously (Paternostre et al., 1996). After this, an aliquot of AmB stock solution was added to get the desired concentration. For faster incorporation to the lipid phase, AmB was added to the liposomal preparation and sonicated until there was a translucent blue solution. The maximal concentration of DMSO in the liposome suspension (4 mg of phospholipids per milliliter of solution) was of 1% (v/v). When using the IOM lipid and egg lecithin preparation, it was assumed that the average weight of these lipids was twice that of sterol to estimate the concentration. This yielded a 4:1 (w/w) ratio, i.e., 30 mol %.

The bilayers were made at the tip of a patch-clamp pipette by introducing the pipette repeatedly through a stable monolayer formed at the air-water interface (Suárez-Isla et al., 1983). A good seal ($\sim$40GΩ) was confirmed by the response to a square voltage pulse. The pipette was filled with the same solution used in the liposome suspension except for the case of ion selectivity determination where 0.2 M KCl was used. Chlorinated silver wires were used as electrodes (Hamill et al., 1981). To determine the concentration threshold of AmB required to observe a single channel, which is a function of the area of the membrane, the tip area of the patch-clamp pipette was estimated as follows. The measured resistance of the electrode ($\sim$10MΩ) was compared to that of electrodes filled with the same solution that were observed by atomic force microscopy. This yielded an estimated tip pore area of $< 1\mu m^2$. Recordings of the AmB channel were obtained at 200 mV for DMPC with cholesterol, lecithin with cholesterol, *E. coli*, and DMPC without cholesterol. For lecithin without cholesterol and lecithin with ergosterol, 100 mV were used because higher potentials induced dielectric disruption of these membranes. Experiments with ergosterol were done under red light and nitrogen to prevent it from becoming reactive. Despite these precautions, the seal sometimes showed ruptures that could be confused with channels. Hence, for this system it was preferable to make sure that a stable seal was observed for at least 5 min before AmB was added to the solution, instead of adding the AmB during the sonication procedure.

Single channel recordings were done using an Axopatch 200A and a DigiData 1200A (Axon Instruments, Union City, CA). The analysis was made using pClamp 8.2 (Axon Instruments) and Origin 5.0 software (MicroCal software, Northampton, MA). Calorimetric measurements were done with a Scanning Calorimeter MC2 (MicroCal, Northampton, MA). Scans were performed in liposomal solution of IOM extraction in the above described buffer solution in a concentration of 4 mg/ml. Temperature was controlled by TC2Bip (Cell MicroControls, Norfolk, CA).

Amphotericin B concentration threshold

An AmB concentration threshold ($[AmB]_{threshold}$) for the formation of channels was defined as the concentration at which channel types I, II, and III (see Table 1) appeared in an interval of time bounded by the expression of these channels with a probability of less than 10%. Channel types IV, V, and VI were not observed at low concentrations, or their probabilities were $<$5%. This concentration was determined from a set of 10 experiments of 6 min each. Once the $[AmB]_{threshold}$ was determined, all the experiments were made at an AmB concentration larger than the threshold. An exception was DMPC, where the $[AmB]_{threshold}$ was used.

Data analysis

The effect of the antibiotic on the membrane is normally observed through the membrane resistance. Channels with different conductivities and dynamical properties contribute to an overall conductance, and it is not possible to determine the particular role of each channel. In this work the overall conductance will be estimated, but without losing the description of the individual properties of channels, namely, the frequency of openings (ν), the average open time (τ), and the open channel probability ($P = \nu \times \tau$) so we can determine their contribution to the antibiotic action. To make sure measurements were made between known events, local probability was defined as the open channel probability in the time interval between the first and last expression of the specific channel, and not in the complete recording time. This is justified because AmB channels are not stable structures (Cass et al., 1970): they form, vanish, and can even be washed away. Local frequency and a local average open time were likewise defined. Local effective probability was defined as the channel conductivity times the local probability.

The global conductance is represented by an effective channel, that is, the average of all the individual channels taking into account their probabilities, now with respect to the total time of experiments, i.e., $G_{eff} = \sum G_i P_i$ and likewise a global probability, $P_{eff} = \sum P_i$, where i denotes the type of channel. In this manner the contribution of each type of channel to the membrane conductance and the molecular properties responsible for this contribution can be seen.

The standard errors associated with frequency, probability, and effective conductivity cannot be obtained from the events distribution but can be obtained from the records distribution, which is small. Therefore, unless a large change in the values of these properties was observed, the differences were not considered meaningful. The open times do not follow a normal distribution, but rather an exponential decay. The standard error is therefore not meaningful, but the average values show the tendency toward shorter or longer events when sterols or temperature are varied.

RESULTS

Conductivity of AmB channels

The AmB channels observed are reported in Table 1, and some examples are presented in Fig. 1. The recordings were made in the absence or presence of 30 mol % of sterols, and at different temperatures. Care was taken that the membrane

TABLE 1 The conductivity of the different types of channels observed in *E. coli* and other phosphatidylcholine-based membranes in the presence or absence of sterols at different temperatures

		Channel conductivity (pS)				
		DMPC*	Egg lecithin		*E. coli* IOM	
Sterol	Channel type	30°C	25°C	15°C	25°C	15°C
0 mol %	I	*nob*	*nob*	3.2 (0.9) [4]	2.6 (0.8) [2]	2.2 (0.4) [3]
	II	10.8 (2.4)	9.9 (1.8) [4]	8.2 (2.4) [6]	6.8 (1.6) [3]	7.4 (2.6) [2]
	III	19.3 (2.8)	20.6 (5.3) [6]	18.7 (4.1) [6]	18.1 (0.9) [7]	16.4 (3.6) [5]
	IV	38.2 (3.6)	35.4 (3.9) [3]	*nob*	35.9 (4.7) [7]	*nob*
	V	52.8 (5.5)	*nob*	50.3 (8.2) [3]	*nob*	*nob*
	VI	*nob*	*nob*	*nob*	65.3 (0.6) [5]	71.4 (11.0) [1]
Cholesterol 30 mol %	I	6.6 (1.6)	3.9 (0.5) [3]		3.3 (0.8) [1]	
	II	10.6 (1.3)	9.7 (1.8) [2]		7.7 (0.8) [4]	
	III	16.4 (0.5)	21.4 (6.1) [3]		17.8 (0.2) [2]	
	IV	*nob*	37.1 (4.3) [5]		27.8 (2.9) [5]	
	V	*nob*	51.2 (7.1) [4]		47.0 (3.0) [3]	
	VI	*nob*	70.3 (9.0) [4]		78.0 (3.1) [3]	
Ergosterol 30 mol %	I		5.2 (0.6) [9]			
	II		11.9 (0.6) [9]			
	III		19.7 (0.4) [6]			
	IV		31.8 (1.5) [9]			
	V		41.4 (0.8) [7]			
	VI		62.9 (1.6) [5]			

200 mV were applied for DMPC with cholesterol, lecithin with cholesterol, and *E. coli*. 100 mV were used for DMPC without cholesterol, lecithin without cholesterol, and lecithin with ergosterol. The buffer used was 2 M KCl, 1mM $CaCl_2$, and 10 mM Hepes at pH 8.0. *Nob*, not observed; ( ), standard deviation; [ ], number of records where the channel was observed.
*Cotero et al. (1998).

was in the liquid crystal phase for all cases. A differential calorimetric scan was performed for the IOM to find the phase transition of the preparation. The results are presented in Fig. 2 and show a broad transition that is completed at 25°C, where the IOM lipids are in the liquid phase. When cholesterol is added, the transition becomes much broader, producing the known plastification of the membrane.

A well-defined spectrum of conductivities allowing for the classification of six types of AmB channels was observed. These different conductivities cannot be assigned as substates of a single AmB channel. The reason for this assumption is that events with overlapping conductivities were very rare and had a strong dependence on the AmB concentration, suggesting that the events were independent. In any case, these types of events were excluded because the opening times of the different overlapping channels could not be well resolved.

The channel conductivities show a substantial dispersion (Fig. 3). This leads to a problem in classifying the channel to obtain open times and probabilities, since their Gaussian distributions overlap. Thus we defined a range of values to be associated with each type of channel that was congruent with the accumulated all-points histograms at all conditions, i.e., at the crossing points of the corresponding normal curves. These ranges are: for channel I, from 1.5 to 4.2 pS; channel II, from 4.3 to 12.5 pS; channel III, 12.6 to 25.0 pS; channel IV, 25.1 to 47.5 pS; channel V, 45.6 to 58.0 pS; and channel VI, 58.1 to 75.0 pS. The numbers of individual experiments where these channels appeared are also presented. As a further test to check if this definition was adequate, *p*-values were computed to determine the level of significance of assigning channels to a particular classification; in all cases $p < 0.01$. The conductivities observed for all channels were very similar with or without sterol in the membrane and different lipid composition; that is, the supramolecular structure of the channels appears to be the same in all cases. However, there was a difference in regard to a previous work (Cotero et al., 1998) where only five types of channels were reported, because very few events of the large channels were observed and the two larger ones were assumed to be a single type. The same conductances for channels in the presence of cholesterol or ergosterol were also reported by Brutyan and McPhie (1996). In all cases, sterols facilitate the expression of channels. In Table 2 we present the $[AmB]_{threshold}$ for all the cases studied. For all types of membrane, large amounts of AmB were needed for the channels to express in the absence of sterols. This was especially true of the bacterial membrane at 25°C, where more than 100μM of AmB was needed. In this case, AmB was added to the solvent containing the lipids previous to evaporation, as has been done in other works (Gagoś et al., 2001; Matsuoka and Murata, 2002). This way, when the liposomes were formed, the antibiotic was already in the lipid phase because of its

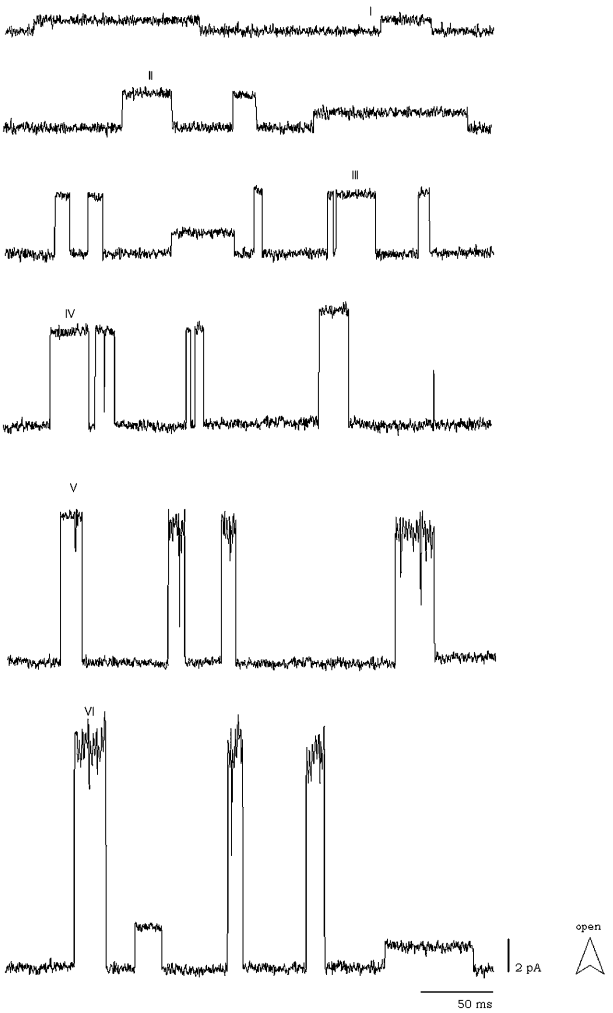

FIGURE 1 Examples of traces showing the six types of AmB channels, in this case in a lecithin membrane with 30 mol % cholesterol and a temperature of 25°C. The buffer solution used was 2 M KCl, 1 mM $CaCl_2$, and 10 mM Hepes at pH 8.0 and 200 mV where applied.

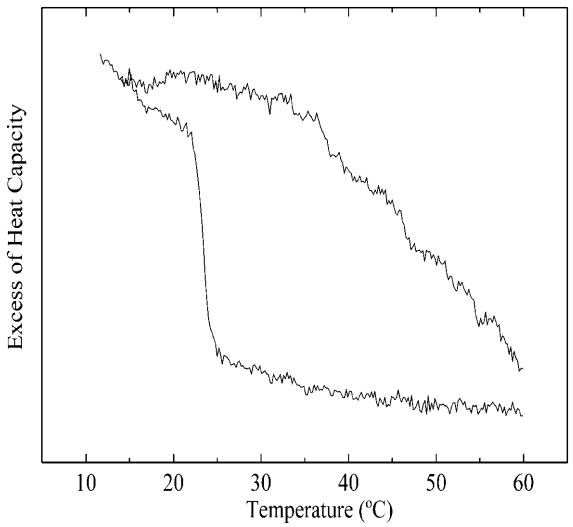

FIGURE 2 Differential calorimetric scanning of the IOM preparation with (30 mol %) or without cholesterol. The concentration of lipid in the solution was 4 mg/ml (2 M KCl, 1 mM $CaCl_2$, and 10mM Hepes, pH = 8.0).

amphiphilic character. Channels were observed only in the first hour after the liposomes were prepared. After this time equilibrium was achieved and the AmB concentration in the lipid phase was reduced. This observation concurs with previous results (Marty and Finkelstein, 1975), which show that AmB molecules can be washed out from the membrane phase. This method allowed the problems of AmB incorporation into the lipid phase to be bypassed so that the expression of the channel could be observed. The results indicate that channels can be formed in bacterial membranes and that the incorporation of the antibiotic is the limiting step. Adding AmB at the time of sonication helps achieve the equilibrium partition of the antibiotic. In fact, adding AmB after the membrane was formed resulted in a smaller number of channels, indicating that achievement of equilibrium takes more than the duration of the membrane patch. And, as will be seen, forcing AmB into the membrane as previously described leads to the expression of the large conductivity channels in a singular way.

To eliminate the possibility that using the mixture of the inner and outer bacterial membranes leads to an artifact in the expression of AmB channels, experiments with an extraction of the inner membrane lipids (IM) of *E. coli* were performed. Similar results were obtained; i.e., it is possible to form AmB channels in the IM. However, since it was easier to work with the IOM preparation, it is the data analysis from the results obtained with this membrane that has been reported.

When cholesterol was added to DMPC there was a three-order-of-magnitude reduction in $[AmB]_{threshold}$. In the case of egg lecithin, the reduction was of two orders of magnitude, and in the case of IOM, one order of magnitude. In the latter case the $[AmB]_{threshold}$ remained quite high despite cholesterol presence. There was a substantial reduction of the threshold when ergosterol was added to lecithin, yet this was difficult to determine due to the poor stability of the seal. As in the case of sterol addition, lowering the temperature of the IOM and egg lecithin membranes down to 15°C led to a reduction of the $[AmB]_{threshold}$ by one order of magnitude in both cases.

The promotion of AmB action by temperature in egg lecithin liposomes and a type of *Mycoplasma* in which the antibiotic action is enhanced by lowering the temperature to 2°C, especially with the first lipid, has already been reported by Archer (1976). This result was explained as produced by the membrane structure, but the author doubted that this increased permeability was due to the same pores as those formed in the presence of sterols.

It is significant that cholesterol, besides decreasing the $[AmB]_{threshold}$, favors the formation of the large conductivity channels in IOM and egg lecithin bilayers (Table 1). Ergos-

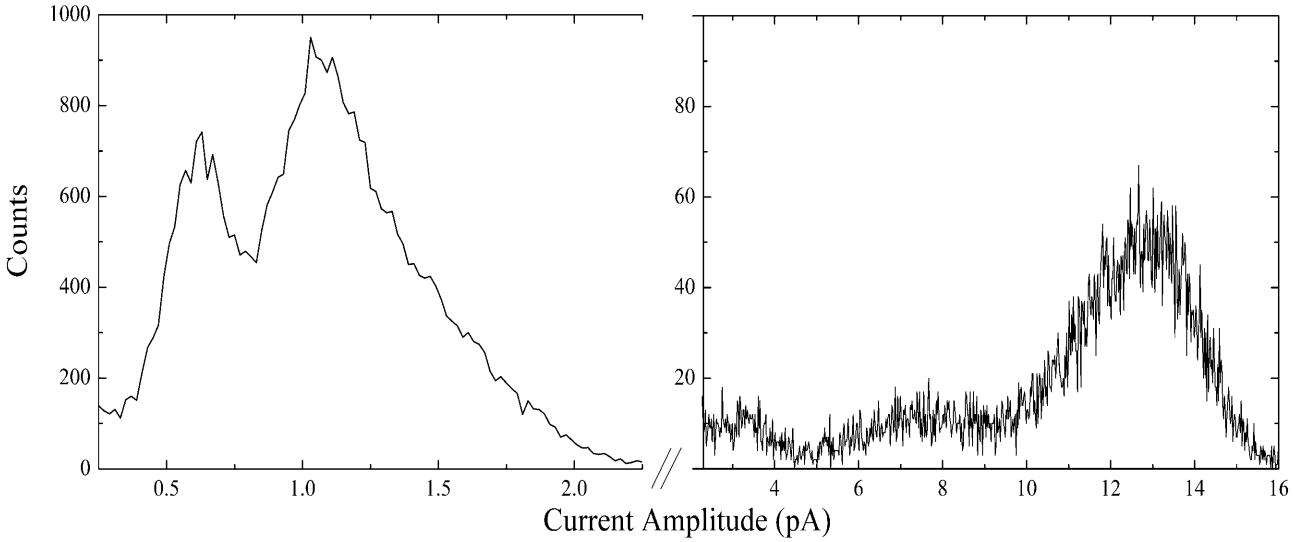

FIGURE 3 Accumulated all-points histogram of all records analyzed in the *E. coli* IOM bilayer at 25°C without cholesterol and AmB concentration of 100 μM. The solution used was 2 M KCl, 1mM $CaCl_2$, and 10 mM Hepes at pH = 8.0. The applied membrane potential was 200 mV. The baseline at 0 current is not shown. In this case channel type V does not appear, but this histogram is shown because it corresponds to those conditions where channels are less likely to appear according to the assumption of sterols requirement.

terol also promotes the formation of the larger channels, but without decreasing the probability of the smaller channels.

Local channel properties

In Table 3 and 4 the local dynamical properties ν, τ, and P of the AmB channels are presented, as well as their local effective conductivities. It can be seen that ν (Table 3) was not affected by temperature, whereas adding 30 mol % of cholesterol increased its value by two orders of magnitude in the IOM bilayer and one order of magnitude in the egg lecithin bilayer. Ergosterol did not increase the frequency. The open times were slightly affected by temperature, but for the three lower conductivity channels there was a significant reduction when cholesterol was added to both membranes. This is certainly related to the increase in frequency. The large conductivity channel, type VI, was quite rare and short lived, except in the IOM at 25°C, where it was long lasting. It should be kept in mind that the AmB was forced in this case.

TABLE 2 Amphotericin B concentration threshold as defined in the text for DMPC, egg lecithin, and the bacterial lipids formed at the tip of a micropipette (<1 μm^2) in the presence or absence of sterols and different temperatures

	AmB concentration threshold (M)				
	DMPC*	Egg lecithin		*E. coli* IOM	
Sterol	30°C	25°C	15°C	25°C	15°C
0 mol %	10^{-4}	10^{-4}	10^{-5}	$>10^{-4}$	10^{-5}
Chol 30 mol %	10^{-7}	10^{-6}		10^{-5}	
Erg 30 mol %		$<10^{-7}$			

The membrane potential and buffer used are those quoted in Table 1. Chol, cholesterol; Erg, ergosterol.
*Cotero et al. (1998).

The combined effect of ν and τ produced the changes in local P shown in Table 4. Lowering the temperature produced an increment in the probability of channels I, II, and III, but with a substantial dispersion in both membranes. The three largest channels remained quite infrequent. Cholesterol addition markedly decreased the occurrence of the low conductivity channels and increased that of the larger channels (Type IV for the IOM and types IV, V, and VI for egg lecithin). Ergosterol addition increased the occurrence of all channels.

The local effective conductivity of the channels reflects their contribution to the potency of the antibiotic in the membrane. Temperature decrement generally increased this effective conductivity in the case of the first three channels, whereas cholesterol addition decreased it and made the large conductivity channels dominant. In the case of the IOM this dominance was restricted to channel IV. Ergosterol acted similarly to cholesterol but also produced larger effective conductivities for the small channels.

It can be seen, based on the local properties, that the bacterial membrane behaved akin to lecithin aside from the threshold values. The addition of cholesterol promoted the formation of channels but reduced the average open times. The resulting local probabilities were certainly increased. That is, the channel bursts were more populated. Temperature decrement produced a similar behavior but this time through the increase of open times. Ergosterol differed from cholesterol because it introduced a more general promotion of all channels.

TABLE 3 Individual local frequencies (ν) and local opening times (τ) of the AmB channels observed in *E. coli* IOM and egg lecithin bilayers in the presence or absence of sterols and different temperatures

		Local frequency (Hz)				Local average open time (sec)			
				30 mol %				30 mol %	
		0 mol%		Cholesterol	Ergosterol	0 mol%		Cholesterol	Ergosterol
Bilayer	Channel type	15°C	25°C	25°C	25°C	15°C	25°C	25°C	25°C
[AmB] (M):		10^{-4}	10^{-4}*	10^{-5}		10^{-4}	10^{-4}*	10^{-5}	
E. coli IOM	I	0.5 (0.1)	0.4 (0.01)	49 (—)		0.43	0.26	$\sim 10^{-3}$	
	II	0.5 (0.2)	0.4 (0.04)	16 (1)		0.64	0.36	$\sim 10^{-4}$	
	III	0.7 (0.2)	0.5 (0.02)	23 (4)		0.42	0.09	$\sim 10^{-4}$	
	IV	*nob*	0.3 (0.08)	32 (9)		*nob*	0.11	0.03	
	V	*nob*	*nob*	21 (4)		*nob*	*nob*	$\sim 10^{-4}$	
	VI	*nob*	0.2 (0.04)	4 (1)		*nob*	0.25	$\sim 10^{-3}$	
[AmB] (M):		10^{-4}	10^{-4}	10^{-5}	10^{-5}†	10^{-4}	10^{-4}	10^{-5}	10^{-5}†
Egg lecithin	I	6.1 (2.2)	*nob*	19.1 (1.3)	1.7 (0.9)	0.04	*nob*	0.04	0.06
	II	1.3 (0.7)	1.1 (0.1)	17.2 (3.9)	3.6 (1.3)	0.39	0.11	$\sim 10^{-3}$	0.39
	III	2.5 (0.9)	1.7 (0.6)	21.5 (5.1)	0.6 (0.2)	0.24	0.24	$\sim 10^{-3}$	0.35
	IV	*nob*	1.8 (0.5)	30.4 (10.8)	1.7 (0.6)	*nob*	0.02	0.02	0.05
	V	15.2 (3.2)	*nob*	25.1 (6.4)	8.0 (2.2)	0.01	*nob*	0.01	0.02
	VI	*nob*	*nob*	20.8 (4.9)	0.6 (0.2)	*nob*	*nob*	0.01	0.01

200 mV were applied for DMPC with cholesterol, lecithin with cholesterol and *E. coli*. 100 mV were used for DMPC withoug cholesterol, lecithin without cholesterol, and lecithin with ergosterol. The buffer used was 2M KCl, 1mM $CaCl_2$, and 10 mM Hepes at pH 8.0. *nob*, not observed; ( ), standard error.
*Incorporation of AmB into the lipid chloroform mixture.
†Incorporation of AmB into the membrane patch external solution.

TABLE 4 Local probability and local effective conductivity of the AmB channels observed in *E. coli* IOM and egg lecithin bilayers in the presence or absence of sterols and different temperatures

		Local probability (%)				Local effective conductivity (pS)			
				30 mol %				30 mol %	
		0 mol%		Cholesterol	Ergosterol	0 mol%		Cholesterol	Ergosterol
Bilayer	Channel type	15°C	25°C	25°C	25°C	15°C	25°C	25°C	25°C
[AmB] (M):		10^{-4}	10^{-4}*	10^{-5}		10^{-4}	10^{-4}*	10^{-5}	
E. coli IOM	I	4.5 (2)	9.5 (5.7)	9.2 (—)		0.6 (0.1)	0.29 (0.03)	0.31 (—)	
	II	19.3 (9)	8.8 (2.8)	0.7 (0.2)		1.7 (0.9)	0.75 (0.15)	0.06 (0.01)	
	III	38.8 (13)	0.5 (0.1)	1.5 (0.3)		5.7 (1.6)	0.09 (0.02)	0.32 (0.02)	
	IV	*nob*	1.5 (0.6)	55.9 (19.2)		*nob*	0.55 (0.24)	17.51 (2.25)	
	V	*nob*	*nob*	0.8 (0.3)		*nob*	*nob*	0.47 (0.11)	
	VI	*nob*	7.2 (1.9)	0.4 (10^{-2})		*nob*	3.5 (1.98)	0.35 (0.03)	
[AmB] (M):		10^{-4}	10^{-4}	10^{-5}	10^{-5}†	10^{-4}	10^{-4}	10^{-5}	10^{-5}†
Egg lecithin	I	20.3 (9.1)	*nob*	7.3 (0.9)	10.2 (2.1)	0.5 (0.2)	*nob*	0.3 (0.1)	0.5 (0.2)
	II	50.7 (18.7)	11.4 (3.9)	3.2 (1.1)	16.8 (3.4)	3.8 (0.9)	1.0 (0.2)	0.3 (0.1)	1.9 (0.9)
	III	53.9 (15.3)	40.3 (10.8)	1.0 (0.3)	10.5 (4.5)	10.1 (2.8)	7.8 (2.6)	0.2 (10^{-2})	2.0 (0.6)
	IV	*nob*	3.4 (1.2)	50.1 (9.3)	15.9 (6.3)	*nob*	0.9 (0.2)	16.5 (2.4)	4.9 (1.5)
	V	9.3 (2.7)	*nob*	22.3 (10.2)	10.7 (4.3)	4.3 (1.5)	*nob*	10.3 (2.8)	4.2 (1.2)
	VI	*nob*	*nob*	11.5 (3.1)	0.5 (0.2)	*nob*	*nob*	6.8 (1.9)	0.3 (0.1)

The membrane potential and solutions are those quoted in Table 3. *nob*, not observed; ( ), standard deviation.
*Incorporation of AmB into the lipid chloroform mixture.
†Incorporation of AmB into the membrane patch external solution.

Effective properties

It can be observed how the different channels affect the membrane conductance and relate it to their individual properties. In Table 5 the probabilities of the different channels over the whole time of observation (i.e., under conditions where the channel could appear) are presented. This is not like the case of the local probabilities and indicates a different bursting activity. From Table 5 we can see that the larger channels V and VI were quite infrequent, except for the IOM at 25°C without cholesterol (this will be discussed later). Cholesterol addition certainly promoted formation of the larger channels; temperature decrement promoted smaller channels. Ergosterol promoted both larger and smaller channels.

Table 6 presents the global properties (P_{eff} and G_{eff}) that will lead to the antibiotic potency and their response to changes in temperature and sterol concentration in the membrane. Student's *t*-test was computed for the addition of sterol or temperature decrement; in the case of these properties the change was considered significant when $p < 0.05$.

Addition of cholesterol to the DMPC bilayer did not affect P_{eff}, and the variation in G_{eff} was not meaningful. At first this seems surprising in view of the results for the IOM and egg lecithin in bilayers. The reason for the discrepancy is that in DMPC the AmB concentration was kept at the threshold values. It can therefore be assumed that it was not possible to increase the amount of AmB in the membrane, preventing the expression of the large conductivity channels.

TABLE 5 Probability of appearance of different channels in *E. coli* IOM and egg lecithin membranes under different conditions

		Probability (%)			
				30 mol%	
		0 mol %		Cholesterol	Ergosterol
Bilayer	Channel type	15°C	25°C	25°C	25°C
[AmB] M:		10^{-4}	10^{-4}*	10^{-5}	
E. coli IOM	I	*nob*	2.09	0.62	
	II	2.19	1.92	0.19	
	III	3.44	0.31	0.51	
	IV	5.49	0.62	11.19	
	V	*nob*	*nob*	0.17	
	VI	*nob*	1.72	0.09	
[AmB] M:		10^{-4}	10^{-4}	10^{-5}	10^{-5}†
Egg lecithin	I	0.15	*nob*	0.59	6.98
	II	3.70	0.43	0.26	12.23
	III	3.94	1.53	0.08	6.88
	IV	*nob*	0.13	4.06	1.37
	V	0.68	*nob*	1.81	0.16
	VI	*nob*	*nob*	0.93	0.09

nob, not observed.
*Incorporation of AmB into the lipid chloroform mixture.
†Incorporation of AmB into the membrane patch external solution.

Adding cholesterol to egg lecithin bilayers led to an increase in G_{eff} that is concomitant with the increase in probability. On the other hand, the increase in probability produced by ergosterol was much larger, whereas the increase in effective conductance is similar to that of cholesterol. This can be understood based on the results observed for the local properties; the larger potency observed in lecithin with cholesterol was due to the promotion of the large conductivity channels, whereas in the case of ergosterol the larger potency was related to a general promotion of all channels. Lowering the temperature in lecithin produced a small but significant increase in the antibiotic potency due to more stable low conductivity channels, which was reflected in an augmented probability.

Surprisingly, the IOM membrane responded better than lecithin to cholesterol addition, reaching the potency attained by ergosterol addition. Looking into the local properties, it can be observed that this is entirely due to channel IV, again the promotion of a large channel. The temperature decrement in the IOM increased the probability but not the effective conductance. Local properties show that in this preparation channel VI presented very long open times. Yet it must be remembered that in this case AmB was forced into the membrane and was out of equilibrium because after one hour the channels were washed away. The prolonged existence of this structure could have been due to the incorporation procedure. In fact, if we take away channel VI, the effective probability and conductance become very similar to those of lecithin at the same conditions. If the incorporation procedure does affect channel expression, the differences between cholesterol and ergosterol must be approached cautiously, since in the latter case AmB was added after membrane formation.

Channel selectivity

There is certain confusion concerning the selectivity of AmB channels. The original proposal assumes two types of channels (Marty and Finkelstein, 1975), one formed with a single barrel spanning the membrane, selective to monovalent cations, and the other formed with two coupled barrels spanning the membrane, selective to monovalent anions. Ermishkin et al. (1976) and Kasumov et al. (1979) proposed, from single channel studies, that they were selective to Cl^-. Kleinberg and Finkelstein (1984), using nystatin, stressed the proposal of the single and double barrel channels being nonideally cation and nonideally anion selective respectively. Hartsel et al. (1994) proposed a 7 to 1 selectivity ratio favoring K^+ over Cl^- and pointed out that the different conditions for the channel formation could be producing the conflicting data. Cohen (1998) proposed the existence of nonaqueous small channels that appeared in the absence of sterol or at low AmB concentrations and permeated only monovalent cations, and aqueous channels that are formed at larger concentrations of AmB permeating mono- and

TABLE 6 Effective probabilities and effective conductances as described in the text for *E. coli* bilayers and other phosphatidylcholine-based bilayers in the presence or absence of sterols and different temperatures

	Without cholesterol		30 mol% cholesterol		30 mol% ergosterol	
Bilayer (T°C)	Effective probability (%)	Effective conductance [pS]	Effective probability (%)	Effective conductance [pS]	Effective probability (%)	Effective conductance [pS]
DMPC						
(30)	3.1	0.5 (0.2)	4.3	0.3 (0.1) $_{p > 0.1}$		
Egg lecithin						
(25)	2.1	0.4 (0.1)	7.7	2.8 (0.6) $_{p < 0.01}$	27.7	3.5 (1.5) $_{p < 0.05}$
(15)	8.5	1.4 (0.4) $_{p < 0.05}$				
E. coli IOM						
(25)	6.6	1.6 (0.8)	12.8	3.4 (1.2)$_{p < 0.05}$		
(15)	11.1	1.4 (0.5)$_{p > 0.1}$				

The membrane potential and buffer used are those quoted in Table 3. *P*-values are also reported to validate the changes in these properties upon the addition of sterols and temperature decrement. IOM, inner and outer membrane of *E. coli*; (), standard deviation.

divalent cations and monovalent anions. Here it was seen how different conditions produced the expression of different channels, which could lead to different observed selectivity. Thus, it is quite convenient to determine the selectivity of K^+ versus Cl^- for each one of the reported channels. I-V curves were obtained for a lecithin with 30 mol % cholesterol. The patch seal was quite unstable probably due to the salt gradient, as reported by Penner (1995), and no more than 100 mV could be applied. It was observed that addition of sucrose, sorbitol, or glucose as osmolites hindered the formation of channels, so it was preferred to leave the osmotic differences present. Under these conditions and for reasons we cannot explain, only four channels were observed. The I-V curves are presented in Fig. 4. Each point for the two smaller channels had more than 1000 events. The third channel had ~200 and the largest one ~40. It was quite difficult to determine the selectivity for several reasons. Because of rectification (Brutyan and McPhie, 1996) it was not known how to associate types of channels for the positive and negative currents; a straight line certainly does not fit the data. At low currents, where rectification is less critical, the conductances clustered and it was difficult to differentiate them. No defined Gaussian distributions could be observed (see Fig. 5) at 0 mV, so all channels were averaged into a single point. At this voltage it was clear that only negative currents were present beyond the noise of the baseline, i.e., all channels were K^+ selective. If the data points are spline-adjusted following the conductivity order, the curves presented in Fig. 4 are obtained. The found average selectivity is ~7 to 1, in agreement with Hartsel et al. (1994). It was also seen that as the channels increased conductivity, there was a decrement in selectivity, in agreement with Cohen (1998).

CONCLUSIONS

It has been shown that AmB channels in very different conditions have similar conductivities; that is, they have the same supramolecular structures. These results confirmed the finding that sterol is not an absolute requirement for channel formation (Cotero et al., 1998). They supported the proposal that the role of sterols rests in the facilitation of the entrance of the polyene into the lipid phase, as well as the proposition that sterol favors the partition coefficient of the AmB into the membrane via structure modulation. Lowering the temperature which, like sterols, structures the liquid crystalline phase of the lipid lamella led to a reduction of the $[AmB]_{threshold}$. But this improvement in the antibiotic incorporation was not as efficient as it was with the addition of sterols. The results of Wang et al. (1998) further support this idea, showing that nystatin, a close analog of AmB, entered a bilayer less markedly when the sterol/lipid ratio produced hexagonal arrangements of the sterol molecules, a clear structural factor that determined selectivity for different sterols in the membrane (Liu et al., 1997). Coutinho and Prieto (2003) also showed in a recent work that the nystatin partition coefficient into liposomes was affected by the liposome size, and thought this due to a different packing of the lipids. They also showed deposition onto the surface of the bilayer, as well as aggregate formation inside the bilayer, in the absence of sterol. Their fluorescence measurements, however, could not discriminate if the mentioned aggregates were channels. Huang et. al. (2002) determined the formation of AmB channels in sterol free and 5 mol % ergosterol and cholesterol phosphatidylcholine supported bilayers. The $[AmB]_{threshold}$ they found for channel formation in these membranes was surprisingly close to the one presented here under the same conditions, except that they used a smaller amount of sterols. This agreement supports their idea that thresholds are determined by the aggregation state of the drug in solution, and not, as was thought, from the [AmB] to membrane surface ratio. By fluorescence, they determined that the thresholds for ergosterol, cholesterol, and sterol-free membranes corresponded to monomeric, aggregated, and highly aggregated states of AmB in solution.

The analysis of the AmB channel properties and their responses to temperature and sterols, two membrane mod-

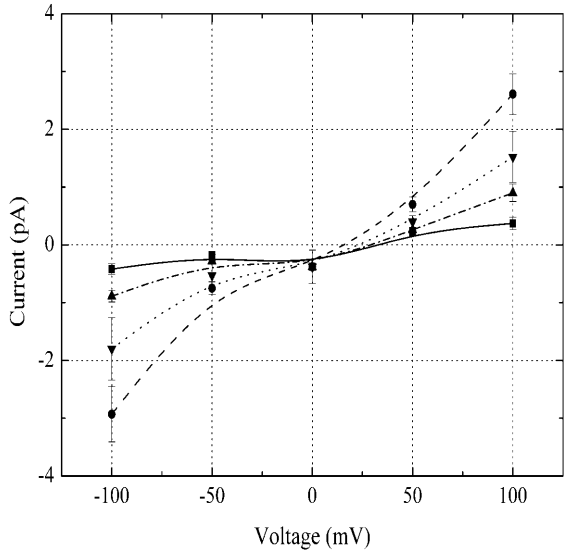

FIGURE 4 I-V curves for AmB channels in a lecithin membrane with a 30 mol % cholesterol at 25°C. The external solution has 2 M KCl, and the micropipette solution 0.2 M KCl. The Nernst potential for the K^+ ion is +52 mV and for the Cl^- ion is −52 mV.

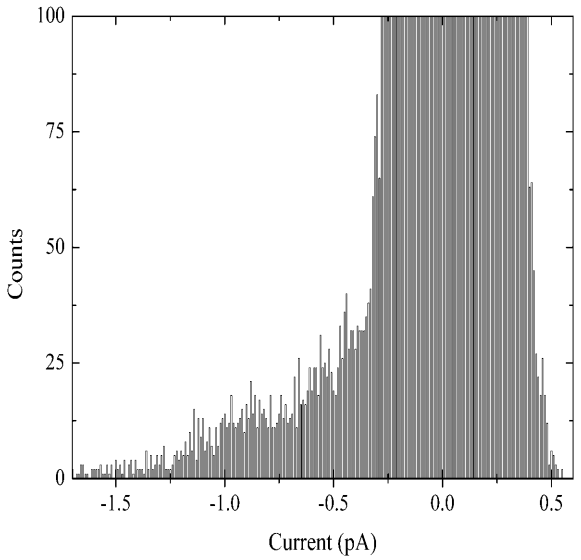

FIGURE 5 Accumulated all-points histogIram of all records at 0 mV for the lecithin membrane with 30 mol % cholesterol at 25°C.

ifier agents, makes an advance in the understanding of the molecular mechanism of channel formation possible. The differences between the behavior of the channels with cholesterol and those with ergosterol are important to understand the different potency of the antibiotic in each case. It is clear that ergosterol allows for greater incorporation of AmB into the membrane, which is correlated to the fact that ergosterol produces a more ordered structure (Urbina et al., 1995). In the present work, the increased presence of AmB in the membrane produced by cholesterol promoted the expression of larger channels. This would agree with Cohen's results (1998), assuming that the smaller channels are nonaqueous channels that convert into larger aqueous channels. This identification was supported by the reduction of K^+ selectivity observed for these channels. However, ergosterol-produced AmB abundance in the membrane led to larger channels without the disappearance of the small conductivity ones. This agrees with the observations of Huang et al. (2002) concerning the incorporation of the monomeric form of AmB into this membrane, i.e., ergosterol would be expected to promote the inclusion of small aggregates. Ergosterol also produced an increment in the open times similar to that produced by temperature decrement, suggesting a stabilization induced by membrane rigidity in both cases. There is one cautionary remark in this last conclusion: since AmB incorporation in lecithin with ergosterol was not made at the time of the sonication step, equilibrium could have not yet been attained and larger channels could appear in time.

Hence, the structure of the membrane modulated by different agents determines the incorporation of AmB, which in enough quantities can form large structures and thus lead to a substantial reduction of the membrane resistance.

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