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# On the role of sterol in the formation of the amphotericin B channel

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**Abstract**

Amphotericin B is an antimycotic agent that has been studied for a long time, both because of its pharmacological action and the interest in understanding how this ionic channel works. It has been proposed that the channel is formed by a barrel of monomers, and that the presence of sterol is needed for the formation of such a barrel. As a matter of fact this need of a sterol has been used as a guiding idea in attemps to design derivatives more efficient in the discrimination of the cholesterol containing membranes, as compared to the ergosterol containing ones, henceforth diminishing the unwanted side effects in its pharmacological use. In this work we show that unitary channels that appear in a cholesterol containing membrane also appear when this membrane is free of cholesterol. We prove this to be the case for two membranes, a biological one, asolectin, and a synthetic one, DMPC. We then advance the idea that the role of sterols in the formation of the amphotericin B channel is related to the effects they have on the structure of the membrane itself, rather than to a direct involvement in the channel formation. We further look into the effect that different cholesterol concentrations in the membrane produce on the single channel properties. 

*Keywords:* Amphotericin B; Sterol; Polyene antibiotic; Ionic channel; Artificial bilayer; Membrane structure

## 1. Introduction

The interest in the amphotericin B channel has lasted for a long time, from the early works of the 70's [1–5] to recent works [6–12]. The reason behind is twofold. First of all the drug is still an important antimycotic agent with many unfortunate side effects [9]. Secondly there is a marked interest in understanding the way this channel works, since in spite of the simplicity of the molecule and of the proposed channel model [13–15] it has quite a few properties associated with more complex channels [16,17].

One of the main characteristics with relevance to both its pharmacological action and the proposed channel structure is the role of sterols. There is plenty of evidence suggesting that the channel formation requires a sterol in the membrane; the fact that the antibiotic is ineffective in bacteria [18], the correlation between sterol content and amphotericin B potency [1–3], and physichochemical studies of the interaction antibiotic-sterol [19,20]. It has been proposed that the sterol molecule is involved in the construction of the channel itself, being intercalated between amphotericin B monomers and acting as a

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'glue' [13,14]. This need of sterols has also been used to explain the difference in channel pharmacological action and conductivity when the membranes contain different sterols, see for example Brutyan and McPhee [10]. With this idea in mind there have been studies to produce derivatives that enhance the discrimination between cholesterol and ergosterol, therefore reducing the very significant effects in medical use, see for example Cheron et al. [21] and HsuChen and Feingold [22]. There is even a recent theoretical work looking into the different binding of amphotericin B to cholesterol and ergosterol [23] and a molecular dynamic simulation of the channel with cholesterol present [11].

On the other hand for a long time there have been reports [24] that there is some formation of amphotericin B channels in membranes free of sterol. However, these channels have been considered to be different from the actual channels responsible for antibiotic activity. In a detailed analysis Cohen et al. [24] advanced a proposal that these structures are protochannels requiring sterol for proper formation, and Bolard [25] suggested a complex interplay between sterols and particular membrane lipids for the formation of effective channels. As a result there is the assumption that the channels formed in the absence of sterol are not functional and recently [10] the need for sterol has been emphasized. Contrary to this, in a very recent work by Hartsel et al. [12] there is clear evidence of an effective macroscopic conductance being produced by amphotericin in the absence of sterols. However, Hartsel et al. [26] have proposed that this conductance is due to a conducting membrane defect at the antibiotic-lipid interphase.

In this work we show that amphotericin B channels can be formed in membranes in the absence of sterols, both in biologically derived and in synthetic membranes. Furthermore, by measuring the molecular properties of the channels in the presence and absence of cholesterol we see that they are the same channels. From the observed effect of cholesterol we advance the proposal that the role of sterols in the structure of the membrane is the phenomenon modulating channel formation and expression. Furthermore, we find agreement with a recent molecular dynamic simulation [11] where the sterols do not interact directly with the channel.

## 2. Materials and methods

### 2.1. Materials

Amphotericin B type I was purchased from Sigma and was stored as a dry powder in the dark at −20°C. Stock solutions in dimethylsulfoxide (1–10 mg/ml) were prepared the same day the experiment took place. The concentration of amphotericin B in aqueous solution was determined by measuring its electronic absorption at 416 nm (extinction coefficient = $1.214 \times 10^5$ $M^{-1}$) [25]. The maximum concentration of dimethylsulfoxide in aqueous solution was 1% (v/v). The lipids used were asolectin (soybean lecithin type IIS from Sigma from which neutral lipids were removed by acetone extraction [27]), DMPC and cholesterol from Avanti Polar Lipids. All were stored at −20°C. The stock solution of DMPC was made in ethanol at a concentration of 20 mg/ml and stored at −20°C. Stock solution of asolectin was prepared in hexane and used the same day. All other reagents were of analytical grade. Buffer solutions contain 2 M KCl, 10 mM HEPES, 1 mM $CaCl_2$ at pH 8. The capillary glass used for the patch clamp micropipettes was borosilicate glass type 7740 purchased from World Precision Instruments, having an internal diameter of 0.84 mm, an external diameter of 1.5 mm and provided with an internal filament. We used a horizontal type puller PN-3 from Narashige Scientific Instruments Laboratory for fabrication of microelectrodes following the procedure described by Hamill et al. [28].

### 2.2. Preparation of liposomes

Small unilamellar vesicles were formed by bath sonication [29] in buffer solution. The concentration of total lipid was 4–4.5 mg/ml and the ratio of sterol/lipid varied from zero to 50% in concentration. The aqueous lipid solution was obtained after evaporation of the organic solvent in a nitrogen atmosphere via rotation, in order to produce a pellet which in aqueous solution will form multilamellar vesicles.

### 2.3. Formation of bilayers at the tip of a patch pipette

We obtained bilayers at the tip of a patch pipette from vesicle suspension using the procedure reported

by Suárez-Islas et al. [30]. After their formation, liposomes were placed in a Teflon camera inmersed in a bath with controlled temperature and after 10 min a monolayer of lipids was produced at the air-water interface. The liposome concentration was large enough to ensure that a stable Gibbs monolayer was formed after 10 min [31]. Afterwards a micropipette filled with the buffer solution was immersed repeatedly in the interface until the formation of a bilayer could be observed as shown by the capacitive response to a train of square waves. Henceforth a typical seal of 70–100 Gigohms was obtained. Single channel recordings were done at room temperature (~22°C) for the asolectin experiments and at 30°C for the DMPC experiments.

For electric register we used an Axopatch-1D amplifier with a CV-4 1/100 headstage, a Digidata 1200 for data acquisition and analysis with Axoscope 1, all from Axon Instruments Inc.

## 3. Results and conclusions

In Table 1 we present the characteristics of amphotericin B channel formation for different concentrations of cholesterol in asolectin membranes. Previous to the discussion of the results we would like to present the reasons for eliminating the possibility that channels observed at zero cholesterol, see for example Fig. 1, could be coming from artifacts, i.e., contamination of asolectin by residual biological channels, leakage in the membrane seal or dielectric breakdown.

Table 1
Conductances of amphotericin B channels at different molar concentrations of cholesterol in an asolectin bilayer

| [Cholesterol] (% molar) | [AmB] (M) | Type | Conductance ($\sigma^a$) (pS) | Events |
|---|---|---|---|---|
| 0 | $10^{-4}$ | I | 2.1 (0.8) | 9 |
| | | II | 5.2 (0.7) | 6 |
| | | III | 8.4 (1.0) | 5 |
| 12.5 | $10^{-6}$ | I | 1.8 (0.9) | 15 |
| | | II | 4.9 (0.6) | 8 |
| | | III | 11.1 (1.8) | 3 |
| 25 | $10^{-6}$ | I | 1.6 (0.5) | 4 |
| | | II | 4.5 (1.0) | 2 |
| | | III | 14.7 (4.1) | 2 |
| 50 | $10^{-8}$ | I | 2.1 (0.8) | 7 |
| | | II | 5.5 (1.6) | 4 |
| | | III | not observed | – |

[AmB]: Threshold of amphotericin B concentration needed to observe channels. Transmembrane potential 200 mV.
[a]Standard deviation.

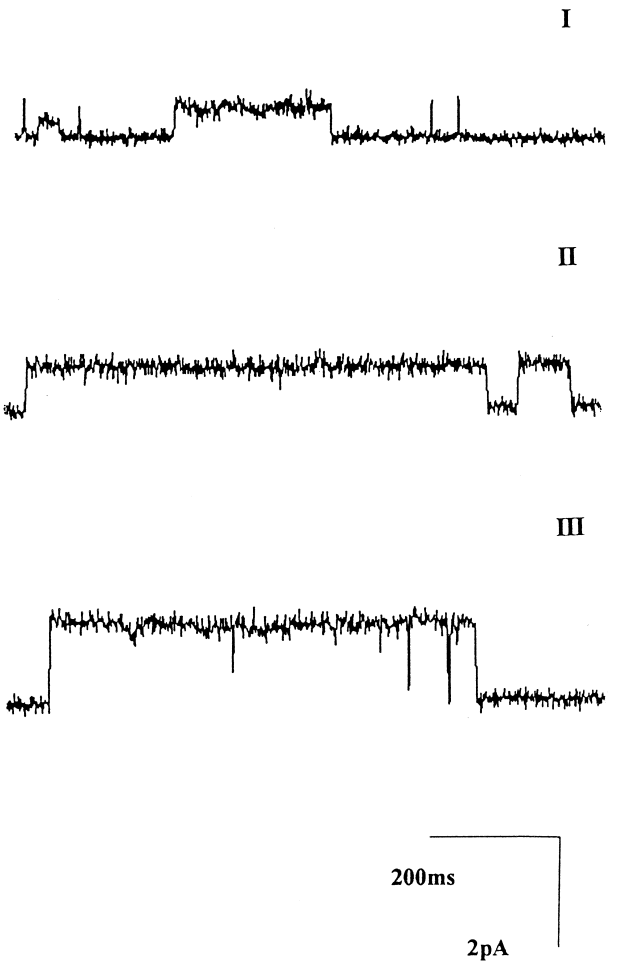


Fig. 1. A typical record showing three types (I, II, III) of amphotericin B channels in the absence of sterol in an asolectin bilayer.

Dielectric breakdown occurs generally at potentials larger than those used here (200 mV) and result in a large leak of current, however, in order to eliminate this possibility we conducted experiments at 100 mV. We observed the same behavior except that the channel with the lower conductivity was at noise level. The possibility of a contaminating channel, either biological or leakage, was ruled out because of the correlation between amphotericin B concentration and the appearance of channels. No channels were observed in the absence of the drug or at large concentrations of it, because of the known

aggregation of the antibiotic in these latter conditions.

We see in Table 1 that there are three types of channels, with conductances ranging from 1.6 to 14 pS. This range of values agrees well with those reported in the literature for amphotericin B channels in different membranes: bovine brain [15], monooleoylglycerol [25], asolectin [16], DOPC and DPhPC [10]. The grouping in three types of channels instead of assuming a single channel with different states comes from the fact that the three cases were observed as unique channels on several occasions. Also we have to remember that the amphotericin B channel has been proposed to have two types of conformation, single and double barreled [14]. The manner in which the drug was incorporated into the membrane in our case allows for both conformations [14,32]. Additionally the single channel, being nonsymmetrical, could have two different conductances, depending on the orientation towards the applied potential. The same criteria were used by Brutyan and McPhee [10] for the characterization of their single barreled channels. We can expect that the lowest conductance value (type I) corresponds to the double barreled channel, and II and III to the different orientations of the single channel. As a matter of fact the ratio between the conductances of these two channels, at zero cholesterol, is 1.6, the same value as the one reported by Brutyan and McPhee [10] at large cholesterol concentration. However, in our case this ratio increases when the cholesterol concentration increases, being nearly 3 at large cholesterol concentrations.

In Table 1 we see that in the absence of sterol amphotericin B can form the three types of channels that appear in the presence of sterol. Appearance of some form of conductance as a result of amphotericin addition, in the absence of sterol, as mentioned in Section 1, has already been reported, but it was thought to be due not to the channel with pharmacological action. In the work of Brutyan and McPhee [10] it is stated that there is need for sterols in the channel formation, but it is also mentioned that the drug is *almost* ineffective in sterol free membranes. In a very recent work [12] there is clear evidence that in the absence of sterols there is a macroscopic conductance as a result of amphotericin B addition. Here we prove in a direct way that the channels formed in the absence and presence of sterols are the same. This being the case, the question remains on what is the role of sterols, and why there is an augmented potency of the drug in membranes containing ergosterol compared to those containing cholesterol. In Table 1 we can see that there are several effects that are sensitive to cholesterol concentration, as was mentioned previously: the conductance ratio between channels II and III, the absence of channel III at high cholesterol, and most importantly the concentration threshold for the formation of channels. The latter point explains the observed increase in conductance as cholesterol concentration is increased. Even if the sterol is not needed for the structural integrity of the channel it is determinant for its formation, affecting quite possibly the partition of the drug between the aqueous and the lipidic media. Before going into further detail in the analysis of the sterol role we would like to consider the following point.

There is the possibility that asolectin, in spite of

Table 2
Conductances of amphotericin B channels at different molar concentrations of cholesterol in a DMPC bilayer

| [Cholesterol] (% molar) | [AmB] (M) | Type | Conductance ($\sigma^c$) (pS) | Events |
|---|---|---|---|---|
| 0[a] | $10^{-4}$ | I | not observed | – |
| | | II | 10.8 (2.4) | 76 |
| | | III | 19.6 (2.8) | 92 |
| | | IV | 38.2 (3.6) | 8 |
| | | V | 52.8 (5.5) | 18 |
| 12.5[a] | $10^{-6}$ | I | not observed | – |
| | | II | 11.0 (0.5) | 26 |
| | | III | 19.3 (5.9) | 20 |
| | | IV | 34.3 (7.5) | 33 |
| | | V | 71.3 (3.3) | 3 |
| 25[a] | $10^{-6}$ | I | not observed | – |
| | | II | 12.7 (3.2) | 48 |
| | | III | 22.9 (3.4) | 69 |
| | | IV | 32.7 (2.6) | 39 |
| | | V | 47.1 (5.9) | 9 |
| 50[b] | $10^{-7}$ | I | 6.6 (1.6) | 95 |
| | | II | 10.6 (1.3) | 57 |
| | | III | 16.4 (.47) | 20 |
| | | IV | not observed | – |
| | | V | not observed | – |

[AmB]: Threshold of amphotericin B concentration needed to observe channels.
[a]Transmembrane potential 100 mV.
[b]Transmembrane potential 200 mV.
[c]Standard deviation.


Fig. 2. A typical record showing four types (II, III, IV, V) of amphotericin B channels in the absence of sterol in a DMPC bilayer.

the treatment used [27], still has some traces of sterol. In order to eliminate this possibility we performed the same analysis in a synthetic lipid, DMPC. As is shown in Table 2, again there is direct evidence of identical channels in the presence and absence of sterols. Furthermore, in this case we investigated in more detail to assess the effect that cholesterol has on the functioning of these channels. Since, as will be discussed below, we think that membrane structure determines some of the channel properties and its formation, we chose to work for the synthetic lipid at a temperature close to the gel-to-liquid-crystal transition, where we expect to have a different structure than that occurring in lipids that have this transition phase below 0°C, as is the case for biological lipids.

In Table 2 we present the accumulated results corresponding to amphotericin B channels in DMPC at 30°C. First of all we see again channels that appear in the membrane containing cholesterol and in the membrane free of sterols, see Fig. 2. There are also differences compared to the asolectin case. Instead of three types of channels there are now five, and the conductances are greater in DMPC. The conductance values for channels IV and V are much larger than any other conductance reported for amphotericin B in the literature. We must make clear that channel I which is quite frequent at 50% cholesterol is absent at the lower cholesterol concentrations. It was not reported here because the DMPC membrane at low cholesterol could not hold 200 mV through it, so it was necessary to apply 100 mV. In addition this membrane system was noisier than asolectin, probably due to the higher temperature and the solution flow to the bath system. Therefore type I channel was at noise level at this concentration and we preferred not to report it.

The fact that we have now more channels, and with substantially different conductances, supports our idea that these channels are quite sensitive to membrane structure. Once again cholesterol concentration has the effect of reducing the amphotericin threshold concentration substantially, so the expected macroscopic decrease in conductance as sterol increases comes from the facilitated formation of the channels, probably due to a better partition of the drug into the membrane.

We can identify the five channels in the following manner. Type I is the double barreled channel. It has lower conductivity, about half that of channel II. This ratio was also observed in the asolectin case, and in the work of Brutyan and McPhee [10]. This factor can be expected both from the double length that this channel has and because the barriers to the passage of anions, to which the double channel is selective, are about twice as large as those for the cations in the single channel. This has been shown to be the case by a simple electrostatical model [33]. Type II and III channels seem to be the two orientations of a single channel. The conductance ratio is $\sim$1.7 and remains almost constant with respect to sterol increase in contrast to the asolectin case. Sim-

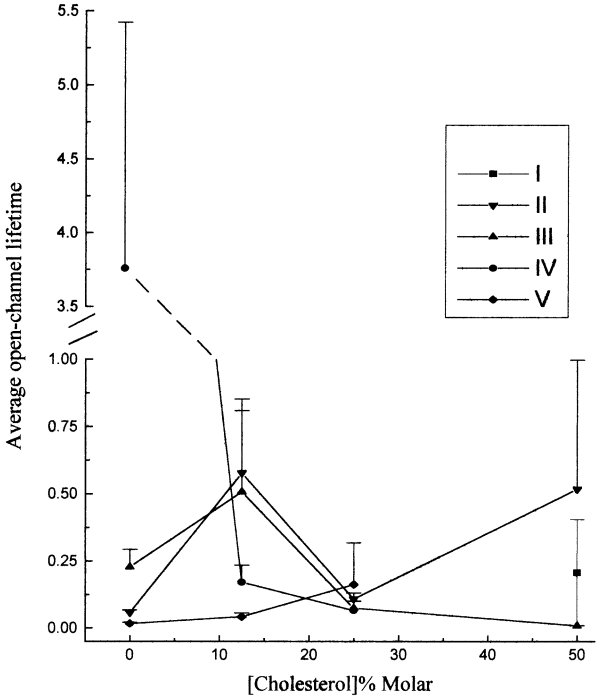


Fig. 3. Amphotericin B open channel lifetime as a function of the cholesterol concentration in a DMPC bilayer. Channels I, II, III, IV and V correspond to those in Table 2. The transmembrane potencial was 100 mV except at 50% of cholesterol where it was 200 mV.

ilarly we think that type IV and type V channels are two orientations of a single barrel with a wider pore. If we look now in more detail into the characteristics of the channels we found that this identification is supported. From Tables 1 and 2 we see that this identification of the channels leads to different values of conductance for the same channel in asolectin or DMPC. We have nonetheless carried out this identification because we assume that channel conductance is dependent on the lipidic system and DMPC at 30°C, being a more ordered membrane, could conduce to higher conductance for the same channel.

In Fig. 3 we present the average open channel lifetime as a function of cholesterol concentration. The lifetime values for all channels in the presence of sterol are similar to those reported by Bruytan and McPhee [10] at 2 M KCl. They also reported that lifetimes were very sensitive (100-fold increase) to change of ergosterol by cholesterol. If we continue in the idea that the effect of sterols is an indirect effect because of affecting membrane structure, then lifetimes should respond to the absence of sterol. This is indeed the case for a channel (type IV) in a marked way (10-fold increase). However, the other channels do not show a clear dependence. The open lifetime for one orientation of the large conductance channel becomes very reduced when cholesterol is increased. This could explain why at large cholesterol concentration it is not observed. The largest conductance channel presents the opposite behavior, its lifespan increases with increasing cholesterol concentration, but it still has a small value at its maximum. Channels II and III behave very similarly, supporting their identification as the two orientations of the same channel. Their discrepancy at 50% cholesterol is now due to the transmembrane potential being 200 mV. The differentiation between the two orientations of the channel when the potential is increased was also reported by Brutyan and McPhee [10]. One could think that this sensitivity to the transmembrane potential intensity could also be responsible for the absence of the channels with the large conductance at the 50% cholesterol concentration. For this reason we performed experiments at 100 mV looking for the existence of the larger conductance channels, but we were not able to observe them.

In Fig. 4 we present the frequency of channel occurrence. Here it is clear that absence of sterol has an

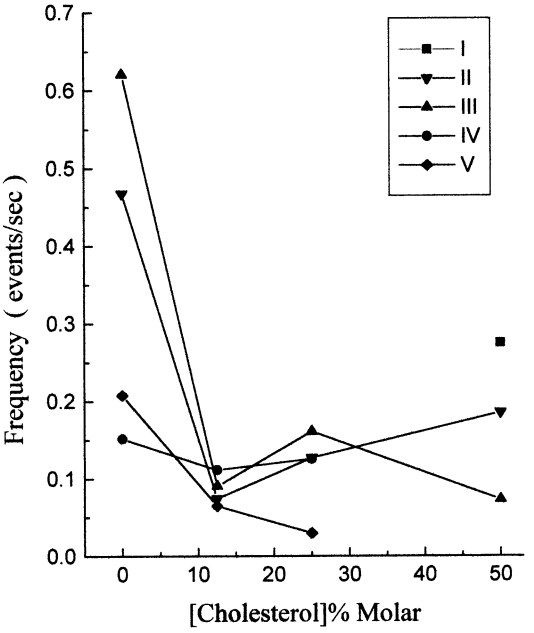


Fig. 4. The frequency of appearance of channels as a function of cholesterol concentration. Channels I, II, III, IV and V are those referred to in Table 2.

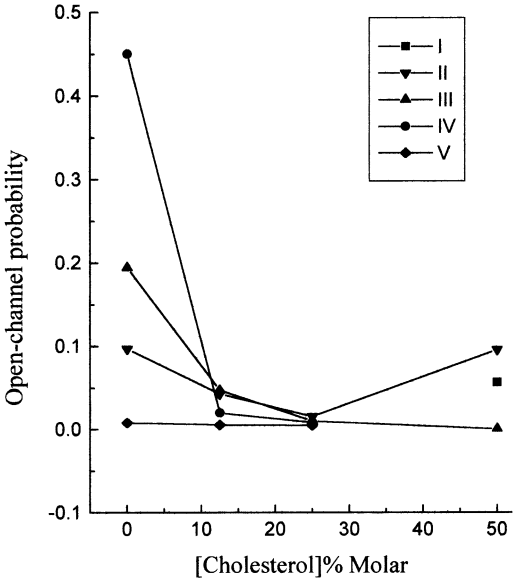


Fig. 5. The probability of finding an open channel, defined as the total time of aperture divided by the total time of observation, as a function of cholesterol concentration.

effect over the channels. There are, surprisingly, more events in the absence of sterol than in its presence. We can also see that the large conductivity channels are still present in a substantial way at 25% cholesterol, hence their absence at 50% is difficult to explain. At 200 mV channels II and III invert their relative frequencies, again in agreement with Brutyan and McPhee [10] on the effect of potential in channel occurrence. In Fig. 5 we see the combined effect of open channel lifetime and frequency, where the effect of cholesterol is clearer, except at 50% where the difference in transmembrane potential is again producing a departure of monotonic behavior. This figure, weighted by the channel conductance, shows the influence that each channel should have on the macroscopic current, and how it is affected by sterol concentration.

Hence we see that identical channels can be formed in the presence and absence of sterols; that indeed the sterol affects the expression of the channel, and therefore explains the observed macroscopic conductance dependence on sterol concentration. The determining role of sterols is the reduction in the concentration treshold for the formation of channels, but once the channel is formed, its expression, lifetime and frequency seem to be favored in the absence of sterols (for DMPC at 30°C).

It seems surprising that a channel, i.e., a molecular entity, would have such different conductances, but there is evidence on the possible formation of different aggregates. As a matter of fact there is a sub-

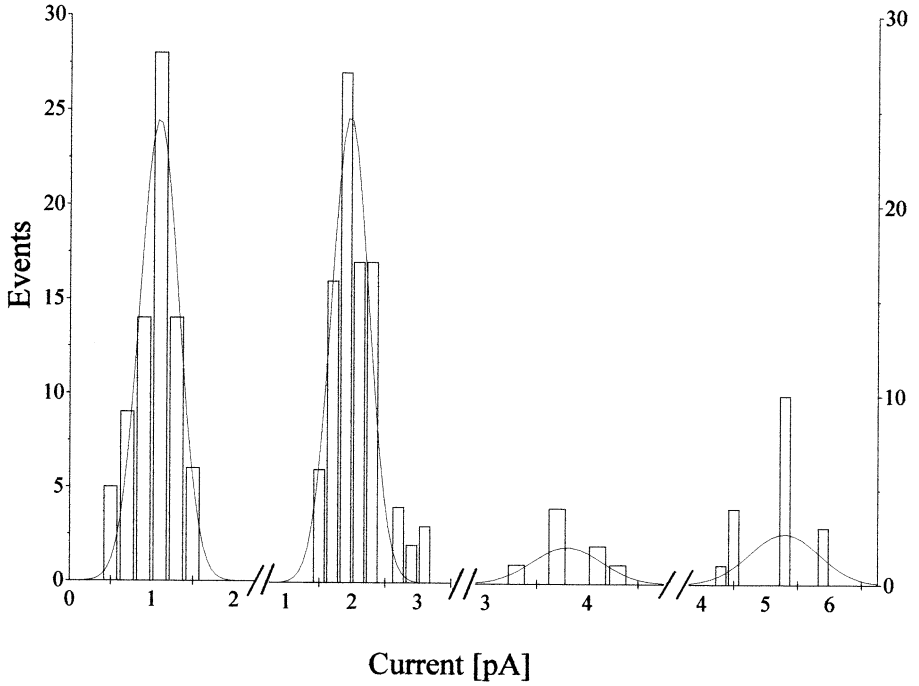


Fig. 6. Collected results for the conductance values of the five types of channels described in Table 2 in the absence of sterol.

stantial dispersion on the measured molarity of the channel, see for example Moreno-Bello et al. [34]. On the same line, it is surprising that the conductance value for a unitary channel presents such a large dispersion. This is emphasized in Fig. 6 where conductance values for different events and different experiments were collected. It is clear that the dispersion is large, leading even to the overlap of the blocks identifying each channel. It seems as if the channel would have many quite similar structures leading to similar conductances. A recent MD simulation [11] found two observations which agree with the results presented here. First of all cholesterol does not interact directly with the channel; they argue that possibly it acts as a screen for the lipids, but there also remains the possibility that cholesterol is not placed even close to the channel. Secondly, they found that the channel does not have a defined structure, but rather a loose and mobile arrangement. In addition to this it has been reported [33], from the construction of a simple electrostatic model for the central pore potential profile for the transfer of an ion, that variation of the channel radii for a defined molarity of the pore does not produce large changes in its profile, hence the observed discrepancy in channel conductance can be explained as a consequence of the loose arrangement of the pores.

An alternative view to the sterol being a needed 'glue' for the channel itself is that the known effects of sterols on amphotericin B potency come from the fact that sterols modify the membrane structure. Even the structural requirements used to explain the effects of different sterols on membrane structure [34,35] are similar to those used to explain the potentiation of the antibiotic action [4,5]. In this way we can also understand many results that have been advanced to support the idea of sterol involvement. For instance, in a recent study it has been reported that ergosterol is more efficient in structuring the DPPC and DMPC membrane than other sterols [36], a finding in agreement with the known fact that the antibiotic is more effective in ergosterol containing membranes. Also a recent work [37] shows that nystatin (a very close analog of amphotericin B) has a substantially larger partitioning (5-fold) in DPPC when it is in the gel phase than in the liquid-crystal phase. This is in agreement with our idea that sterols, by affecting the membrane structure, produce a different threshold for the formation of amphotericin B channels as a function of cholesterol. In the same way, recent results of Hartsel et al. [12] on the promotion of amphotericin B induced $K^+$ permeability by osmotic stress can be thought as the effect of a more structured membrane produced by osmotic pressure.

These findings are important since we have to rethink the molecular role of sterols and therefore our search for the understanding of the functioning of the antibiotic. A functioning which seems to be very dependent on membrane structure explains the large variation of effects that it presents in several systems.

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