On the One-Sided Action of Amphotericin B on Lipid Bilayer Membranes

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ABSTRACT The one-sided action of the polyene antibiotic, amphotericin B, on phospholipid bilayer membranes formed from synthetic phosphatidylcholines (DOPC and DPhPC) and sterols (ergosterol and cholesterol), has been investigated. We found formation of well-defined ionic channels for both sterols and not only for ergosterol-containing membranes (Bolard, J., P. Legrand, F. Heitz, and B. Cybulska. 1991. *Biochemistry.* 30:5707–5715). Characteristics of these channels were studied in the presence of different salts. It was found that the channels have comparable conductances but different lifetimes that are ~100-fold less in cholesterol-containing membranes than in ergosterol-containing ones. Channel blocking by tetraethylammonium (TEA) ions shows that TEA blockage of channels in the presence of cholesterol increases their lifetimes in analogy to the lengthening of lifetimes of protein channels blocked by local anesthetics (Neher, E., and J. H. Steinbach. 1978. *J. Physiol.* 277: 153–176). However, the effect of the blocker on single-channel conductance is very close for both sterols. The data support the classical model of amphotericin B pore formation from complexes initially lying on the membrane surface as nonconducting prepores. We explain the antibiotic's cytotoxic selectivity by differences in the lifetimes of the channels formed with different sterols and suggest that phosphatidylcholine-sterol membranes can be used as a tool for rapid estimation of polyene antibiotic cytotoxicity.

INTRODUCTION

Polyene antibiotics (PA) amphotericin B (AmB) and nysatin (Nys) are mostly known as important antifungal drugs as well as one of the first model systems for transmembrane ionic channel structures (Szoka and Tang, 1993; Ermishkin et al., 1976).

To fit the large body of data showing AmB and Nys similar action on the cellular and model membranes, Marty and Finkelstein (1975) proposed that the two-sided effect of these PA in lipid bilayer membranes (BLM) results from the formation of anion-selective symmetric pores made from two "half pores" in opposite monolayers. For certain bilayers, especially with relatively thin hydrocarbon interior, however, the one-sided half-pores (cation-selective) themselves can be conductive (see also Van Hoogevest and De Kruijff, 1978).

A theory (model) of one-sided PA activity is needed for explanation of pharmacological effects, since drugs are necessarily added from one side only and there is no evidence about formation of anion-selective symmetric pores in the cell membranes.

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Actually, these PAs cause different effects with different cells. The most probable explanation of the cytotoxicity of AmB and Nys is that of the membrane's shunting by ion and metabolite fluxes through one-sided PA-sterol half pores. The higher affinity (of about eight to nine times) of AmB to ergosterol (Erl) vs cholesterol (Chl) (see Szoka and Tang, 1993) may explain the higher toxicity toward Erl-containing fungal cells. However, there are other suggested mechanisms such as nonspecific membrane damage by amphiphilic drug molecules, the formation of nonbilayer structures, induction of lipid peroxidation, etc. (Bolard, 1986; Hartsel et al., 1993; Brajtburg et al., 1990).

In this work we report and compare the properties of AmBs one-sided action on Erl- or Chl-containing BLMs formed from synthetic phosphatidylcholines. The results obtained are consistent with PA-sterol asymmetric half-pore formation theory. The possibility of using DPhPC- or DOPC-sterol BLMs to test rapidly effectiveness and toxicity of the PAs and their chemical derivatives is also discussed.

MATERIALS AND METHODS

Lipid bilayer membranes were formed as described by Montal and Mueller (1972) across a round aperture (0.1–0.3-mm diam)

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in a thin (13 μ) Teflon partition clamped between two symmetrical cell compartments designed to minimize any unstirred areas in the membrane bathing solution. 0.5% hexadecane (Aldrich Chemical Co., Inc, Milwaukee, WI) dissolved in *n*-pentane (Baxter Healthcare Co., McGaw Park, IL) was used for the aperture pretreatment.

Synthetic and chromatographically pure 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, Inc., Pelham, AL) and cholesterol or ergosterol from Sigma Chemical Co. (St. Louis, MO) were used. Monolayers were spread from a 1% solution in *n*-pentane. The phospholipid/sterol composition was the same, 5:1 (wt/wt), in all experiments for ergosterol- or cholesterol-containing BLMs (Chl-BLM or Erl-BLM).

If not stated otherwise, the analytical grade salt solutions were buffered at pH 6.3 with 4.7 mM MES (2-(*N*-morpholino)ethanesulfonic acid) at room temperature (22–24°C).

Amphotericin B powder (registry lot no. 61H4039; Sigma Chemical Co.) dissolved to 10 mM in dimethyl sulfoxide (DMSO) stock was stored refrigerated in the dark for ~1 wk. For the fresh dilutions of the AmB stock solution the same saline was used as in BLM bath. The final concentration of DMSO in membrane bathing solutions usually was < 0.05%.

For electrical measurements a pair of Ag-AgCl-KCl-agar–agar bridges assembled within standard pipette tips (Bezrukov and Vodyanoy, 1993) were used. The voltage clamp circuit was constructed using an OPA 128LM (Burr Brown Co., Tucson, AZ) amplifier with output connected to a Unitrade/Sony 75 ES digital magnetic tape recorder (Unitrade, Philadelphia, PA) and WX 4301 recorder (Western Graphtec, Inc., Irvine, CA).

For AmB short-living channels in Chl-BLMs, conductance and average lifetime were determined using a laboratory-written computer program for statistical signal analysis and methods described by Vodyanoy et al. (1993). The parameters of the long-living channels in Erl-BLMs were determined directly from the chart records.

BLM thickness was estimated from membrane capacitance measurements using a method described earlier (Kleinberg and Finkelstein, 1984).

Circular Dichroism (CD) measurements were made in a Jasco J-500C spectropolarimeter (Easton, MD) at room temperature.

RESULTS

Observations on Ergosterol-containing Membranes

DOPC and DPhPC with 9-*cis*-octadecenoic and 3,7,11, 15-tetramethylhexadecanoic fatty acids, respectively, were used because the BLM formed from these phospholipids have good stability and relatively thin hydrophobic interiors, which is essential for one-sided AmB or Nys effectivity (Kleinberg and Finkelstein, 1984). For example, the measured capacitances of DPhPC/Erl and DOPC/Erl BLMs were 0.80 μF/cm^2 and 0.74 μF/cm^2, respectively, which correspond to ~23.3- and 25.1-Å membrane hydrocarbon thickness. These membrane thickness data are in good agreement with those reported by Kleinberg and Finkelstein (1984) for BLMs formed from Erl-containing monoglycerides (9-*cis*-octadecenoic and 9-*cis*-hexadecenoic) and facilitate comparison of our results.

AmB added in concentrations under 10^{-4} M from one side of sterol-free DPhPC or DOPC BLMs (total ~1% DMSO) was ineffective in terms of changing membrane conductance. Addition of ergosterol induced ability AmB to form channels.

Fig. 1 *A* shows typical current traces (ion channels) obtained after addition of 6×10^{-8} M AmB to only one bathing solution of DPhPC:Erl BLM formed in 2 M KCl. There are remarkable differences among the current traces depending on the sign of applied mem-

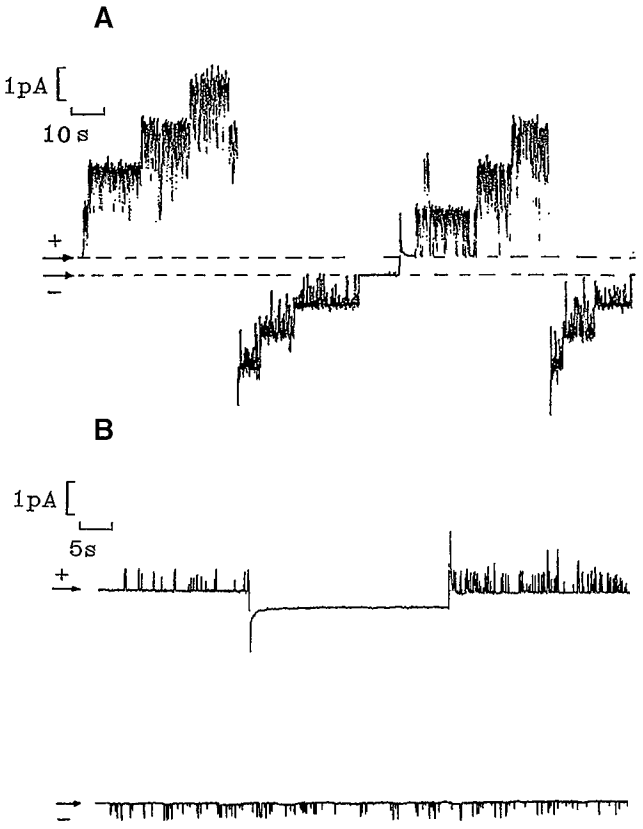

FIGURE 1. BLM continuous current records at positive or negative 100 mV membrane potential. (*A*) Final concentration of 6×10^{-8} M AmB added to only one bathing solution of DphPC-ergosterol membrane in 2 M KCl, 4.7 mM MES, pH 6.3. The arrows with + or − signs, and dashed line show the leakage current across membrane in either direction. Sign of potential is given from the side of drug addition. The number of multiple current levels increases under applied positive and decreases under negative potentials. Note also that the magnitudes of multiple current levels are different depending on current direction. (*B*) Final concentration of 2×10^{-7} M AmB added to only one side of DOPC-cholesterol BLM in MES-buffered 1 M KCl, pH 6.3. The lower current record is taken after increasing antibiotic concentration to 5×10^{-7} M on the same side of the same membrane (induced current is not in its steady state; the number of channels is increasing in time). Other symbols as in *A*.

brane potential: (*a*) the number of the discrete current levels increases (decreases to zero) when the sign of applied membrane potential is positive (negative) on the drug addition side; (*b*) the positive current levels are ~1.6 times higher than the negative ones. The first observation has already been reported for channels from one-sided addition of nystatin (Kleinberg and Finkelstein, 1984); the second observation can also be seen (see Fig. 8 *A*, ibid) but was not noted by the authors presumably because of a relativly low potential used (70 mV).

The data in Fig. 1 *A* indicate that the number of open channels depends on the applied potential and decreases at its negative values. However, if the antibiotic concentration increases in the bathing solution, ion channels could be observed even if the applied membrane potential is kept negative on the AmB addition side. Therefore, applied membrane potential regulates the number of channels but is not a necessary prerequisite for these channel formations.

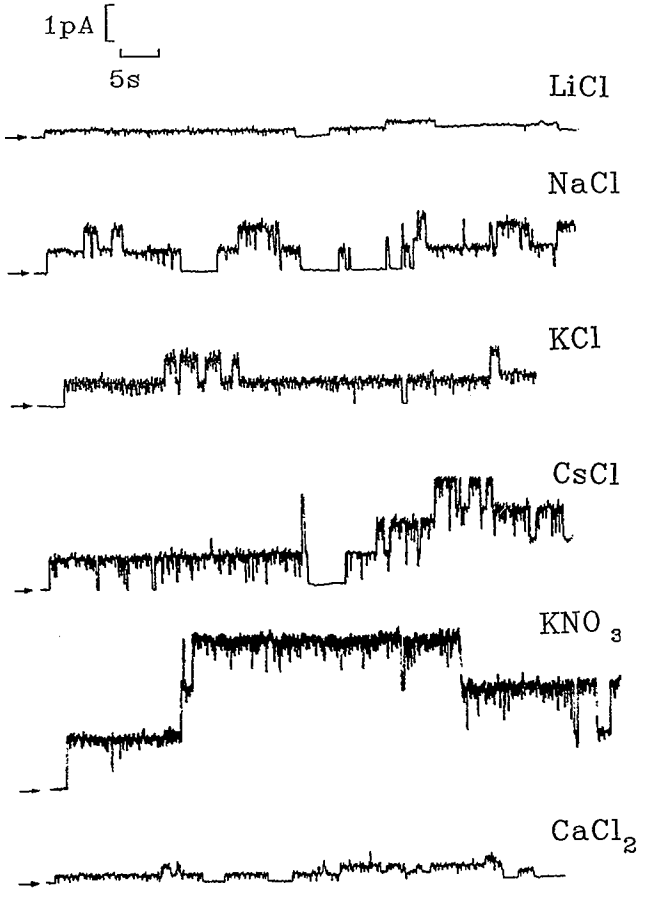

FIGURE 2. BLM current records of ~1-min duration in the presence of 0.8–1.0 × 10^{-7} M amphotericin B added to only one bathing solution, under 100 mV applied potential (positive on the side of drug addition). Arrows show the current levels through the unmodified DPhPC-ergosterol membranes in 1 M LiCl, NaCl, KCl, CsCl, KNO_3, respectively, buffered with 4.7 mM MES, pH 6.3, and unbuffered 1 M $CaCl_2$ (pH 6.3).

In DPhPC-Erl membranes the channel conductance is about 15 pS in 2 M KCl at 100 mV potential, positive on the side of drug addition. The mean life time of the channels is ~18 s.

Fig. 2 shows several examples of ion channel records formed in DPhPC-Erl BLMs in 1 M solutions of different salts. As can be seen from the picture, the channels have basically one conductive state, with or without switching to the closed state and different durations, determined by the type of salt in the membrane bath. In chloride solutions of univalent cations, channel conductances decrease in the order CsCl > KCl > NaCl > LiCl. For the potassium nitrate and halides, AmB one-sided channel conductance decreases in the order KNO_3 > KI > KCl > KF (channel conductances in 1 M KF and 1 M KI are 3.0 and 12.3 pS, respectively, records not presented). Therefore the relative passage rates of alkaline and halogen ions through these channels correlate with the order of their mobilities in water (Hille, 1992).

Replacing DPhPC with DOPC in Erl-BLMs leads to only a slight decrease of one-sided AmB channel conductance but a ~10× decrease in the mean lifetime of channels: ~12.8 pS and ~1.3 s in 2 M KCl, respectively.

In an early report (Brutyan, 1982) on Erl-containing BLMs formed from ox brain lipids or a mixture of egg lecithin with synthetic phospholipids in 2 M KCl with AmB added to one side of the membrane, we described the formation of poorly defined ion channels of ~10 pS conductance and 100 ms duration. The use of heterogeneous phospholipid mixtures for BLM formation, as well as low amounts (5%) of ergosterol, was the cause of our failure to record well-defined one-sided AmB ion channels, such as seen now in BLMs made of phosphatidylcholines and sterols.

Observations on Cholesterol-containing Membranes: Comparisons

When AmB was added to only one bathing solution of Chl-containing DOPC or DPhPC BLMs, the current again changes discretely between the baseline, a well-defined level (Fig. 1 *B*) and multiples of that level (see also records in Figs. 3, 5 *B*, and 6 *A*). A number of short-duration current bursts are seen, corresponding to AmB channels in Chl-BLM, that did not reach the discrete levels because of the limitation of recorder resolution time.

Again, the single channel conductance, as in Erl-BLMs, depends on the sign of the potential with about the same asymmetry coefficient (~1.5 in 1 M KCl), as calculated from maximum current levels, at +100 and −100 mV potentials (Fig. 1 *B*). Comparison of Fig. 1, *A* and *B* shows, that AmB increases Chl-BLM current

more effectively, when the applied potential is positive on the drug addition side, as with Erl-BLM. It is important to note here that positive potential corresponds to the physiological situation where antibiotic is applied from outside the cell.

Fig. 3 presents a few examples of one-sided AmB ion channel records in DPhPC:Chl BLMs at increasing KCl concentrations, or in other 1 M salt solutions. Comparison of Fig. 2 and Fig. 3 shows that the replacement of Erl by Chl in DPhPC BLM leads to a slight difference in channel conductances (for the given salts and concentrations). However, the lifetimes of the channels for any of salts are significantly shorter for Chl-BLMs. For clarity, Fig. 4 compares the open channel conductances and mean lifetimes in DPhPC membranes with different sterols bathed in different KCl concentrations. As can be seen, in both type of membranes, the channel conductances (higher in Erl-BLMs) increase linearly with KCl concentrations up to 3 M. The lifetime of the channels, however, shows ~100-fold difference in magnitude (much longer in Erl- than Chl-containing BLMs), decreasing with increasing KCl concentration. Changing KCl concentration and sterol results in a similar variation of conductance and channel lifetime in DOPC membranes.

The results shown in Fig. 4, i.e., the observed decrease of channel lifetime with increasing salt concentration, is a consequence of Debye screening of the charged groups of the antibiotic molecules that have been described as one of the main sources for channel

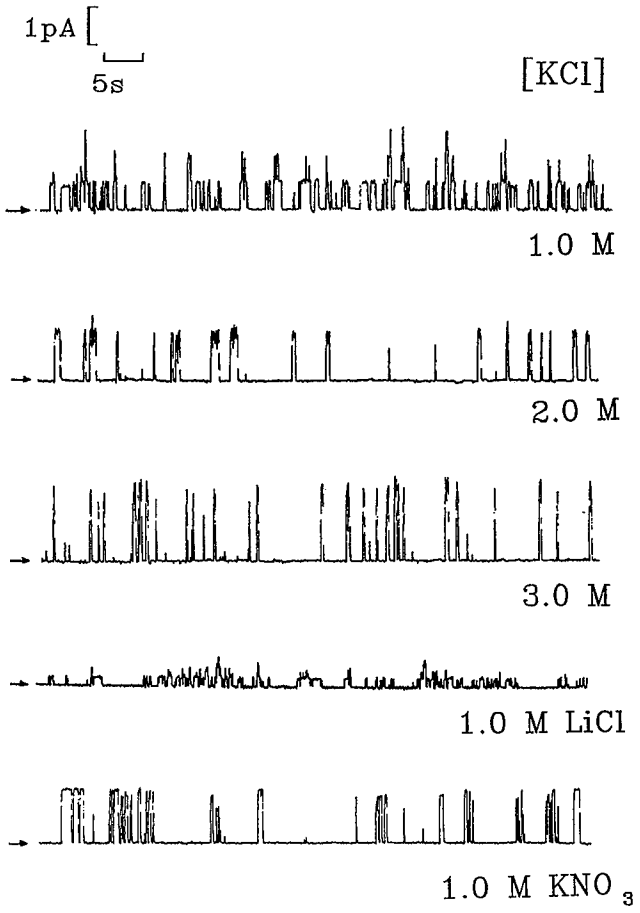

FIGURE 3. BLM current records of ~1-min duration in the presence of 1–2 × 10^{-7} M amphotericin B added to only one bathing solution, under 100 mV applied potential (positive on the side of drug addition). Arrows show the current levels through the unmodified DPhPC-cholesterol membranes in 1–3 M KCl and in one molar solutions of LiCl and KNO_3, respectively, buffered with 4.7 mM MES, pH 6.3.

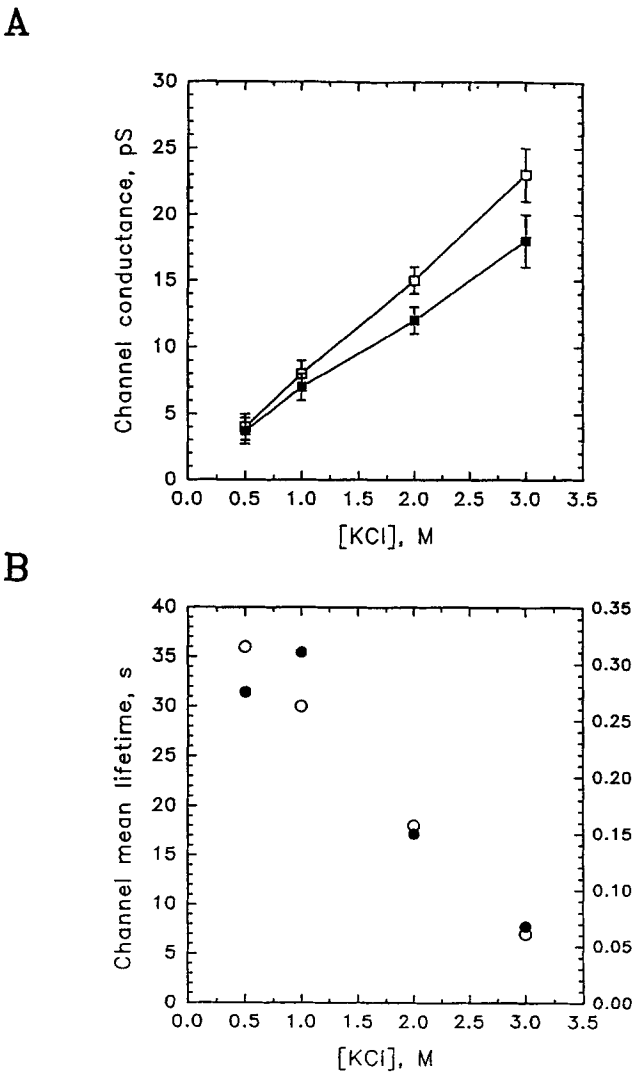

FIGURE 4. Comparison of AmB one-sided channel conductances (*A*) and mean lifetimes (*B*) in ergosterol (*open symbols*) and cholesterol (*filled symbols*) containing BLMs at increasing KCl concentrations in membrane bath. Parameters of ion channels formed at 100 mV (positive in drug addition side) were compared. (*B*) The left scale and open circles and right scale and filled dots correspond to ergosterol- and cholesterol-containing membranes, respectively. Note ~100× differing scales.

complex stabilization (Andreoli, 1974; De Kruijff and Demel, 1974).

Among the various salts tested, there was a significant tendency to saturation of channel conductance only with KNO_3, where the maximum conductance was observed. This was seen with both Erl- and Chl-BLMs. The channel conductivity sequence for Chl-DPhPC membranes, also was determined in chlorides of alkali cations and in potassium halides and was found to be the same (see in previous section) as for Erl-BLMs.

In earlier reports it was shown (Brutyan, 1982; Kolomytkin et al., 1988) that tetraethylammonium (TEA), which is an effective blocker of AmB symmetrically formed channels (Borisova et al., 1979), also blocks one-sided AmBs-induced BLM current. However, TEA is effective only if added to *cis*-AmB side. This suggests that one-sided channels have nonidentical entrances from different sides of membrane and that only the *cis*-AmB entrance is identical with the entrances to the symmetrical pore, which is consistent with the classic AmB-sterol pore (Marty and Finkelstein, 1975; Andreoli, 1974; De Kruijff and Demel, 1974; see for review Hartsel et al., 1993).

The effectiveness of 5 mM TEA (chloride salt) in blocking one-sided channels in Erl- or Chl-containing DPhPC membranes was also tested. The addition of 5 mM TEA to the *trans*-AmB side, as was expected, practically did not change induced currents, independent of the type of sterol that was incorporated. Addition of 5 mM TEA to the *cis*-AmB solution (at positive 100 mV) of multichannel Erl-DPhPC BLMs that had conductivities up to 2×10^{-5} S/cm^2 in 1 M KCl, caused the steady state current to drop, within seconds, to a new stationary level 7.5–8 times lower than before TEA addition. When analogous experiments were carried out with Chl-DPhPC BLMs, the effectiveness of TEA blocking membrane current was lower than its effectiveness on Erl-BLM. However, this difference does not point to the differences in channel structure with different sterols. Rather, it is due to another effect of the blocker on AmB formed channels, i.e., its sterol-dependent ability to stabilize the blocked channel complex structure and to increase blocked channel lifetime.

Fig. 5 *A* shows that after addition of 5 mM TEA to the *cis*-AmB side of Chl-BLM, current in the positive direction dropped only about twofold. At the same time, the negative current was increased more than twice. This result could be explained, if TEA addition increased (*a*) the number of channels or (*b*) the channel lifetime. Either leads to an underestimate of TEA's ability to block positive current. Possibility *a* would suggest an increase in the amount of antibiotic bound to the membrane upon TEA addition, for example via changing the equilibrium between AmB formed aggregates and monomers in membrane bathing solution.

In separate experiments CD spectra, sensitive to the state of AmB aggregation (Bolard et al., 1991), showed no change when TEA was added to solutions of AmB. No decrease was seen in the intensity of positive 332-

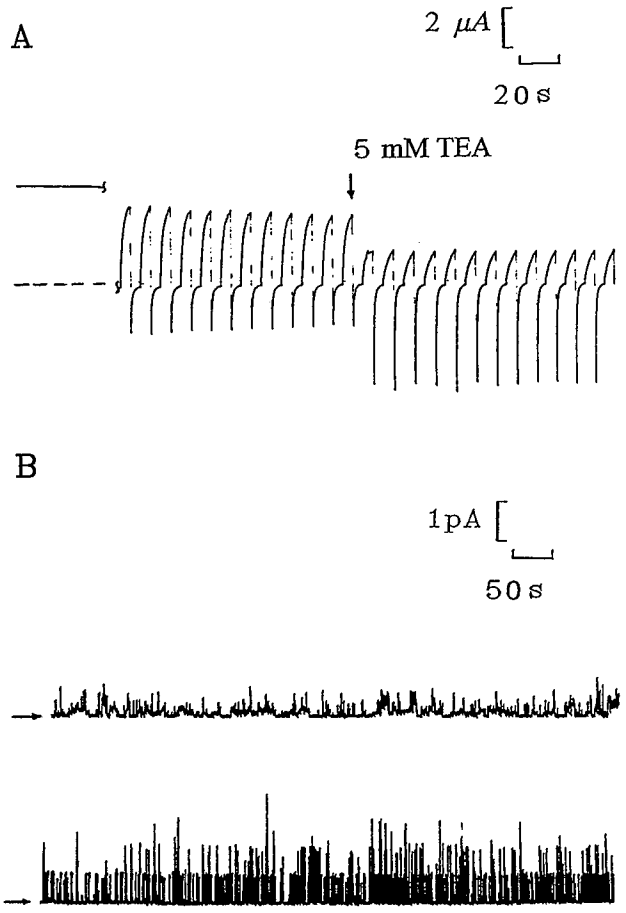

FIGURE 5. Current records of the DPhPC-Chl BLMs with amphotericin B added to only one bathing solution. (*A*) After establishment of steady state current at +100 mV applied potential (level shown on the left), the membrane was subjected to 200 mV peak-to-peak and 0.1 Hz sign-alternating squared symmetric signal (current response is shown after few seconds interruption). During application of potentials of each polarity current relaxed to ~75% of the steady state level. Then 5 mM TEA was added (*arrow*) to the 1 M KCl bathing solution that contained 3×10^{-6} M AmB. The dashed line corresponds to zero current. The membrane surface is ~5.9×10^{-4} cm^2, however, the contribution of the membrane capacitance current to the total current is negligible in presented current scale. Note the ~1.5 factor asymmetry in limits of instantaneous currents with changes in sign of potential and the more than 30× difference in current levels to which the positive and negative currents can relax before TEA addition. The positive current decreases by ~2× while negative current increases by a factor of 2.3 upon TEA addition (see explanations in the text). (*B, top*) The 5 mM TEA concentration is applied only to the positive (100 mV) side of membrane in 1 M KCl. Then amphotericin B introduced to 4×10^{-7} M concentration to the same side. (*Bottom*) The same final 4×10^{-7} M concentration of AmB without TEA.

nm peak for 1×10^{-6} M AmB in 1 M KCl, 4.7 mM MES, pH 7.2, buffer, upon addition of 5–10 mM TEA, for spectra measured immediately after 2–3 s of mixing. This observation argues against possibility *a*. Possibility *b*, the increase of blocked channel lifetime, is seen in Fig. 5 *B* (see below).

First, Fig. 5 *A* shows that currents created by high concentrations of AmB added to one side have the same properties as observed for single-channel currents: switching the sign of the 100 mV potential creates instantaneous currents, 1.5 times greater in the positive direction; subject to a +100 mV potential after each 10 s of its application, the membrane passes a current more than 30 times greater than the current under −100 mV applied potential. Therefore, at higher AmB concentrations (comparable to those effective on cell membranes) the same channel structures are presumably responsible for induced membrane currents.

In Fig. 5 *B*, for comparison, with the same time scale resolution, AmB one-sided ion channel current records are shown in 1 M KCl with (Fig. 5 *B*, *top*) and without (Fig. 5 *B*, *bottom*) 5 mM TEA added to membrane bath before the drug introduction. In the presence of TEA a number of long lasting (~seconds) current jumps are seen with 1–1.3 pS conductances. None are seen without TEA. These channels could correspond to TEA-blocked channels with increased lifetimes, compared with ordinary channels without blocker (~7 pS and ~0.25 s mean lifetime). If so, then TEA may block the channel current by a factor of about six to seven times, similar to what was seen for Erl-BLMs.

To sum up, the main differences in one-sided AmB action on Erl- or Chl-BLM particularly come from the orders of magnitude differences in ion channel lifetimes (see Fig. 4).

In a series of experiments we compared the one-sided AmB threshold concentrations needed for the single ion channel formation in Erl- or Chl-containing DPhPC BLMs. The diluted antibiotic was added to bath in large volumes (~ml), to avoid artifacts from locally concentrated AmB that could interact with the membrane during stirring. The freshly dissolved antibiotic was added to 1 M KCl solution while imposing a +100-mV potential. After stirring, the membrane current was recorded for ~15 min. Under these conditions single ion channel current events for both Erl- and Chl-containing BLMs were observed at a $\sim 4 \times 10^{-8}$ M final bathing concentration of antibiotic. In this study, a difference in effectiveness of AmB channel-forming ability in the presence of different sterols within a factor ~2 would not have been noted.

According to the above data, it becomes clear, that the different AmB effectivities on Erl- or Chl-containing membranes (thus the AmB selective cytotoxicity) mainly come from the differences in the ion channel's lifetimes. There is no significant difference in channel conductances (see Fig. 4 *A*), nor is there a difference in the amount of drug bound to the membranes (since about the same AmB concentration forms channels in both cases).

It should be noted that a difference in the binding of AmB to Chl- vs Erl-containing liposomes, as monitored by changes in AmB CD spectra, has been shown (Bolard et al., 1991). However, the interpretation of the CD data did not consider the extent to which sterols may come out of the liposome membranes (as well as from BLMs) to create nonchannel AmB-sterol aggregates in the bathing solution.

When AmB at low concentrations is added to both sides of sterol-containing DOPC or DPhPC BLMs, then two-sided channels of comparatively long duration (average lifetimes of minutes) can be formed (Fig. 6). Because of the potential dependence of one-sided AmB action, it is possible to choose experimental conditions, when preferentially only one-sided channels can be formed, rather than two sided, although some AmB is added to both sides of membrane (Brutyan, 1994). For example, even at a final 3×10^{-7} M AmB on both sides of Chl-DPhPC membrane, an applied 100 mV potential prevents the formation of two-sided channels, though numerous one-sided (positive) channels are observed. The symmetric channels could be observed only after decreasing applied potential, or keeping the membrane without applied potential. The higher applied potential can inhibit the formation of symmetric AmB channels as more antibiotic is added to both sides of membranes. However, such a possibility is limited, when the AmB concentration added to both sides is high enough.

As can be seen in the records in Fig. 6, in 2 M KCl solution the average conductances of two-sided symmetric channels are 3.3 and 4.0 pS in Erl-DPhPC and Chl-DOPC membranes, respectively. There have been earlier reports (Ermishkin et al., 1977; Borisova and Kasumov, 1978) of 3.5 and 6.5 pS ion channels with ~minutes duration in 2 M KCl in, respectively, ergosterol- or cholesterol-containing BlMs formed from ox brain phospholipids and *n*-heptane. Therefore, in contrast to one-sided AmB channels, no significant changes in conductance and duration are observed for two-sided channels for various BLMs. This difference between channel types probably comes from the fact that two-sided channels have more stabilizing factors, and thus are comparatively more stable structures.

The conductance of the one-sided channels at 100 mV is about five times greater than that of symmetric channel (Fig. 6 *B*). A 5:1 ratio in conductance for one- and two-sided nystatin channels has been reported (Kleinberg and Finkelstein, 1984) in ergosterol-containing BLMs formed from acetone-extracted soybean

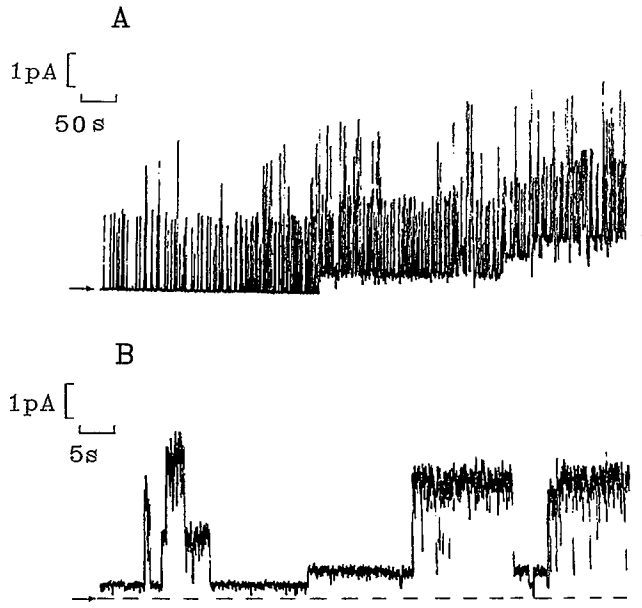

FIGURE 6. One-sided and two-sided amphotericin B channel current records simultaneously formed in cholesterol- (*A*) and ergosterol- (*B*) containing BLMs (in 2 M KCl, 4.7 mM MES, pH 6.3, and 150 mV) (applied potential). Arrows and dashed line show the levels corresponding to unmodified membrane current leakage. (*A*) The initial current is due to channels formed in membrane from one-sided addition of AmB at 3×10^{-7} molar concentration. Immediately before the beginning of the record, the same concentration of antibiotic was stirred into the solution on the other side of the membrane. The first switching of two-sided channels is seen after ~6 min. Membrane composition: DOPC/cholesterol 5:1 (wt/wt). (*B*) Several current traces are seen. Two nearest to dashed line traces correspond to two two-sided channels with 3.3 pS conductance each. One medium-current channel on the left (~11 pS) and others (~20 pS) correspond to one-sided channels due to insertions from either side. The difference in conductance is from the different sign of applied potential with respect to the side of insertion. This record was taken after addition of 6×10^{-8} M AmB to both side of BLM and subsequent partial wash-out of antibiotic from the "positive" solution after two-sided channel formation. Membrane composition: DPhPC/ergosterol = 5:1 (wt/wt). Note the more than five times smaller conductance of AmB two-sided channels, compared with one-sided channel conductance. The conductances of one-sided channels (~20 pS) are higher than those in Fig. 1 *A* at 100 mV (~15 pS), because of one-sided channel conductance potential dependence (not considered in this paper).

lipids, suggesting similarities in action on membranes of AmB and Nys, antibiotics with close chemical structures.

DISCUSSION

Investigation of the one-sided action of AmB on BLMs formed from pure DOPC and DPhPC shows that the drug is almost ineffective in sterol-free membranes. We observed little or no change in BLM conductance even in 8×10^{-5} M solution, a concentration larger than that reported to be effective on sterol-containing cell membranes (Itoh et al., 1990). This observation strongly supports a sterol requirement for the AmB effectivity. It also suggests that AmB cytotoxicity occurs by the formation of specific antibiotic-sterol structures rather than by nonspecific damage (Hartsel et al., 1993) of the membranes. Of course, there could be nonspecific membrane damage mediated without interaction with sterol by amphiphilic AmB molecules probably at concentrations higher than their effective cytotoxic concentrations. It should be noted that in previous studies, data about significant one-sided AmB effectivity on lipid bilayers formed from sterol-free and chromatographically pure lipids are absent. This is important because even trace amount of sterol could produce an artifactual result because of the high-order sterol dependence.

We show that when AmB is added only to one side of sterol-containing DOPC or DPhPC BLM, the formed ion channels have similar properties for both Chl- or Erl-containing membranes: the same single-channel conductance asymmetry; same sequence in channel conductances, about the same asymmetric blocking by TEA; similar potential dependence and sterol requirement of drug effectivity. These data allow one to conclude that in both Chl- and Erl-containing BLMs one-sided AmB forms ion channels with the same molecular structure, consistent with the classical AmB-sterol channel model.

These findings contradict data by Bolard et al. (1991) on the inability of AmB to form one-sided channels of similar structure in Chl- and Erl-containing BLMs, or to their new model of the conductivity unit consisting of self-associated symmetric head-to-tail antiparallel dimers. In terms of this new model, it is impossible to account for most of the present observations, especially those connected with asymmetric properties of one-sided channels. In addition, the AmB molecules in the symmetric dimer form, as considered, could easily be transferred to the *trans*-side of the membrane, a fact that was not previously noted.

In phosphatidilcholine-sterol membranes the channel conductances differ only slightly, but the channel mean lifetime in Erl-BLM is about one hundred times longer than in Chl-BLM. At the same time the concentrations needed for single ion channel formation event recording are about the same for both Erl- or Chl-containing BLMs. Therefore, at relatively low drug concentrations, when the antibiotic exists mostly as small aggregates or monomers in the bathing solution, the amount of the drug bound to the membrane from the solution is about the same for both Erl- and Chl-containing membranes. Given this information we conclude that the difference in AmB effectivity in Erl- ver-

sus Chl-containing membranes (hence the AmB's selective cytotoxicity) comes mainly from a difference in open-channel lifetimes. Therefore, these model membranes can be used to compare and estimate rapidly the potential effectivity of AmB, other PA, or their new chemical derivatives for selective cytotoxic activity.

One may compare the $g_{chan} \times t_{chan}$ (channel conductance and mean lifetime, respectively) products measured in same experimental conditions for Erl- or Chl-containing BLMs. For example, from the data in Fig. 4 and the linearity of dependences of channel conductances and lifetimes on salt concentration, a ratio, R, of these products can be calculated by extrapolation to lower (physiological) salt concentrations to give $R = 1.3 \times 10^2$. According to Cheron et al. (1988), AmB concentrations causing 50% of Candida albicans growth inhibition and 50% of hemoglobin release from erythrocytes are 0.03 and 1.7 μg/ml, respectively. The ratio of these two concentrations is 56, which is only 2.3 times less than R. However, upon closer fitting of these two numbers, there are other factors to take into account: cell membrane potential, the cellular membrane lipid and free sterol composition, etc. For comparison of the efficacy of different PAs (or their chemical derivatives) with different sterols a factor of concentration must be taken into account, e.g., the concentrations required for single channel formation. In vitro assays on the comparative toxicity of PAs, on fungal and mammalian cells (Cybulska et al., 1984; Cheron et al., 1988), are complicated by several different parameters: external conditions, growth state of the cells, cell-inherent drug resistance, and many others.

The observed one-sided potential-dependent action of AmB strongly suggests that the selective cytotoxicity of the antibiotic through its sterol specificity is at least partly connected with the difference in cell membrane potentials. These differences can be one of the causes of nonuniform distribution of AmB in different tissues after systematic administration (Szoka and Tang, 1993).

The 23–25-Å membrane hydrocarbon thickness measured from the capacitance of the BLMs used is about the same as the AmB-sterol half-pore length. If the antibiotic forms a number of water-filled nonconducting half-pores inside the BLM (Marty and Finkelstein, 1975), then it seems likely that such BLM, destabilized across almost the whole length of its hydrocarbon interior would show an increase of membrane leakage current. Such leakage was never seen in the current records of AmB treated BLMs, used in this work. It is more likely that the pre–half-pores are not bounded inside of the BLM, but are lying on membrane surface, in form of flat mosaic structures of AmB molecules, and only sometimes dip into the BLM hydrocarbon interior to become conductive. This mechanism could also explain the observed relative time delay of symmetric channel formation and its kinetics (Marty and Finkelstein, 1975), since two penetration events must occur at the same time and on opposite sides of the BLM.

Remaining within the framework of the classical AmB-pore model, it is natural to expect that the conductance of the half-length one-sided channel could be about only two rather than five times the two-sided channel conductance (Fig. 6 *B*). In classical models of pores, a special role was assumed for the ring of hydrogen bonds at the junction of semi-pores (at C_{35} carbon of antibiotic), connected with the channel complex stabilization and induction of the electrostatic barrier for cations. The latter suggestion also requires us to explain the observed cation–anion interaction in AmB two-sided channels (Brutyan and Ermishkin, 1983; Ermshkin and Brutyan, 1983) (though in Borisova et al., 1986 an alternative reason for a barrier was discussed). Probably, the lack of hydrogen bonding at C_{35}-OH leads to the observed shorter lifetimes and anomalously larger conductance of one-sided AmB channels compared with two-sided channels, as well as to the difference in ion selectivity of two types of channels. Thus, much of the data on AmB action that appears to be conflicting (Hartsel et al., 1993) can be explained in terms of classical AmB-sterol pore formation theory.

The nature of the potential-dependent mechanism of one-sided AmB channel opening (closing) remains unclear. Over a wide pH range, the zwitterionic AmB is net neutral. It was proposed that the response to applied membrane potential comes from its action on bound cations in a nonconductive channel complex (Brutyan, 1994). But even in $CaCl_2$ solution (divalent Ca^{2+} is not permeable through AmB channels, although channels could be seen in $CaCl_2$ solution [Fig. 3]) or in both high- and low-pH solutions when AmB is charged, the same sign of membrane conductivity stimulation was observed (results are not shown). Perhaps there is a polarization along the lactone ring of the antibiotic molecule and the applied potential can either increase the intramembrane (or especially membrane surface) antibiotic concentration, or force a surface pre-pore aggregate of AmB into the hydrocarbon interior in an open-channel conformation.

According to classical models of amphotericin B-sterol pore complex, due to the unequal proximity of charged positive and negative groups of antibiotic from the channel axes, the induced sum of electrostatic potential at the entrance of the pore is negative, favoring TEA^+-binding at the channel entrance. Also, as has been shown by Borisova and Kasumov (1978), the properties of amphotericin B (though for only two-sided antibiotic action) channels (lifetime, conductance, ion selectivity, possibly the antibiotic-sterol stoichiometry of the entire complex and thus diameter of the pore) depend on the type of sterol molecule form-

ing the channel. Referring to the above mentioned physical basis, the observed difference in increasing lifetimes of TEA^+ blocked channels, formed with ergosterol or cholesterol, may be explained by different extents of TEA^+-channel interactions.

An alternative mechanism for the explanation of TEA^+-blocked channel lifetime lengthening could be considered, according to which the blocked open channel complex with excess total positive charge (coming on TEA^+) penetrates deeper toward the membrane, or is held inside the membrane by the transmembrane potential. Further investigation will be necessary to understand which one of these mechanisms (electrostatic stabilization or channel complex viscous movement toward the bilayer) plays the key role in increasing of the channels lifetime.

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