A Selective Cholesterol-Dependent Induction of H^+/OH^- Currents in Phospholipid Vesicles by Amphotericin B†

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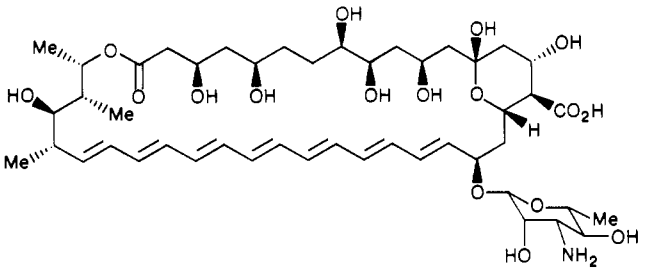
ABSTRACT: The effect of amphotericin B on the proton/hydroxide permeability of small unilamellar vesicles has been investigated by using potential-dependent paramagnetic probes. Amphotericin B at 1–10 molecules/vesicle causes a modest 4–8-fold increase in the background H^+/OH^- permeability of egg phosphatidylcholine (egg PC) vesicles. However, in the presence of cholesterol, amphotericin B promotes a dramatic increase in the H^+/OH^- permeability of more than 2 orders of magnitude. Surprisingly, this is not observed in vesicle membranes containing ergosterol. In membranes composed of 5–15 mol % ergosterol, amphotericin B is even less effective at promoting H^+/OH^- currents than in pure egg PC vesicles. The K^+ current promoted by amphotericin B in vesicles formed from egg PC and from egg PC plus cholesterol or ergosterol was measured. No significant sterol dependence was found for the K^+ current. These results strongly suggest that different mechanisms, or amphotericin B/sterol complexes, are responsible for the induction of H^+/OH^- and K^+ currents. These results have important implications for understanding the therapeutic and toxic effects of amphotericin B.

Amphotericin B (AmB)[1] is an important member of the polyene macrolide antibiotic family, and it is the only antibiotic that is generally used to treat systemic fungal infections. It



amphotericin B

is frequently used in chemotherapy in combination with other agents. In addition, the methyl ester of AmB is currently of considerable interest because it is observed to block replication and infection by human immunodeficiency virus in vitro (Schaffner et al., 1986). This antibiotic appears to act at the level of the plasma membrane, ultimately inducing lysis of the target cell. AmB is quite toxic to eukaryotic organisms, including humans, but does not appear to be toxic to organisms with sterol-free cell membranes, such as bacteria. The activity of AmB against fungal cells is believed to result from its preferential association with membranes containing ergosterol [for a review, see Bolard (1986)].

AmB is observed to form channels in model membranes, and a popular model for its lytic activity proposes that AmB associates with sterols to produce a transmembrane pore. This transmembrane pore is thought to be a cylindrical aggregate structure built up from approximately eight 1/1 AmB/sterol units (Finkelstein & Holz, 1973; Andreoli, 1973; De Kruijff & Demel, 1974). In planar bilayer systems, optimal channel activity is observed when AmB is added to both faces of the membrane. In this case, the conduction is anion selective. When it added only to one side of the membrane, the channel activity is diminished and is cation selective. The association of two "half-pores" from each side of the bilayer is proposed to explain these results.

Several questions are raised by this model. AmB does not appear to permeate model membranes; if this is the case in biological membranes, only low conductance cation-selective channels should be found (Holtz, 1979). It has been demonstrated that fewer than one AmB per lipid vesicle can release all of the internally trapped K^+ ions (Bolard, 1986). According to the sterol pore model, this would require an extremely high cooperativity for AmB aggregation in the membrane. So far, there is little evidence that this type of cooperativity exists. Other studies show that sterols are not even necessary for AmB-induced permeability changes (Vertut-Croquin et al., 1983; Archer, 1976; HsuChen & Feingold, 1973; Sessa & Weissman, 1967).

Clearly, characterizing and quantitating the channel activity of AmB is an important step in elucidating its mechanism of action. As a result, many measurements of solute and ion leakages induced by AmB in membrane vesicles and cells have been made (Bolard, 1986). To date, quantitative estimates of electrogenic ion fluxes and proton currents induced by AmB in lipid vesicles have not been made. If AmB forms a water-filled pore, relatively high proton permeabilities might be expected, as is the case for the linear polypeptide channel gramicidin (Levitt, 1984). The rigid structure of AmB and its polyol face also suggest that AmB could have some activity as a proton conductor.

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[1] Abbreviations: AmB, amphotericin B; EPR, electron paramagnetic resonance; egg PC, egg phosphatidylcholine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; SUV, small unilamellar vesicle; DMSO, dimethyl sulfoxide; TPB^-, tetraphenylborate anion; CD, circular dichroism; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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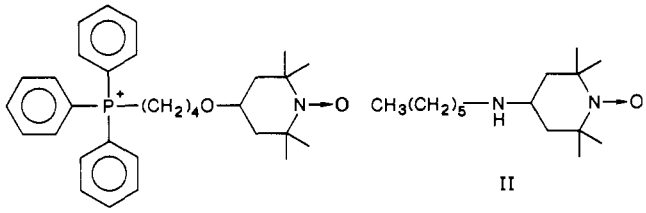
In the present paper, we make quantitative estimates of the K^+ and H^+/OH^- currents induced by AmB in lipid vesicles. We use two paramagnetic probes, I and II. The spin-labeled



I II

phosphonium I measures transmembrane voltages resulting from ion movements that charge the vesicle membrane capacitance. The secondary alkylamine II directly monitors the transmembrane pH gradient in vesicle systems (Cafiso & Hubbell, 1983). We present evidence for a selective, proton conductive state of AmB in lipid vesicle systems. We also establish methodologies using voltage-sensitive spin probes that can be used to quantitate ion currents and characterize ion channels in phospholipid vesicles.

Materials and Methods

Materials. The phosphonium I and *n*-hexylamine nitroxides II used here were synthesized as previously described (Cafiso & Hubbell, 1978b; Flewelling & Hubbell, 1985). Egg phosphatidylcholine (egg PC) was isolated from fresh hen eggs according to the procedure of Singleton et al. (1965) and stored in chloroform at −20 °C under argon. AmB, valinomycin, and phloretin were obtained from the Sigma Chemical Co. (St. Louis, MO). The protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was purchased from Calbiochem (La Jolla, CA).

Preparation of Lipid Vesicles. To form lipid vesicles, egg PC or sterol/egg PC was dispersed in the appropriate buffer at a concentration of approximately 200 mg/mL and sonically irradiated to form small unilamellar vesicles as previously described (Castle & Hubbell, 1976). AmB was added to the vesicles following sonication and vortexed extensively. This suspension was allowed to equilibrate for at least 4 h at 4 °C in the dark under Ar and then was centrifuged at 2000 rpm in a Sorvall SS-34 rotor for 5 min to remove any particulates. In general, only small amounts of AmB and lipid could be pelleted and most remained as SUV's. The membrane concentrations of AmB were determined by dissolving a small aliquot of the vesicle sample in DMSO and measuring the absorbance at 416 nm (ϵ = 121 400 at 416 nm in DMSO; Vertut-Croquin et al., 1984). The phosphorus content was assayed as described previously (Bartlett, 1959) to determine the AmB/lipid ratio. At the concentrations of lipid used in our EPR experiments, greater than 98% of the AmB was membrane bound; for CD measurements, greater than 95% of the AmB was membrane bound (Witzke & Bittman, 1984). As described previously, tetraphenylborate (TPB^-) was added at concentrations up to 20 μM to the undiluted suspension to enhance the transmembrane equilibration of the phosphonium spin-label I (Cafiso & Hubbell, 1982).

EPR Measurements of H^+/OH^- Permeability. Proton currents were determined in sonicated vesicles containing AmB by utilizing the alkylamine nitroxide II to measure time-dependent pH gradients. Proton gradients were established across phospholipid vesicles by diluting a vesicle suspension into a buffer solution with a rapid mixing device fitted to the EPR spectrometer [see Cafiso and Hubbell (1982)]. Probe II undergoes ΔpH-dependent partitioning changes that can be used to quantitatively determine ΔpH (Cafiso & Hubbell, 1978b). When we establish pH gradients under weakly buffered conditions, time-dependent changes in the partioning of II are observed. These changes in partitioning can be used to measure the vesicle H^+/OH^- current as described previously (Cafiso & Hubbell, 1983). In our experiments, we estimate the initial H^+/OH^- current, i_0 (at $t = 0$ and $\Delta\psi = 0$), from $(\partial\Delta pH/\partial t)_{t=0}$, the initial rate of change in ΔpH measured with II (Cafiso & Hubbell, 1983). This current is related to the buffer capacity B, the Faraday constant F, and the inner and outer vesicle radii r_i and r_o by

$$i_0 = \frac{r_i^2 FB(\partial\Delta pH/\partial t)_{t=0}}{3r_o} \quad (1)$$

EPR Measurements of K^+ Permeability. Label I was used to monitor transmembrane voltage changes across lipid vesicles as described previously (Cafiso & Hubbell, 1980; Cafiso, 1987). To ensure accurate potential measurements, we measured the binding of the label to both the interior and exterior vesicle surfaces as described previously (Cafiso & Hubbell, 1982; Cafiso, 1987). To create transmembrane potentials, the vesicle suspension was made in 100 mM K_2SO_4, buffered with 10 mM MOPS, pH 7.5. Vesicles were then rapidly diluted into isoosmotic sucrose (7.2%) or arginine sulfate so that the final internal/external K^+ concentration ratio was 14/1. When ion activities and H^+/OH^- movement are taken into account, a K^+ equilibrium potential of 63 mV is expected (Kornberg et al., 1972). Transmembrane voltages that developed in the presence of AmB upon creating the K^+ gradient were used to measure the K^+ currents. From the initial slope of the voltage–time curve, $(\partial\Delta\psi/\partial t)_{t=0}$, the initial K^+ current, i_0, at $\Delta\psi = 0$ was estimated by using $i_0 = c(\partial\Delta\psi/\partial t)_{t=0}$. We used a value for c, the specific membrane capacitance, of 0.9 $\mu F/cm^2$, which corresponds to the capacitance of solvent-free planar bilayers (Montal & Mueller, 1972).

CD Measurements. CD spectra were obtained with a home-built simultaneous absorption and CD spectropolarimeter. Absorption spectra were taken with a Hewlett-Packard 8452A diode array spectrophotometer.

Results

Amphotericin B Induces H^+/OH^- Currents in Phospholipid Vesicles. When a weakly buffered pH gradient is created across egg PC vesicles, the amplitude of the high-field resonance of II becomes time dependent. As demonstrated previously, the increasing amplitude of this resonance represents a decrease in the partitioning of the probe to the membrane that accompanies a decrease in ΔpH (Cafiso & Hubbell, 1983; Perkins & Cafiso, 1986). The pH gradient decreases because of a transmembrane H^+/OH^- flow that occurs in pure lipid membrane vesicles. Because of the large ratio of the external volume to the trapped vesicle volume ($V_0/V_i \approx 100$), changes in ΔpH occur primarily as a result of pH changes in the vesicle interior. This background H^+/OH^- current occurs in the absence of AmB and is described in detail elsewhere (Cafiso & Hubbell, 1983; Perkins & Cafiso, 1986).

When vesicles are incubated with AmB, the magnitude of this H^+/OH^- current increases. Figure 1 shows the decay of the high-field resonance for probe II in egg PC vesicles made with cholesterol or ergosterol and with added AmB. A decay for egg PC vesicles with added FCCP is also shown. The extent of the ΔpH decay clearly differs when the effect of AmB in cholesterol is compared with that of FCCP. AmB completely dissipates the pH gradient; FCCP does not. This is consistent with the observation that FCCP, under these conditions, does not lead to significant changes in the cation

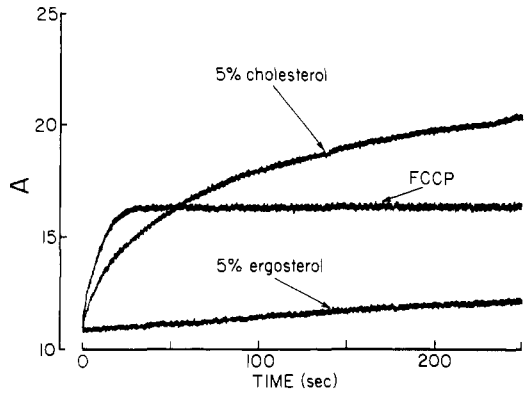


FIGURE 1: Recording of the high-field resonance of 50 μM spin-labeled *n*-hexylamine II following the establishment of a pH gradient at $t = 0$ across sonicated vesicles. The time dependence represents a change in the partitioning of the probe that accompanies a change in ΔpH. The initial H^+/OH^- currents were measured from the probe phase partitioning as described in the text. Tracings are shown for vesicles formed from egg PC containing 1 μM FCCP and from egg PC plus 5 mol % ergosterol or cholesterol. The ergosterol and cholesterol samples contain AmB at a macrolide/lipid concentration of 1/900. Vesicles contained 5 mM citrate and 125 mM Na_2SO_4 and were mixed into an isoosmotic solution containing 10 mM MES to establish a pH gradient of −1.96 (pH_{in} = 3.88; pH_{out} = 5.84). The lipid was at a concentration ≈26 mM. The total amplitude of 50 μM aqueous spin on the *y* axis is 55.1.

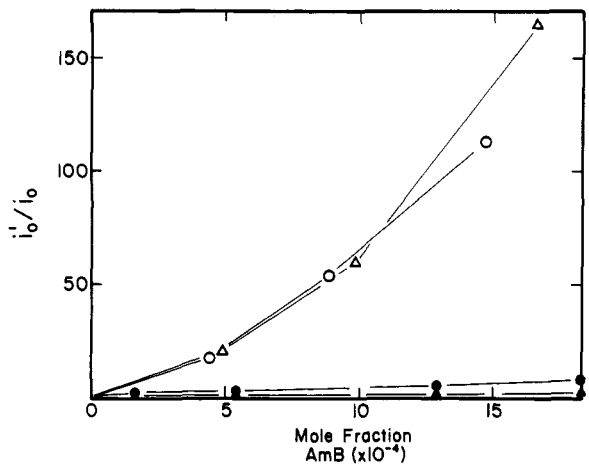


FIGURE 2: Ratio of the initial ($\Delta\psi = 0$) H^+/OH^- currents in sonicated vesicles in the presence and absence of AmB, i_0' and i_0, respectively. Vesicles were formed from (●) pure egg PC, (▲) 5 mol % ergosterol, and (△) 5 and (○) 20 mol % cholesterol. AmB produced a smaller effect in PC with 15 mol % ergosterol (not shown) than in 5 mol % ergosterol. The conditions used were the same as those in Figure 1. The background H^+/OH^- currents in the absence of AmB (in pA/cm^2; 10^{-12} C·cm^{-2}·s^{-1}) are as follows: egg PC, 13; egg PC + 5 mol % ergosterol, 24; egg PC + 15 mol % ergosterol, 28; egg PC + 5 mol % cholesterol, 20; egg PC + 20 mol % cholesterol, 13. For reference, the presence of 1 μM FCCP alone, in 5 mol % cholesterol vesicles, produced a value for i_0'/i_0 of 150.

permeability, whereas AmB does (see below). A more quantitative comparison of these H^+/OH^- currents is shown in Figure 2. Here, the initial H^+/OH^- currents with and without sterol are plotted as a function of the AmB concentration. The most striking feature of these measurements is the dramatic increase in the H^+/OH^- current induced across vesicles containing cholesterol. In cholesterol-containing vesicles, AmB increases the initial H^+/OH^- current, i_0, by more than 2 orders of magnitude at AmB/lipid ratios of 1/1000. This effectiveness is observed over an extremely wide range of cholesterol levels (from 5 to 20 mol % cholesterol). A large increase in the H^+/OH^- current is not seen in vesicles formed with ergosterol, even when sterol concentrations are

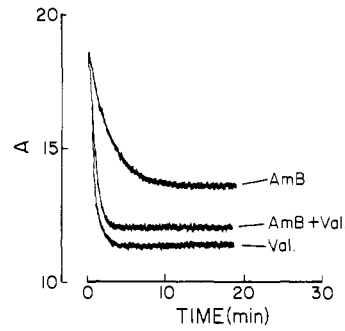


FIGURE 3: Time dependence of the high-field resonance of 20 μM spin-labeled phosphonium I following the establishment of a K^+ gradient across lipid vesicles containing AmB and/or valinomycin. The decrease in the amplitude of this resonance represents a polarization of the lipid vesicle membrane. This time-dependent voltage change was used to calculate the K^+ currents induced by AmB in the vesicle system as described in the text. The K^+ gradient was formed by mixing and diluting the vesicle suspension (in 100 mM K_2SO_4, 10 mM MOPS, pH 7.5) with an osmotically balanced sucrose or arginine sulfate solution. The valinomycin and AmB concentrations used were 5 μM and 1/900 lipids, respectively. The lipid concentration was approximately 13 mM. The total amplitude of 20 μM aqueous spin on the *y* axis is 23.7.

varied from 5 to 15 mol %. In fact, in vesicles containing ergosterol, AmB was less effective at promoting H^+/OH^- currents than in pure egg PC vesicles.

The observed increases in H^+/OH^- conduction appear to be insensitive to changes in the membrane dipole field. We previously demonstrated that phloretin can be used to modulate the membrane dipole field in sonicated vesicles. The addition of 5 mol % phloretin to the vesicle membrane had no significant effect on the magnitude of the initial AmB-induced H^+/OH^- current. These are phloretin levels that were previously observed to result in about an 80-mV decrease in the dipole field (Perkins & Cafiso, 1987).

K^+ Currents Induced in Vesicles by Amphotericin B. When K^+ gradients are established across phospholipid vesicles (balanced against isoosmotic sucrose or arginine sulfate), time-dependent changes in the binding of I are observed in the presence of AmB or valinomycin that accompany the development of a transmembrane potential. Shown in Figure 3 are tracings of the high-field resonance of I following the establishment of a 14:1 K^+ gradient (inside/outside) in sonicated vesicles composed of egg PC. We estimate that the ratio of K^+ activities under our experimental conditions is 11.8. In the presence of valinomycin we estimate an equilibrium potential from this partitioning change of 63 ± 4 mV (inside negative), which agrees with the expected value. As shown in Figure 3, the addition of AmB alone to these vesicles results in a change in the partitioning of probe I. The potential estimated from this partitioning change is 54 ± 3 mV (inside negative), slightly less than that expected on the basis of K^+ equilibrium in this system. The addition of valinomycin in combination with AmB (Figure 3) leads to slightly higher estimates of potential than with AmB alone.[2] Results identical

[2] The fact that AmB does not completely polarize our vesicle systems to a K^+ equilibrium could be accounted for by the movement of counterions such as SO_4^{2-}. However, the substitution of larger counterions, such as citrate or pyrenetetrasulfonate, did not alter the equilibrium potential. This argues against counterion movement. Breakage of a portion of the vesicles also does not appear to take place, since the addition of valinomycin can bring the value of $\Delta\psi$ much closer to its equilibrium value. A small part of the discrepancy in the equilibrium $\Delta\psi$ values is due to the fact that AmB modulates the zero voltage binding constants of probe I. The difference in $\Delta\psi$ could be explained if a population of lipid vesicles remained unpolarized in the presence of AmB.

with those shown in Figure 3 are obtained in the presence of 5 mol % cholesterol or ergosterol (data not shown). In each of these cases, the partitioning and estimated potentials vary with the magnitude of the K^+ gradient and do not appear in the absence of the gradient.

An estimate of the currents from the data shown in Figure 3 requires that time-dependent changes in the partitioning of I not be rate limited by its transmembrane movement. As described previously, tetraphenylborate (TPB^-) at extremely low ion/lipid ratios can dramatically lower the energy barrier to the transmembrane movement of I (Cafiso & Hubbell, 1982). We used TPB^- here at levels sufficient to ensure that the partitioning changes in Figure 3 were representative of transmembrane potential changes. From the data in Figure 3, the initial rate of change in $\Delta\psi$ was obtained and used to estimate the K^+ current. For egg PC vesicles, the zero-voltage current induced by AmB was $\approx$700 pA/cm^2 (10^{-12} C·s^{-1}·cm^{-2}) at an AmB/lipid ratio of 1/600. Almost identical results were found for sterol-containing vesicles where currents of 1100 and 540 pA/cm^2 were obtained in 5 mol % ergosterol and cholesterol, respectively, at this concentration of AmB. These currents were seen to decrease slightly at higher sterol concentrations.

CD Spectra. CD spectroscopy has been used extensively to provide information on the aggregation state and conformation of AmB. We obtained CD spectra of AmB in pure egg PC vesicles or egg PC vesicles with 5 mol % sterol. The AmB/lipid ratio was approximately 1/600 (the conditions of our ion transport experiments, above). The CD spectra observed in the presence of sterol were in excellent agreement with those obtained previously for AmB/sterol complexes, with the cholesterol spectrum being slightly less intense (Vertut-Croquin et al., 1983; Gruda & Bolard, 1987). The major features of these sterol/AmB spectra are alternating positive and negative bands between 320 and 420 nm. At least two separate spectral species of these complexes, termed type II and type III (Bolard, 1986), are thought to be associated with ion permeation. Our spectra of AmB in vesicles containing 5% sterol show a superposition of these two forms, as would be expected at these AmB/lipid ratios (Vertut-Croquin et al., 1983). The monomeric species, which is only weakly optically active, is probably also present. In pure PC vesicles, most of the AmB remained as a monomeric form (>93%), with a small contribution from the proposed dimer/micelle aggregate (Vertut-Croquin et al., 1983; Ernst et al., 1981). This species is characterized by an intense excitonic CD doublet centered at about 340 nm.

Discussion

Several intriguing features of the conductance properties of AmB in lipid vesicles are observed here. There appears to be a striking selectivity for cholesterol over ergosterol when H^+/OH^- currents are examined. AmB induces large increases in the background H^+/OH^- current, but only in the presence of cholesterol. Not only does ergosterol not promote these increases, it clearly inhibits them. This opposing behavior has not been previously observed for these sterols. Generally, cholesterol and ergosterol are observed to modulate the behavior of membranes and AmB activity in a similar fashion. At present, we do not understand the reasons for this striking sterol specificity. It might reflect the formation of a unique AmB/cholesterol complex (or AmB conformer) or result from differences in the properties of vesicles formed from ergosterol versus cholesterol. Experiments to resolve this question are currently under way.

Another surprising feature of the cholesterol-dependent H^+/OH^- permeability is that it is not accompanied by a similar increase in the conductivity of K^+. In fact, the AmB-induced increases in K^+ currents measured here do not even appear to be sterol dependent. This observation strongly suggests that the induction of K^+ and H^+/OH^- currents in vesicles by AmB occurs via two distinct molecular mechanisms. While we do not yet know if these results are peculiar to sonicated vesicle systems or egg PC, the lack of a sterol-dependent cation conductance argues against the sterol-dependent pore hypothesis for AmB activity.

Several molecular structures could promote H^+/OH^- conduction. Proton/hydroxide ions might be conducted in a water-filled pore formed by AmB, as they are in the gramicidin pore (Levitt, 1984). In this case, the existence of a distinct aggregate for AmB in cholesterol-containing membranes would be required to explain the sterol specificity. We also expect this pore to facilitate cation conductance, which is difficult to reconcile with the lack of a sterol effect on cation conductances. It is possible that AmB, or small levels of contaminants, acts as a carrier in the presence of cholesterol; however, the inability of dipole field changes to modulate H^+/OH^- conductances argues against a carrier mechanism. Another intriguing possibility is that the hydroxyl backbone of AmB, perhaps combined with associated water molecules, facilitates conduction through a hydrogen-bonded chain. Hydrogen-bonded chains are proposed as mechanisms for achieving proton conduction through proteins (Nagle & Tristam-Nagle, 1983).

The CD data we obtain are in agreement with other published spectra (Bolard, 1986). These data provide no evidence for a detectable, single CD conformer that is present in membranes containing 5 mol % ergosterol, cholesterol, and pure egg PC. Yet these environments are observed to produce similar K^+ currents. The only major AmB species that is present in all of these membranes is the membrane-bound monomeric form. The obvious, but by no means definitive, conclusion is that the monomeric form alone accounts for the observed K^+ currents. Most CD studies indicate that AmB forms spectroscopically similar complexes with ergosterol or cholesterol. However, Gruda and Bolard (1987) recently demonstrated by CD that a unique cholesterol/AmB complex is formed in solution. We did not observe this species in vesicles, but a small population in this conformation would be difficult to distinguish from other strong optically active transitions and might account for the H^+/OH^- conduction we observe here. In any case, we do not presently know whether the proton conductance is due to an aggregate structure or the AmB monomer.

AmB is believed to be more toxic to fungal cells due to the presence of ergosterol in the cell membrane. Since ergosterol does not promote H^+/OH^- conduction, H^+/OH^- currents cannot account for the antifungal activity of AmB (this conclusion presumes that the results obtained here hold for biological membrane systems). It is worth noting that the H^+/OH^- current (which is seen in the presence of cholesterol) might conceivably account for the high toxicity of AmB to humans. Indeed, many of the side effects of AmB are similar to those of protonophoric poisons such as dinitrophenol (i.e., high fever, tachycardia, nausea, anorexia, and rapid respiration; Goodman & Gilman, 1985). We are currently carrying out experiments directed at measuring AmB induced H^+/OH^- currents in biological membrane systems.

Finally, the measurements made here establish methodologies using paramagnetic probes that can be used to quantitate the activity of ion channels in lipid vesicle systems. We are

now using these methods to establish and measure membrane voltages in the presence of voltage-gated ion channels such as alamethicin.

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