Pores Formed in Lipid Bilayer Membranes by Nystatin

Differences in Its One-Sided and Two-Sided Action

ALAIN MARTY and ALAN FINKELSTEIN

From the Departments of Physiology and Neurology, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT Nystatin and amphotericin B induce a cation-selective conductance when added to one side of a lipid bilayer membrane and an anion-selective conductance when added to both sides. The concentrations of antibiotic required for the one-sided action are comparable to those employed on plasma membranes and are considerably larger than those required for the two-sided action. We propose that the two-sided effect results from the formation of aqueous pores formed by the hydrogen bonding in the middle of the bilayer of two “half pores,” whereas the one-sided effect results from the half pores alone. We discuss, in terms of the flexibility of bilayer structure and its thickness, how it is possible to have conducting half pores and “complete pores” in the same membrane. The role of sterol (cholesterol and ergosterol) in pore formation is also examined.

INTRODUCTION

The polyene antibiotics nystatin and amphotericin B are known to increase the ion and nonelectrolyte permeability of sterol-containing biological and artificial membranes (for a review, see Kinsky, 1970). They have been shown to create aqueous pores of about 4-Å radius in thin lipid membranes (Holz and Finkelstein, 1970), and a molecular model for the structure of such pores has been proposed (Finkelstein and Holz, 1973). Furthermore, the data obtained on biological membranes such as those of red blood cells (Cass and Dalmark, 1973) and Acholeplasma laidlawii (de Kruijff et al., 1974) suggest that they also act there by creating pores of about the same size. Thus, nystatin-treated human erythrocytes are slightly permeable to glucose and impermeable to sucrrose (Cass and Dalmark, 1973), and nystatin-treated Acholeplasma laidlawii show a decreasing permeability to molecules of increasing size from urea to ribose, with glucose being impermeant (de Kruijff et al., 1974).

There are three apparent differences, however, between what is known about the action of nystatin and amphotericin B on lipid bilayer membranes.
and their reported action on biological membranes: (a) The antibiotics generally have to be added to both sides of a bilayer membrane to be effective (Cass et al., 1970), whereas they act from one side on biological membranes. (b) Nystatin induces an anion-selective conductance in bilayer membranes (Cass et al., 1970), whereas it induces a cation-selective conductance in erythrocyte membranes (Cass and Dalmark, 1973). (c) The nystatin concentrations necessary for the two-sided action on bilayer membranes are much lower than those concentrations effective biologically (see Cass and Dalmark, 1973 on red blood cells and de Kruijff et al., 1974 on Acholeplasma).

This paper shows that there is no contradiction between these two sets of data. We find that on bilayers formed from two monolayers (Montal and Mueller, 1972), nystatin and amphotericin B produce a cation-selective conductance when added to one side and an anion-selective conductance when added to both sides. We propose that the former results from an aggregate of polyene molecules that spans the membrane to form a cylindrical “half pore,” whereas the latter results from the association of two of these half pores and corresponds to the model proposed by Finkelstein and Holz (1973).

**MATERIALS AND METHODS**

Membranes were formed following the method described by Montal and Mueller (1972). Two Teflon chambers were mechanically clamped together and separated by a “Saran wrap” partition (~12 μm thick) containing a circular hole (0.1–0.2 mm in diameter). The level of the solution in each half chamber was controlled with a syringe. The surface area of each chamber was either 31 or 7 cm². The aqueous subphase was usually 0.01 M KCl, although experiments were performed with KCl concentrations up to 1 M. An excess of a 0.1–1% solution of lipid in hexane or petroleum ether was spread on the surface of the subphase in each chamber, the solvent was allowed to evaporate, and the membrane was then formed by raising the levels of the solutions on both sides of the partition. Routinely, after the membrane was formed, the KCl concentration in one chamber was increased to about 0.04 M. This enabled us to continuously monitor ion selectivity (via the membrane potential at zero current). Membrane formation was followed electrically by observing the increase in the capacitance between the two recording electrodes. Before use, the partitions were coated with Vaseline by dipping them into a suspension of Vaseline in petroleum ether.

The electrical measurements were made through a pair of calomel electrodes connected to an apparatus similar to that used by Montal and Mueller (1972), which could be switched from the voltage-clamp to current-clamp mode. Most experiments were done with nystatin, although we have reasons to believe that amphotericin B behaves in a very similar way, as will be discussed later. Nystatin (Squibb Mycostatin)

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1 As will be discussed later, we might have been able to obtain on bilayers formed by the more classical brush technique results similar to those presented here for bilayers formed directly from two monolayers. Because of the uncertainties associated with the presence of hydrocarbon solvent (e.g. decane) or tocopherol in the former preparation, we felt that results obtained on membranes lacking these components would be less ambiguous.
was added to one or both aqueous phases, after the membrane was formed, from stock methanol solutions (0.1–4 mg/ml); the solutions in each chamber were stirred with magnetic fleas. Amphotericin B, also added from methanol solutions, was a gift from Miss Barbara Stearns of the Squibb Institute for Medical Research, Princeton, N. J. (Methanol alone added to a concentration of 2%, the maximum ever achieved in our experiments, was without effect on membrane conductance.)

We used a variety of lipids to form membranes: brain lipids (deproteinized chloroform:methanol extracts of ox-brain white matter [Mueller et al., 1963]), red cell lipids (deproteinized butanol extracts of human red cell ghosts [Maddy, 1966]), egg lecithin (Sylvana Chemical Company, Orange, N. J.), and glycercydioleate (Pfaltz & Bauer, Inc., Stamford, Conn.). In some preparations of brain lipids and red cell lipids, cholesterol was removed by either silicic acid chromatography or acetone extraction. Removal was confirmed by thin layer chromatography. Cholesterol or ergosterol was added back to these preparations to achieve a molar ratio of sterol to phospholipid of about 1:1. Cholesterol (Eastman Kodak Co., Rochester, N. Y.) was recrystallized twice from ethanol; ergosterol (Matheson Coleman and Bell, East Rutherford, N. J.) was used either directly as obtained from the manufacturer or recrystallized once from ethanol. Hexane and petroleum ether were used as supplied by Fisher Scientific Co., Pittsburgh, Pa.

RESULTS

Addition of nystatin to one side of the membrane to a concentration of 5–100 μg/ml induces a cation-selective conductance. The membrane potential is approximately 50 mV for a 10:1 activity ratio of KCl. A very disturbing observation was the great variability among membranes with respect to the concentration of polyene necessary to induce a given steady-state conductance. (Occasionally, the conductance rose to a peak after the addition of nystatin and then decayed to a smaller stable value. This appeared to be due to a transient increase of nystatin concentration near the membrane: if a parafilm barrier was placed vertically near the Saran wrap partition to prevent access of nystatin to the membrane until a homogeneous concentration was reached in the solution, and then this barrier was removed, no transient effect was observed.) We have the feeling that if the conditions for the mixing-in of nystatin were kept constant, more reproducible data would be obtained, although why this should be so is unclear. As an extreme example of the scatter of the conductance versus concentration data, one membrane formed with red blood cell lipids gave a conductance of $8 \times 10^{-4} \Omega^{-1}/\text{cm}^{2}$ for a concentration of nystatin of 40 μg/ml, whereas another membrane gave a conductance of $10^{-4} \Omega^{-1}/\text{cm}^{2}$ for a concentration of 20 μg/ml. For comparison, the data obtained on intact red blood cells give a conductance of about $2 \times 10^{-4} \Omega^{-1}/\text{cm}^{2}$.

2 This is based on values obtained for ratios of about 4:1, assuming a logarithmic dependence of membrane potential on concentration ratio. We saw no significant effect of lipid composition on these values.
for a concentration of 20 μg/ml (Cass and Dalmark, 1973). This happens to correspond roughly to the average of the conductances obtained with this concentration in our system (Fig. 1).

When nystatin is added symmetrically, a reproducible anion-selective conductance is induced which agrees in magnitude with the data obtained previously on bilayers formed by the brush technique (Cass et al., 1970). One may ask why the one-sided cationic effect is not observed upon symmetrical addition. It appears that the concentration range necessary to produce the one-sided effect is much higher than that required for the two-sided effect (Fig. 1), so that an amount which gives a large anionic conductance when applied symmetrically would give a trivial cationic effect when applied to one side. Thus a conductance of 10⁻⁸Ω⁻¹/cm² is obtained with concentrations of...
about 0.4 \( \mu g/ml \) on two sides and concentrations of about 10 \( \mu g/ml \) on one side.

It seems possible, therefore, that the anion- and cation-selective conductances created by nystatin act independently of each other, and that for a symmetrical addition of polyene, the cationic conductance \( (g_c) \) does exist but is shunted by the much higher anionic conductance \( (g_a) \). (Indeed, in a few cases, a low symmetrical concentration of nystatin induced first a cationic and then an anionic conductance, presumably because of a transient one-sided effect. Further symmetrical addition of nystatin gave only a stable anionic conductance.) As a test for the independence of \( g_c \) and \( g_a \), we performed experiments in which a large concentration of nystatin was first added to one side to induce a cationic EMF \( (E_c) \) at a stable conductance \( (g_c) \), and then a much smaller concentration was added to the opposite side. The total resistance \( (R) \) and the membrane potential \( (V) \) (at zero current) were recorded as the anionic conductance developed with time. If \( g_c \) and \( g_a \) are independent, then from the equivalent circuit in Fig. 2:

\[
V = \frac{g_a E_a + g_c E_a}{g_c + g_a}.
\]

Since \( R = \frac{1}{(g_c + g_a)} \),

\[
V = E_a + R g_s (E_a - E_c)
\]

\( E_a \) is the anionic EMF and is equal to the membrane potential at the end of the experiment, when \( g_c \) is negligible compared to \( g_a \). If \( g_c \) remains constant as \( g_a \) develops, Eq. 2 predicts a linear relationship between the membrane potential, \( V \), and the total membrane resistance, \( R \); this is seen experimentally in Fig. 2.

We have investigated qualitatively the effect of sterols on our preparation. It is generally believed that the presence of sterol in the membrane is a prerequisite for the action of the polyene antibiotics. We have observed, however, one-sided cationic and two-sided anionic effects on plain lecithin membranes and on decholesterolized brain lipids membranes. From the few experiments we performed with these lipids, it appeared that cholesterol did not potentiate the one-sided action of nystatin; ergosterol, however, did greatly potentiate the one-sided action of nystatin on decholesterolized brain lipids. On the other hand, cholesterol (and ergosterol) greatly enhanced the two-sided action of nystatin on decholesterolized brain lipids membranes. The concentrations of nystatin required for the two-sided effect on decholesterolized brain lipids were about a factor of 10 larger than those required on cholesterol-containing brain lipids (molar ratio 1:1).

With amphotericin B, an anion-selective conductance of \( 3 \times 10^{-4} \Omega^{-1}/cm^2 \)
Figure 2. The conversion of the selectivity of a nystatin-treated membrane from cationic to anionic. The membrane was formed from an equimolar mixture of decholesterolized ox-brain lipids and ergosterol, and separated 0.01 M KCl solutions. The membrane resistance was greater than $10^{10}$ $\Omega$ (diameter of hole in partition = 0.15 mm). Nystatin was then added to one side to a concentration of 8 $\mu$g/ml. Within a minute the resistance fell to $6 \times 10^8$ $\Omega$. KCl was then added to the same side to a concentration of 0.05 M and a potential difference of $-34$ mV (0.05 M solution negative) appeared across the membrane. After 16 min the membrane resistance stabilized at $3 \times 10^8$ $\Omega$ ($5.4 \times 10^4$ $\Omega$ cm$^2$) and the potential difference was still $-34$ mV. This is taken as $t = 0$ in the figure. At this time nystatin was added to the opposite side to a concentration of 1.0 $\mu$g/ml. Over the next 7 min the membrane resistance continuously decreased to $1.2 \times 10^8$ $\Omega$, and the membrane potential simultaneously rose to $+10$ mV. The nystatin concentration was then increased to 2.0 $\mu$g/ml. Over the next 20 min the membrane resistance fell to $2.8 \times 10^7$ $\Omega$ and the potential rose to $+26$ mV. Nystatin concentration was increased again to 4 $\mu$g/ml. The membrane resistance continued to fall to $3 \times 10^4$ $\Omega$ and the membrane potential asymptoted at $+35$ mV. (Similar results are obtained with membranes containing cholesterol instead of ergosterol. The asymmetry in concentrations of nystatin from one and two sides are much more pronounced in cholesterol-containing membranes than in ergosterol-containing membranes. In one experiment, for example, on a membrane formed from red cell lipids, a concentration of nystatin of 42.6 $\mu$g/ml on one side produced a resistance of $2 \times 10^7$ $\Omega$ [cation selective]. Subsequent addition of nystatin to the opposite side to a concentration of only 0.33 $\mu$g/ml produced a rapid fall in resistance to $2 \times 10^4$ $\Omega$ [anion selective].)

was obtained with symmetrical concentrations of the order of 0.02 $\mu$g/ml on glyceryldioleate-cholesterol (molar ratio 1:1) membranes, and a corresponding cation-selective conductance with a one-sided concentration of about 12 $\mu$g/ml; the concentration gap between the one-sided and two-sided effect may be greater than for nystatin, but we did not investigate this point. Since the data obtained with symmetrical additions of amphotericin B on these membranes are comparable to the data obtained on membranes formed by
DISCUSSION

The results presented in this paper demonstrate that there are no major discrepancies between the action of nystatin (and amphotericin B) on lipid bilayers and its action on biological membranes. Thus, at comparable concentrations, nystatin added to one side of either bilayers or plasma membranes produces cation-selective permeability. The apparent differences noted in the introduction between the action of nystatin on lipid bilayers and its action on biological membranes arose from the comparison of the two-sided action on bilayers to the one-sided action on plasma membranes. We now see, however, that these differences between the two-sided and one-sided effects of nystatin occur on lipid bilayers themselves. Thus, the question is how can nystatin induce cation selectivity when added to one side of a membrane and anion selectivity when added to both sides of the same membrane?

Since much larger concentrations are required for the one-sided effect than for the two-sided effect, it is possible that the former is produced by a minor contaminant. There are at least four points that argue against this: First, since the same range of concentrations is effective on both bilayers and plasma membranes, one would have to assume that all of the biological action reported for nystatin and amphotericin B is attributable to a contaminant. Second, the ergosterol dependence of the one-sided effect is consistent with this effect being polyene produced rather than being the result of a contaminant. Third, as we pointed out in the introduction, the radius of the pores produced by the one-sided action of nystatin on plasma membranes is very similar, if not identical, to the radius of pores produced by its two-sided action (at much lower concentrations) on bilayers. It would be most for-

In previous investigations of bilayers formed by the brush technique with either d, L-α-tocopherol, or decane in the membrane-forming mixture, we rarely went to the high concentrations of nystatin (>10 μg/ml) used in the present experiments to elicit the one-sided effect. We therefore cannot compare the one-sided nystatin sensitivity of those membranes with the ones used in the present experiments. It is interesting, however, that in those cases where one-sided nystatin-induced conductance was observed with membranes formed by the brush technique, it was usually cation selective.

If the ideas presented in this section are correct, similar differences should be demonstrable between the two-sided and one-sided action of nystatin and amphotericin B on plasma membranes.

Our failure to see cholesterol dependence might suggest that a contaminant is responsible for the one-sided action of nystatin. However, given the spread of our data and the few experiments we performed, we cannot rule out that cholesterol enhances the one-sided action of nystatin in our system just as it does on liposomes and biological membranes.

Because of the technical difficulties involved, we have not performed nonelectrolyte sieving experiments on bilayer membranes treated with nystatin from one side. We can assume, however, on the basis of the other similarities, that nystatin acting from one side on bilayers forms pores of the same radius as those formed from one side on plasma membranes.
tuitous (or perverse) if a contaminant created such similar-sized pores. Finally, it is unlikely that both nystatin and amphotericin B, which are produced by different microorganisms, contain the same (or similar) contaminant responsible for the one-sided induced conductance.

We believe that there is another, and much more interesting, explanation for the simultaneous existence of one-sided and two-sided action of nystatin. Finkelstein and Holz (1973) have proposed a model based on the three-dimensional structure of amphotericin B\(^7\) in which two half pores (lined by OH groups to give them a polar interior) combine to build up a conducting unit (Figs. 3 and 4 A). This model implies that some polyene molecules are oriented perpendicular to the membrane and aggregate to form half pores. The two half pores are held together in the middle of the bilayer through hydrogen bonds between the hydroxyl groups located at the end of each polyene molecule. The other end of each molecule has the very polar amino sugar and carboxyl groups which are anchored in the aqueous phase. Since the hydroxyl group is itself polar, it is conceivable that it could be directly in contact with the aqueous solution on the opposite side, a half pore spanning now the entire membrane (Fig. 4 B). We think that such half pores are responsible for the one-sided effect. Indeed, the long dimension of an amphotericin B molecule (28Å, of which 22Å is the lactone ring) is comparable to the thickness of the hydrocarbon core of lipid bilayers (Lecuyer and Dervichian, 1969; Small, 1967). (This thickness is about half the length of two completely extended phospholipid molecules.)

One might object that the thickness of the hydrocarbon region cannot at the same time be equal to one and two lengths of a nystatin or amphotericin B molecule. We think, however, that this thickness can vary, the lipid molecules being stretched in the vicinity of a complete pore and compressed in the vicinity of a half pore (compare Fig. 4 A and B). Alternatively, a half pore may span only half of the hydrocarbon region, but the opposite half of the bilayer is sufficiently disrupted so that it no longer acts as a significant permeability barrier (Fig. 4 C). (De Kruijff and Demel [1974] have tried to reconcile the one- and two-sided actions of nystatin and amphotericin B by suggesting that in plasma membranes and liposomes half pores can form from both sides [and then come together to form a complete pore], even though the antibiotic has been added to one side. We think that this is unlikely for two reasons: First, since nystatin action is rapidly reversed when the antibiotic is removed from the medium [Cass and Dalmark, 1973], it is difficult to see why half pores on the inner half of the plasma membrane would remain stable in the face of zero concentration of antibiotic within the cell. Second, since one-sided nystatin action leads to cation selectivity whereas two-sided action leads

\(^7\) The three-dimensional structure of nystatin is very similar to that of amphotericin B.
Finklestein and Holz, 1973.)

to anion selectivity, clearly the one-sided and two-sided channels are not identical, in contrast to the suggestion of de Kruijff and Demel.)

The simplest interpretation of experiments such as the one described in Fig. 2, where nystatin is added first to one side and then to the opposite side, is that molecules of nystatin bind to half pores and convert them into complete pores. However, Fig. 2 shows that the cationic conductance does not change significantly during the appearance of the anionic conductance. That could mean that only a very small fraction of half pores are converted into complete
pores, but then the unit conductance of half pores would have to be much smaller than that of complete pores. A second possibility, and one which we tend to favor, is that there is a large reservoir of nonconducting half pores (e.g. Fig. 4 D), an insignificant proportion of which is converted into complete pores in the experiment of Fig. 2. An alternative way of describing the phenomenon is to say that half pores have a much shorter life time than complete pores. Both the large concentrations of antibiotic necessary to produce one-sided action and the presumed length of the half pore suggest that these structures should be much less stable than the complete pore. (See p. 116 of Cass et al., 1970 for experimental support of this statement.) In fact it is possible to imagine various multimeric structures penetrating from one side that finally can become stabilized by linking up with similar structures from the opposite side. The marginal stability of these half pores, the suggested changes in thickness of the bilayer in the region of the pore, and the inherent detergent properties of large concentrations of nystatin may be responsible for the variability in concentration of antibiotic necessary to produce a given conductance by one-sided action. If our proposed model is correct, we might expect that the effectiveness of nystatin from one and two sides is a sensitive function of membrane thickness. This could be tested on a series of membranes formed from a single phospholipid (and cholesterol) whose chain length is systematically increased from C_{14} to C_{25}.

The molecular basis for the ion selectivity remains open. To explain the anion selectivity of the complete pore, Finkelstein and Holz (1973) suggested that the OH dipoles within the pore were so aligned as to impart a positive electrostatic potential to the interior of the pore with respect to the bulk.
aqueous media. Assuming that this is correct, then one hypothesis to explain the cation selectivity of the half pore is that the OH dipoles in the ring at one end of the pore are so aligned as to impart a negative electrostatic potential to the entrance of the pore, thus favoring the entry of cations into the channel. One thus has two ion-selective barriers in series, with the cation barrier (at the entrance) predominating over the anion barrier (within the pore). In complete pores, the cation-selective barrier would be removed by the hydrogen bonding of those hydroxyls. Alternatively, the cation-selective barrier may be due to the orientation of the polar groups of the lipids in the disrupted half of the bilayer, if the model for the half pore in Fig. 4 C is appropriate. There is also the possibility that we do not have a (expletive deleted) clue about the ion-selectivity mechanism.

Finally, the finding that sterols are not absolutely required for the action of nystatin and amphotericin B is also consistent with the model of the structure of the pore. The only role that this model assumes for sterol molecules is to pack the polyene molecules together, and lipid molecules can certainly replace them to some extent in this function.

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REFERENCES

Cass, A., and M. Dalmark. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nature* (Lond.). 244:47.

Cass, A., A. Finkelstein, and V. Kressel. 1970. The ion permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* 56:100.

Finkelstein, A., and R. Holz. 1973. Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *In Membranes* vol. 2. Lipid Bilayers and Antibiotics. G. Eisenman, editor. Marcel Dekker, Inc., New York. 377.

Holz, R., and A. Finkelstein. 1970. The water and nonelectrolyte permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* 56:125.

Kinsky, S. C. 1970. Antibiotic interaction with model membranes. *Annu. Rev. Pharmacol.* 10:119.

De Kruijff, B., and R. A. Demel. 1974. Polyene antibiotic-sterol interactions in membranes of *Acholeplasma Laidlawii* cells and lecithin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes. *Biochim. Biophys. Acta.* 339:57.

De Kruijff, B., W. J. Gerritsen, A. Oerlemans, R. A. Demel, and L. L. M. van Deenen. 1974. Polyene antibiotic-sterol interactions in membranes of *Acholeplasma Laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochim. Biophys. Acta.* 339:20.

LeGeyter, H., and D. G. Devvichian. 1969. Structure of aqueous mixtures of lecithin and cholesterol. *J. Mol. Biol.* 45:39.

At present we know of no a priori reasons to assign the OH dipole orientations as we have suggested. The proposal is merely an attempt to suggest a rationale for the experimental observables.
Maddy, A. H. 1966. The properties of the protein of the plasma membrane of ox erythrocytes. *Biochim. Biophys. Acta* 117:193.

Montal, M., and P. Mueller. 1972. Formation of biomolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. U.S.A.* 69:3561.

Mueller, P., D. O. Rudin, H. T. Then, and W. C. Wescott. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67:534.

Small, D. M. 1967. Phase equilibria and structure of dry and hydrated egg lecithin. *J. Lipid Res.* 8:551.