BRIEF COMMUNICATION

EVIDENCE THAT NYSTATIN MAY NOT FORM CHANNELS IN THIN LIPID MEMBRANES

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ABSTRACT The mechanism of nystatin-induced conductance increases in planar bilayer membranes formed from lecithin/cholesterol (1:1) has been examined by spectral analysis of kinetic fluctuations. Lorentzian components of the power spectrum, characteristic of channel activity, were not observed for nystatin-doped membranes. Instead, the noise associated with this system ($\sim 10^{-27} A^2/Hz$) was in better agreement with that expected of a carrier mechanism involved in the transport of single charges in uncorrelated fashion. The implications of this result with respect to previous models of nystatin-induced conductance increases are discussed.

The polyene antibiotics, nystatin and amphotericin B, are known to increase markedly the permeability of lipid membranes to ions and nonelectrolytes, including water (1). Considering properties of the induced permeability, various authors have suggested that these antibiotics form channels of diameter 4–8 Å across the membrane (2–4), but discrete current jumps that might be attributable to a single channel have never been observed (5). Such single events have been recorded in the case of other antibiotics such as gramicidin A (6), alamethicin (7), and monazomycin (8). This has led to the conclusion that the mean channel lifetime may be less than 100 ms, if the electrical size of the single channel is near that reported for other antibiotics (10^{-11} mho) (5), and certainly less than 10^{-14} A-s.

However, experiments performed in this laboratory using the method of spectral analysis of kinetic fluctuations (9) have led to the possibility that a significant reinterpretation of the channel hypothesis may be necessary. Although increases in current flow across nystatin-doped bilayer membranes were observed, in agreement with earlier studies (1–4), detailed attempts to detect associated current fluctuations were unsuccessful. Wanke has reported the presence of $1/f$ ("one over f") noise in the power spectrum of nystatin-doped bilayers (10). However, this result would be expected if the macroscopic membrane current were drifting, i.e. had not reached an equilibrium steady state at the time of data collection. As our methods allowed resolution of power spectrum components down to 10^{-28} A^2/Hz, it is possible to suggest an upper limit

for the unit channel current flow versus mean channel lifetime for nystatin in the range of the unit electron charge-second. This estimate is based on differential subtraction of instrumentation and background noise in control bilayers from the noise present in nystatin-doped bilayers. The residual noise is within the range expected from shot and thermal effects and is in better agreement with the noise expected of a carrier than with that of discrete channels.

The apparatus used was similar to that previously described (11), with the following modifications: The bilayer system was located inside a Faraday cage floating on an air table, thus eliminating virtually all sources of electrical and mechanical environmental noise. Bilayers were formed from a 1:1 mixture of lecithin and cholesterol in *n*-decane. A Keithley 427 current feedback amplifier with a response time of 30 μs at a gain of 10^9 A/V was employed for current measurement (Keithley Instruments Co., Inc., Cleveland, Ohio). Analog-to-digital conversion and spectral analysis under real-time conditions were performed by a Ubiquitous digital spectrum analyzer (Nicolet Scientific Corp., Northvale, N.J.) with an input dynamic range of 50 dB and an internal dynamic range of 80 dB. The power spectrum was derived from the Hanning-weighted fast Fourier transform and the average taken from 512 spectra, each computed from 1,024 points obtained at a rate of 2,560 samples/s.

Fig. 1 shows the power spectra of the background membrane noise, before and after the addition of nystatin and alamethicin. The macroscopic membrane current was 2.4×10^{-9} A at +50 mV in the nystatin-doped membrane (10^{-8} M symmetrically in the surrounding bathing medium) and approximately 10^{-12} A at the same potential in undoped membranes. In all cases the membrane was allowed to undergo equilibration for at least 4 h before noise measurements. The alamethicin spectrum is shown as a demonstration of fluctuations induced by a known channel-forming antibiotic.

If the kinetic process involved in the permeability-inducing mechanism is assumed to involve a single principal rate-determining step, then the power spectrum will contain a predominant Lorentzian that is linearly summed to the other noise sources (9, 12). If a discrete on-off mechanism for pore formation is assumed, we may express the variance as

$$\sigma^2_{\text{total}(t)} = \sigma^2_{\text{nys}(n)} + \sigma^2_{\text{background}(b)}, \tag{1}$$

which can be expressed in terms of its power spectral distribution as

$$G_t(f) = 4\sigma_n^2[1/(2\pi f_c)]/[1 + (f/f_c)^2] + G_b(f). \tag{2}$$

The spectral distribution of the nystatin-induced fluctuations is given by

$$G_n(f) = G_t(f) - G_b(f) = 4\sigma_n^2[1/(2\pi f_c)]/[1 + (f/f_c)^2]. \tag{3}$$

The cutoff frequency, f_c, is beyond possible resolution at the low current levels evidenced in our data due to the presence of considerable capacitative noise at higher frequencies. Nonetheless, considerable information may be derived from the plateau

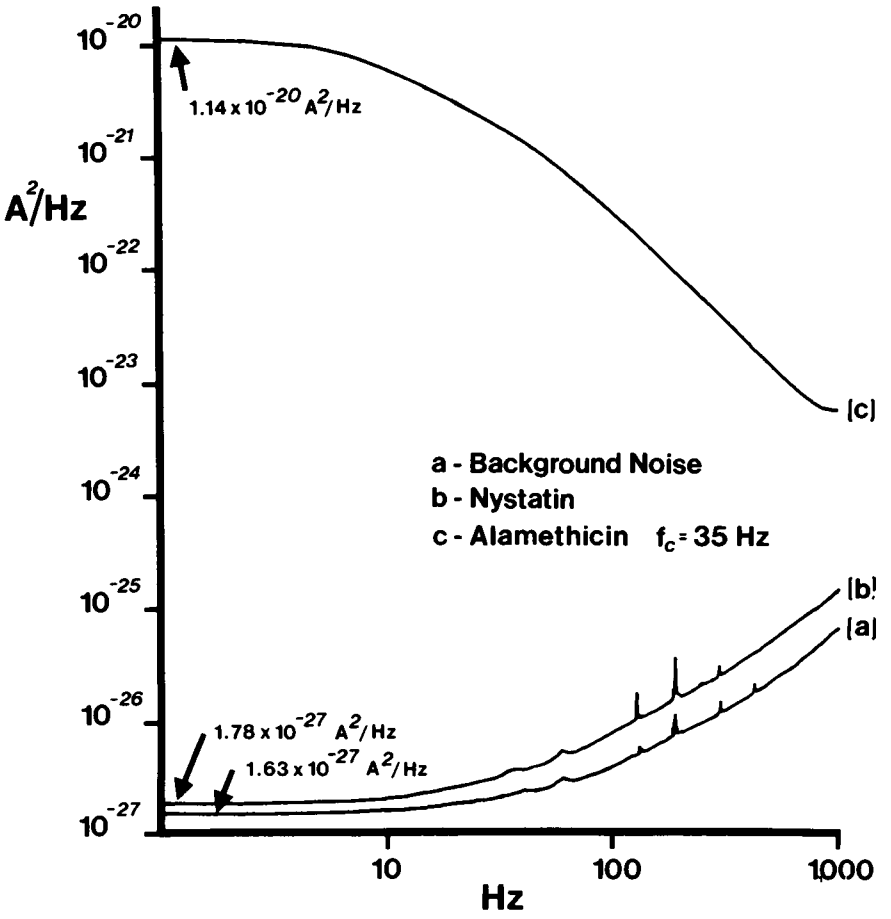

FIGURE 1 Power spectrum of current noise, $G(f)$, versus frequency for lecithin-cholesterol membranes in 1 N NaCl at V_m = 50 mv and 25°C. The upper spectrum is taken from an alamethicin-doped membrane (2×10^{-7}M) at a macroscopic current of 6 nA. The two lower curves are the spectral densities (512 averages) for a nystatin-doped bilayer (10^{-8}M) with a macroscopic current of 2.4 nA, and an undoped membrane with a macroscopic current of approximately 1 pA.

expected of a Lorentzian at lower frequencies. That is, as $f \rightarrow 0$ we find

$$G_n(0) = 4\sigma_n^2/2\pi f_c = 4\sigma_n^2\tau \quad (\tau \equiv (2\pi f_c)^{-1}), \tag{4}$$

where τ is the mean "open channel" lifetime. Rearranging, the variance for the nystatin fluctuations can be expressed as

$$\sigma_n^2 = G_n(0)/4\tau \tag{5}$$

The maximum possible value for $G_n(0)$ may be obtained by differential subtraction of the spectra obtained at $f \rightarrow 0$ for nystatin-doped bilayers and that obtained for undoped bilayers. Our data indicates this to be less than 10^{-27} A^2/Hz based on

512 averaged spectra. Standard canonical statistics applied to one step discrete, on-off conductance fluctuations in membranes (12) relate the unit channel current to the variance by the equation

$$\sigma_n^2 = \gamma^2 N P_o(1 - P_o) \tag{6}$$

where P_o is the probability that a given membrane-bound nystatin molecule will be part of an open channel, N is the total number of membrane-bound nystatin molecules divided by the number required per open channel, and γ is the unit channel current. Because the steady-state current exhibits a 4th to 10th order concentration dependence (1–3), and considering that our data were obtained at lower currents in the dose-response region, it is reasonable to conclude that P_o is close to zero. That is, the proportion of membrane-bound nystatin actually conducting is minute. With this approximation, we can estimate the unit channel current as

$$\gamma = G_n(0)/4\tau\bar{\imath} \quad (\bar{\imath} = \gamma N P_o) \tag{7}$$

where $\bar{\imath}$ is the mean macroscopic current. Even in the case of a multistep kinetic system, Eq. 7 is approached in the limit as $P_o \rightarrow 0$. (For a detailed analysis leading to this result, see ref. 12, Appendix B). Our data suggest an estimate for the unit channel current versus mean channel lifetime ($\gamma\tau$) of 10^{-19}–10^{-20} C (A-s). Parallel results were also obtained with the antibiotic, amphotericin B. It is of interest to note that the minimum value of unit current during a transport process is limited by the quantal nature of charge to 1.59×10^{-19} C. Further, uncorrelated transport of single charges will give rise to shot noise described by the relation

$$G(f) = 2q\bar{\imath} \tag{8}$$

where q is the fundamental electron charge. Nyquist, or thermal agitation noise, will also induce electrical fluctuations of the same order of magnitude at room temperature (298°K) and the membrane potential used (50 mV). These noise sources should account for around 1.5×10^{-27} A^2/Hz, within the same order of magnitude of the doped bilayer. Thus, our power spectra results are consistent with the transport of single charges in uncorrelated fashion.

Given these considerations, it is worth examining some possibilities for the physical mode of ion transport. First, perhaps only one ion is transported during the lifetime of a single transmembrane pore. Holz (2), Andreoli (3), and de Kruijff and Demel (4) have proposed space-filling models of membrane pores implying an ionic radius of at least 4 Å. The nonelectrolyte permeability of nystatin- and amphotericin B-doped bilayers supports this suggested radius. However, the electrical properties of other channel-forming antibiotics might, by analogy, suggest for this size channel a unit channel current in 1.0 N NaCl at 50 mV of at least 10^{-13} A. Our data indicate a limit of charge transfer of about 10^{-19} C/channel, suggesting (from this model) that the mean channel lifetime should be less than 1 μs. Such rapid transitions would imply that extremely low molecular activation energies exist between the open channel state and at least one closed channel state. At the other extreme, our methods, due to

sampling time, would fail to detect fluctuations arising from channels having a mean open lifetime of >200 s approximately. Such long-lived channels, however, should be relatively easy to detect by direct observation of the macroscopic membrane current. The absence of such transitions, both in our experiments and in others (5), leads us to consider this an unlikely possibility.

Another possibility is that nystatin and amphotericin B act as carriers. This would be consistent with our data, but leaves unexplained the nonelectrolyte permeability characteristics reported by others in terms of the usual ligand-complexation theories for carriers. The possibility must be considered, however, that the antibiotics may interact to form a hydrophobic shell by a different mechanism from those that form ligands such as valinomycin and X-537A.

A speculative but interesting possibility is that nystatin and amphotericin B transport ions and molecules by a means borderline between a channel and carrier. That is, a cluster of these antibiotics may interact in the membrane to form a partial pore until some molecular or ionic entity enters. At this point, the pore "folds in" around the molecules, preventing further entrance of ions or molecules and effectively closing the channel. Presumably, this structure would then transport the species to the other side and release it. Such a hybrid model would be at least partially consistent with the molecular model ideas of others (2–4) and might explain the failure to detect electrical fluctuations common with other ion channels such as the alamethicin spectrum in the figure.

In any case, the data reported elsewhere (1–4), combined with the experimental observations presented here, suggest that the actual transport process cannot be adequately described by any transport mechanism previously proposed.

Note added in proof:

Since completion of this work, I. N. Ermishkin et al. have reported (*Nature (Lond.)*. **262**: 699, 1976) the direct observation of nystatin and amphotericin B-induced channel activity in bilayers formed from brain phospholipids and cholesterol. Using lecithin-cholesterol (2:1) bilayers bathed in 3 M KCl, we have attempted, without success, to replicate their results. The reason for this discrepancy is not known, but may be related to the choice of phospholipid.

W. O. R. was supported by a National Science Foundation predoctoral fellowship. This research was supported by grants from the National Science Foundation and the National Institutes of Health.

Received for publication 9 August 1976 and in revised form 6 December 1976.

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