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The nature of the conductance increase induced by filipin in cholesterol-containing planar lipid bilayers

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The effects of the polyene antibiotic filipin on the conductance and permeability of planar lipid bilayers were investigated under voltage-clamp conditions. The membrane conductance of lipid bilayers containing no cholesterol was not affected by filipin. In the presence of cholesterol containing lipid bilayers, filipin induced a 10^4–10^5-fold increase in transmembrane conductance. This conductance increase was dependent on the ionic species present in solution, decreasing in the following order: $G_{CsCl} > G_{NaAc} > G_{KCl} > G_{NaCl} > G_{CaCl_2} > G_{Na_2SO_4} > G_{BaCl_2} > G_{MgCl_2}$. Reversal potential measurements in simple biionic conditions revealed the following relative permeability sequence: $P_K > P_{Cl} > P_{Na} \approx P_{ac} \approx P_{Ba} > P_{Cs} > P_{Mg} \approx P_{Ca} > P_{sulphate}$. The filipin-sterol mediated increase in membrane conductance was independent of the membrane potential. The increase in membrane current following a step alteration in membrane potential occurred instantaneously and had no dependence on the previous value of the holding membrane potential. We propose that the filipin-sterol complex forms ion channels in lipid membranes. These channels are found in a single configuration (open state) and select preferentially monovalent cations or anions over divalent ions. Our experimental results are discussed in relation to the effects of other polyene antibiotics on the membrane permeability, and also in relation to experimental problems previously reported with the use of filipin in planar lipid bilayers.

Introduction

The use of substances capable of forming ion channels in both artificial and biological membranes has provided important information regarding ionic permeation processes. Among different substances that increase the membrane conductance are the polyene antibiotics [1–4]. Polyene antibiotics belong to a class of antifungal agents produced by the bacteria of the genus *Streptomyces*. The basic structural feature of those antibiotics is the presence of a large polyhydroxylic lactone ring consisting of 23–37 atoms with 4–7 conjugated double bonds (Ref. 5, see Fig. 1).

The alteration in membrane permeability induced by two polyene antibiotics, nystatin and amphotericin B, has been partially characterized [3,4,6]. However, the effects of filipin (another polyene antibotic) on membrane permeability are not clear. Studies from two different laboratories have shown that filipin does not induce changes in the electrical resistivity of planar lipid membranes nor alteration in their ionic permeabilities [3,4]. On the other hand, it has been reported that filipin is capable of inducing significant alteractions in the electrical properties of bilayers [7] as well as ionic fluxes across the membrane of liposomes [8]. Moreover, there is an important disagreement in relation to the main ionic species responsible for the alterations in membrane permeability induced by those antibiotics. While nystatin and amphotericin B promoted a predominant increase in anionic permeability [3,4], filipin has been mainly characterized as a (Ca^{2+}) transporter [7,8]. Since there is a strong structural similarity between those different polyene antibiotics, the elucidation of these discrepancies is conceptually relevant for the under-

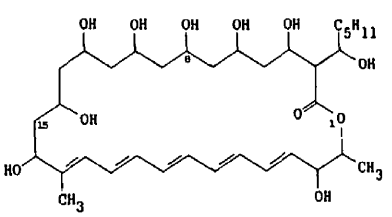

Fig. 1. Proposed molecular structure of filipin.

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standing of general principles involved in structure-function relationships in ion channels.

Filipin is the most simple molecule among different polyene antibiotics [6], and considerable structural information on the interaction between filipin and different lipids have recently emerged [10]. In view of these features, filipin can be regarded as a simple model for the study of structure-function relationships in ion channels. Our main goal in this study was to investigate the ionic nature of the filipin-induced permeability changes in planar lipid bilayers under controlled transmembrane voltage, and in simple biionic conditions. We have found that the filipin-sterol complex is indeed able to increase the conductance and permeability of lipid bilayers. We also show that the conductance and permeability changes induced by filipin depend on the ionic species present in solution. The filipin-induced conductance increase is not voltage-dependent and we propose that the filipin-sterol complex in the membrane exists in a single open (conductive) configuration.

Materials and Methods

Lipids. In an earlier phase of this study, we used membranes with different phospholipid compositions. We found that in order for filipin to increase the membrane conductance, cholesterol must be present in the lipid bilayers. The experiments that we are reporting were performed in membranes with the following composition: 48 mM 1-palmitoyl 2-oleoylphosphatidylethanolamine, 12 mM 1-palmitoyl 2-oleoylphosphatidylcholine, and 30 mM cholesterol. The synthetic phospholipids were purchased from Avanti Lipids (U.S.A.). Cholesterol was obtained from Sigma. The lipid mixture was dissolved with decane that had been previously purified using an alumina column containing the acid, basic, and neutral forms (in that order) of the aluminas (Sigma).

Membrane formation. Bilayers were formed by painting, with the lipid mixture, a 0.15 mm hole in a polystyrene partition separating two different compartments. The process of bilayer formation ('thinning') was monitored by measuring the increase in transmembrane capacitance and also by visual inspection. The bilayer capacitance was routinely checked during the course of the experiments. For each bilayer, the membrane area was measured using a calibrated graticle placed inside the ocular of the microscope and also, by measuring the total bilayer capacitance. A typical value for the specific membrane capacitance of our bilayers was 0.4 $\mu F/cm^2$.

Electrical measurements. One side of the bilayer was kept at the ground potential while the applied membrane potential and transmembrane currents were measured on the other side. A patch clamp amplifier in the virtual ground configuration (EPC-7, List Instruments) was used for measuring transmembrane currents. Both sides of the bilayer were connected to the amplifier by means of 3 M KCl-agar bridges. In this way, junction potentials were negligible (less than 1 mV). Transmembrane currents were displayed and measured from an oscilloscope screen or using a strip-chart recorder. In some experiments, a microcomputer with an AD converter (Labmaster, Scientific Solutions, U.S.A.) was used to digitize the transmembrane currents for later analyses.

Solutions. Different salt solutions were used and will be properly identified in the text. All analytical grade salts were from Sigma. All solutions were buffered with 5 mM Hepes to pH 7.00 using an appropriate base. All solutions were prepared with deionized milli-Q water (Millipore Corporation, U.S.A.). Experiments were performed at room temperature (21–23°C).

Experimental procedure. Once the lipid bilayer was formed, its area, capacitance and resistance were determined. Filipin (Sigma) was then added to both sides of the bilayer from a 20 mM stock solution in ethanol. Both sides of the bilayer were thoroughly stirred until a steady-state level of membrane conductance at a given membrane potential was achieved. This steady level of membrane conductance remained constant during the duration of a single experiment (typically 15 min). The filipin concentration in the experimental chambers was 150 μM. It is important to mention that filipin was stored in the freezer in small aliquots. Fresh ethanol solutions of filipin were routinely prepared.

Determination of relative ionic permeabilities from reversal potential measurements. The following equations were used to calculate the relative permeabilities of different ions to the filipin-sterol complex:

$$V_{rev} = RT/F \ln \frac{P_x/P_y[X]_1 + [Y]_1}{P_x/P_y[X]_2 + [Y]_2} \quad (1)$$

where V_{rev} is the experimentally determined reversal potential, R, T, and F have their usual meanings, and P is the ion permeability. The expressions in brackets denote the concentrations of different ions (in simple biionic conditions) across the membrane. It should be mentioned that when comparing the relative permeability of a cation to an anion, the pairs {X] and [Y] in the numerator and denominator must refer to concentrations present in opposite sides of the membrane ($[X]_1$ and $[Y]_2$, and $[X]_2$ and $[Y]_1$, respectively).

For the calculation of the relative permeability of a divalent ion to a monovalent one, the above equation becomes more complex [10] and is given by:

$$V_{rev} = RT/F \ln \frac{P_z'/P_w[Z]_1 + [W]_1}{P_z'/P_w[Z]_2 \cdot \exp(V_{rev}F/RT) + [W]_2} \quad (2)$$

In that equation, P_w is the permeability of a monovalent ion, and P_z' is defined by $P_z \cdot (1 +$

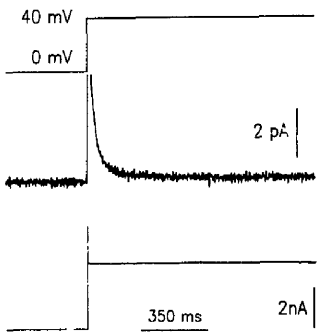

Fig. 2. Recording of transmembrane currents in different experimental conditions. The upper recording represents the change in transmembrane voltage. The middle recording is the transmembrane current in a bilayer containing no filipin. The lower recording shows the change in transmembrane current at 40 mV, promoted by adding filipin to both solutions facing the membrane. 125 mM NaCl solution on both sides of the membrane. Notice the different transmembrane current gains in the middle and lower recordings.

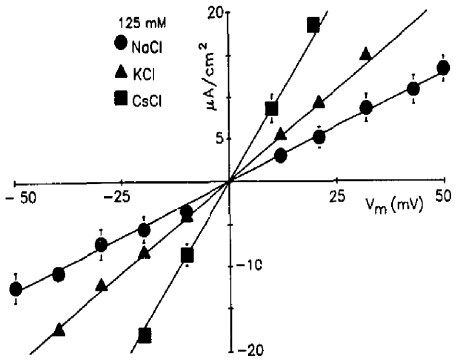

Fig. 3. Current-voltage relationships in membranes containing filipin in different salt solutions. Each point represents the mean ± S.D. of several different observations. S.D. bars were omitted when they were smaller than the size of the symbols. All lines in this and in Figs. 4 and 5 were drawn following linear regression analyses. In all cases, the correlation coefficients of these linear regression analyses were higher than 0.995.

$\exp(V_{rev}F/RT)^{-1}$, where P_z is the permeability of the divalent ion. As mentioned before for Eqn. 1, when referring the relative permeability of an anion to a cation, the numerator and denominator in the above expression must express ionic concentrations on opposite sides of the membrane.

Results

In several different experiments, filipin was added to solutions bathing membranes with different lipid compositions. Provided that cholesterol was absent from those membranes, no increase in transmembrane conductance and/or permeability were observed. This effect was independent of the filipin concentration in solution (up to 2 mM). However, in the presence of cholesterol, the effects of filipin on the membrane conductance were dramatic. Fig. 2 shows the results of a typical experiment. In this figure, the upper recording represents a step increase in transmembrane potential from 0 to 40 mV. The middle recording shows the transmembrane current in the absence of filipin. Lipid bilayers have a typical specific resistance of 10^8 Ω cm² (in this figure, the specific membrane resistance was $6 \cdot 10^7$ Ω cm²). The lower recording shows the transmembrane current after filipin had been added to both solutions facing the bilayer: the specific membrane resistance decreased to $1.9 \cdot 10^3$ Ω cm². Upon addition of filipin, the membrane conductance increased 30000-fold.

Despite this remarkable increase in membrane conductance induced by the filipin-sterol complex in the membrane, this conductance has a quantitative dependence on the nature of ions present in solution. Fig. 3 shows current-voltage relationships in different solutions containing only monovalent ions. It can be seen, that the conductance of the filipin-cholesterol complex has an ohmic behaviour and is relatively small in NaCl solutions, increasing to higher values in KCl and CsCl solutions, respectively.

Filipin has been used to promote the influx of Ca in epithelia [11]. Fig. 4 shows that in presence of divalent cations, filipin is also able to increase the membrane conductance. The dashed line in this figure shows the current voltage relationship in 125 mM NaCl solutions. The conductance increase promoted by filipin in

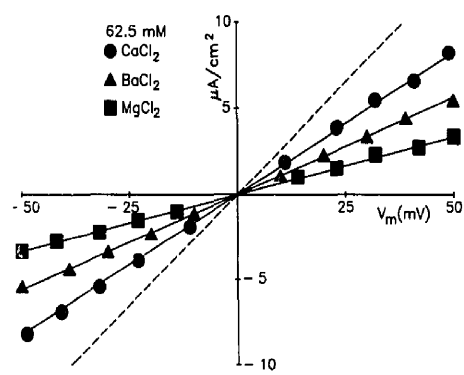

Fig. 4. Current-voltage relationships in membranes containing filipin in solutions containing the same concentration of different divalent cations. Each point represents the mean ± S.D. of several different observations. S.D. bars were omitted when they were smaller than the size of the symbols. The dashed curve is the conductance of a filipin-treated bilayer in presence of symmetrical 125 mM NaCl (Fig. 3).

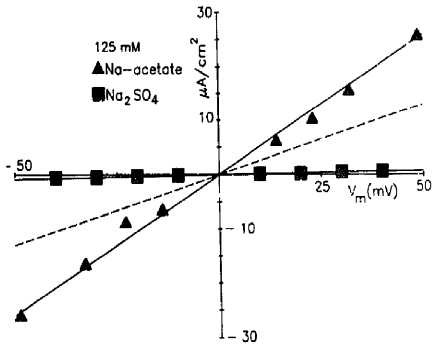

Fig. 5. Current-voltage relationships in solutions containing the same concentration of different anions. Each point represents the mean ± S.D. of different determinations. S.D. bars were omitted when they were smaller than the size of the symbols. The dashed line is the conductance of the membrane in presence of 125 mM NaCl (see Fig. 3).

cholesterol containing membranes was relatively low in $MgCl_2$, increasing in $BaCl_2$ and $CaCl_2$ solutions.

In Fig. 5, we show the effects of different anions on the current-voltage relationship in the presence of filipin. From this figure, it can be seen that the filipin-induced increase in membrane conductance is highly dependent on the type of anions present in solution. In the presence of Na^+ as the common cation between different solutions, there is a dramatic increase in membrane conductance following replacement of sulphate ions by Cl^- ions (dashed curve) and acetate.

From Figs. 3–5, it is evident that both anions and cations are able to affect the filipin-induced increase in membrane conductance. We now ask the question about the selectivity nature of this conductance increase. In order to answer this question we performed several experiments under simple biionic conditions. One of those experiments is illustrated by Fig. 6. In this figure, circles represent a control current-voltage relationship

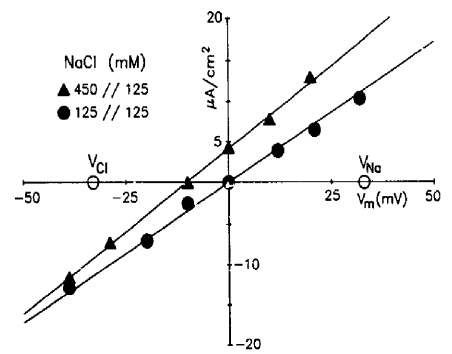

Fig. 6. Measurement of the reversal potential in bionic conditions. The equilibrium potential for Cl^- and Na^+ ions are indicated on the x-axis. See text for explanation. The experimental points are membrane current measurements from a single bilayer experiment.

in presence of symmetrical 125 mM NaCl concentrations. After this control curve was obtained, the concentration of NaCl on only one side of the bilayer was increased to 450 mM. The experimental points pertaining to the latter condition are displayed as triangles. It can be seen that the reversal potential, i.e., the membrane potential where the transmembrane current is 0 was shifted by 10 mV in the hyperpolarizing direction. By applying Eqn. 1, we estimated the relative permeabilities of Na to Cl ions (P_{Na}/P_{Cl}) in this experiment to be 0.49. Following the same strategy illustrated in Fig. 6 but in the presence of other anions and cations (biionic conditions) we came to the following relative permeability sequence (the numerical values are the means of 3–6 different determinations of reversal potentials; $P_{Cl} = 1.0$):

$$P_K(3.2) > P_{Cl} > (1.0) > P_{Na}(0.52) \approx P_{ac} \approx P_{Ba} > P_{Cs}(0.27)$$

$$> P_{Mg}(0.19) \approx P_{Ca} > P_{sulph}(0.17)$$

For the sake of comparison with the previous sequence, the relationship between the transmembrane conductances induced by filipin in different salt solutions follows the sequence:

$$G_{CsCl} > G_{NaAc} > G_{KCl} > G_{NaCl} > G_{CaCl_2} > G_{Na_2SO_4} > G_{BaCl_2} > G_{MgCl_2}$$

We now turn our attention to the question: Is the filipin-induced membrane conductance dependent on the transmembrane potential? Fig. 7 shows a plot of the (membrane conductance/maximum membrane conductance) as a function of transmembrane voltage in the range of −50 to +50 mV. In the experiment shown in this figure, the maximum membrane conductance induced by filipin was 160 $\mu S/cm^2$ at 50 mV. There is no alteration of the membrane conductance induced by filipin as a function of the transmembrane potential.

In Fig. 8, we examine the voltage dependence of the time course of the transmembrane current after two

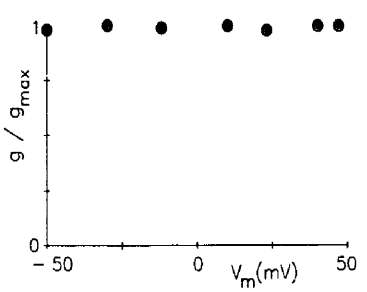

Fig. 7. Relationship between the membrane conductance at a given voltage and the maximum membrane conductance (g/g_{max}) at different membrane potentials. The experimental points were obtained in the presence of the usual concentration of filipin and symmetrical 62.5 mM $CaCl_2$ solutions.

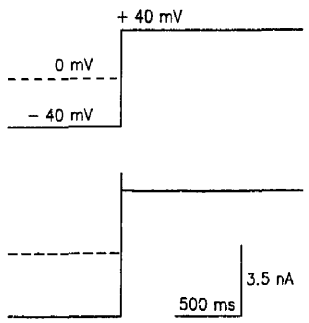

Fig. 8. The upper trace in this figure shows two different voltage clamp protocols (from 0, dashed line, or −40 (solid line) to +40 mV). The lower recording superimposed transmembrane currents. The dashed line in the lower recording represents the current level at 0 mV. The solution was 125 mM NaCl.

different voltage jumps (from 0 or −40 to +40 mV). It can be seen that the transmembrane current attained the same steady-state level at +40 mV independent of the previous holding membrane potential (0 or −40 mV). In addition to that, we did not observe inactivation of filipin induced membrane currents. The capacitive current, that flows across the planar bilayer as a consequence of a transmembrane voltage step, hampers the analysis of the initial first few milliseconds of transmembrane current following the voltage step. However, it is important to stress that in planar lipid bilayers, under experimental conditions similar to ours and using different channel forming substances, the time course of the conductance increase following a transmembrane potential step is of the order of several seconds or even minutes [12,13]. This is definitely not the case with filipin. Within our temporal resolution, we can conclude that: (1) There is an instantaneous increase in transmembrane current following a change in transmembrane voltage and, (2) This current change is independent of the previous membrane holding potential.

Discussion

In this study, we demonstrated that provided cholesterol is present in the lipid membrane, filipin induces a decrease in membrane resistivity. A possible explanation for the discrepancy between our results (see also Refs. 7 and 8) and previous ones [3,4,6] might be related to the storage conditions of filipin. In earlier phases of this work, we were not successful in obtaining reproducible quantitative results in different bilayer experiments. Soon we learned that once filipin is in solution, its capability to increase membrane conductance vanishes rapidly in a couple of days. If small aliquots of filipin are stored in the freezer and fresh ethanol solutions are prepared on a routine basis, then the experimental results are highly reproducible. Another difficulty that has been alluded to, concerns the integrity of the plasma membrane after filipin addition [3–7]. In our hands, we did not observe any unusual problems in keeping the membrane stable and responsive after filipin addition. A possible cause for this discrepancy might reside in our use of synthetic phospholipids that form extremely stable bilayers.

Filipin is known to promote cell death in fungi but not in bacteria [14]. This differential effect of filipin (as well as of other polyene antibiotics) on different microorganisms was correlated with the presence of high levels of ergosterol in the plasma membrane of fungi but not in bacteria. Since that correlation has been made, filipin has been used in cytochemistry as a specific probe for the presence of cholesterol in the plasma membrane of cells from higher animals. However, recent studies have seriously challenged that specificity. In fact, it has been shown that cholesterol and phospholipids are able to compete for filipin binding [9]. In relation to our experimental results, we are convinced that the binding of filipin to phospholipids does not produce any measurable increase in membrane conductance and/or permeability. This finding supports the notion that the filipin-cholesterol complex is actually mediating the increase in membrane conductance.

Unfortunately, there is no detailed information on the permeability and conductance selectivities of cholesterol containing membranes treated with other polyene antibiotics like nystatin and amphotericin B. Nevertheless, it is still possible to recall some common features shared between those two polyene antibiotics [3,4,6]: (1) Both antibiotics promote an increase in membrane conductance only in the presence of sterol containing lipid bilayers; (2) The conductance increase induced by those antibiotics has a large power dependence on antibiotic concentration; (3) There is a good quantitative agreement between the decrease in membrane resistivity promoted by amphotericin B and nystatin; (4) Both nystatin and amphotericin B were considered slightly selective for anions. Our experimental results with filipin-cholesterol complex are in basic agreement with items 1–3 mentioned above. However, item 4 does not necessarily hold, at least for filipin. The anionic or cationic preference of the filipin-sterol complex depends heavily on the type of ions present in solution. Among different ions tested, we found that the filipin-sterol complex is three times more permeable to K^+ than to Cl^- ions. In addition to that, the valence of the ion can be decisive in determining its relative permeability to the filipin: SO_4^{2-} is six times less permeable than Cl^- and it was found to be the least permeable ion tested.

While we are not in a position to propose a detailed energetic picture responsible for the ion conduction and selectivity properties of the filipin-cholesterol complex

in planar bilayers, our experimental results provide some first important insights on those properties. First, all current-voltage relationships were found to be linear. This is suggestive of a symmetrical energy profile for ion translocation across the membrane. Second, we conclude that the filipin-cholesterol complex prefers to conduct monovalent ions to divalents. Once a divalent ion is present in solution, the membrane conductance is reduced significantly. A possible explanation for this effect is that there are sites inside the conduction pathway of the filipin complex that bind very effectivley divalent ions thus, reducing their transit time through the membrane with a consequent reduction in conductance.

From the point of view of the permeability sequence, the situation is similar to that found for the conductance sequence: in general, monovalent cations or anions are more permeable than divalent ions. However, when those two different sequences are analyzed simultaneously, it becomes clear that each ion experiences a unique energetic barrier for translocation across the membrane. In considering Cs as an example, we find that a relatively low permeability is associated with a relatively high conductance. An energetic profile that could explain this feature is associated to the fact that it is difficult for a Cs ion to enter the filipin-sterol complex. However, once inside that complex the affinity of filipin for that cation is presumably so low that the ion is quickly released out of the filipin-sterol complex. It is thus in principle possible to explain the different conductance and permeation sequences by invoking different affinities for the ions to the filipin-sterol complex and by the energy barriers necessary for the ion to enter and exit the filipin permeation pathway [15,16].

It was proposed that polyene antibiotics are capable of forming ion channels in membranes [6,7]. The morphological features observed in electron micrographs are suggestive of pore formation [2]. However, the distinction between an ionophore and an ion channel can be quite subtle in functional terms [15]. A strong argument favouring the idea that filipin is capable of forming ion channels would be the detection of discrete opening and closing events with defined conductance levels. We were not able to detect these single channel events with filipin concentrations as low as 0.15 μM. With this concentration, the increased membrane conductance induced by filipin is in the pSiemens range, which is the usual conductance level found in ion channels. This failure in detecting discrete opening-closing events of single channels can be attributed to the fact that the filipin-sterol complex in planar membranes is always in the open configuration (see below). However, we noticed that in order for the filipin-sterol complex to promote an increase in membrane conductance, the bilayer has to assume its bimolecular configuration. In the absence of a typical bilayer, as evaluated from capacitance and optical measurements, filipin is not able to increase the transmembrane conductance. We believe that this is an important argument favouring the idea that filipin is able to form ion channels in membranes since, an ionophore would in principle retain its essential electrogenic properties in the absence of a bilayer structure provided the environment still remains essentially hydrophobic.

An interesting feature concerning the mode of action of polyene antibiotics in lipid bilayers is that appreciable delays in the activation of transmembrane currents were not detected following a step change in membrane potential. This conclusion applies for a wide range of membrane holding potentials. Also, the conductance of filipin treated bilayers is independent of the membrane potential. We suggest that the filipin-sterol complex is able to form ion channels in planar lipid bilayers and that these channels are permanently in the open configuration. This feature is sufficient enough to explain the efficacy of filipin in exterminating microorganisms that contain cholesterol-rich plasma membranes: it instantaneously dissipates different electrochemical gradients across the plasma membrane leading to cell lysis and death [14].

One of the most important challenges concerning ion channels, is the study of structure-function relationships. In this regard, one possibility that we would like to explore in the future is the modification of the chemical structure of the filipin-sterol complex in such a way as to make it voltage-dependent, i.e., creating another kinetic state such that the relative distribution between different states becomes dependent on the transmembrane voltage. Said accomplishment would represent an important step toward the understanding of structure-function relationships in ion channels and more specifically, in relation to polyene antibiotics.

Acknowledgements

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References

1 Van Zutphen, H., Van Deenen, L.L.M. and Kinsky, S.C. (1966) Biochem. Biophys. Res. Commun. 22, 393–398.
2 Kinsky, S.C., Luse, S.A. and Van Deenen, L.L.M. (1966) Fed. Proc. 25, 1503–1510.
3 Andreoli, T. and Monhan, M. (1968) J. Gen. Physiol. 52, 300–325.
4 Cass, A., Finkelstein, A. and Krespi, V. (1970) J. Gen. Physiol. 56, 100–124.
5 Norman, A.W., Spielvogel, A.M. and Wong, R.G. (1976) Adv. Lipid. Res. 4, 127–170.
6 Finkelstein, A. and Holz, R. (1973) in Membranes: a Series of Advances, Vol. 2 (Eisenman, G., ed.), pp. 377–408, M. Dekker, New York.
7 Van Zutphen, H., Demel, R.A., Norman, A.W. and Van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 241, 310–330.

8 Spielvogel, A.M. and Norman, A.W. (1975) Arch. of Biochem. Biophys. 167, 335–344.
9 Milhaud, J., Mazerski, J. and Bolard, J. (1989) Biochim. Biophys. Acta 987, 193–198.
10 Cukierman, S. (1985) Cardiovasc. Res. 19, 754–762.
11 Wong, R.G., Adams, T.H., Roberts, P.A. and Norman, A.W. (1970) Biochim. Biophys. Acta 219, 61–72.
12 Hoch, D.H., Romero-Mira, M., Ehrlich, B.E., Finkelstein, A., DasGupta, B.R. and Simpson, L.L. (1985) Proc. Natl. Acad. Sci. USA 82, 1692–1696.
13 Raymond, L., Slatin, S.L. and Finkelstein, A. (1985) J. Membr. Biol. 84, 173–181.
14 Lampen, J.O. (1966) in Biochemical Studies of Antimicrobial Drugs (Newton, B.A. and Reynolds, P.E., eds.), pp. 111–130, Cambridge University Press, London.
15 Läuger, P. 1973) Biochim. Biophys. Acta 311, 423–441.
16 Cukierman, S., Yellen, G. and Miller, C. (1985) Biophys. J. 48, 477–484.