# VOLTAGE-DEPENDENT LIPID FLIP-FLOP INDUCED BY ALAMETHICIN

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ABSTRACT Alamethicin appears to allow voltage-dependent lipid exchange ("flip-flop") between leaflets of a planar bilayer. In membranes with one leaflet of phosphatidyl serine and one of phosphatidyl ethanolamine, the shape of the nonactin current-voltage curve accurately reports the difference in surface potential between the two sides of the membrane. The surface potential is itself a good measure of membrane asymmetry. Alamethicin added to the bathing solutions of an asymmetric membrane does not *per se* reduce the membrane asymmetry, but turning on the alamethicin conductance by application of a voltage pulse does. Immediately after application of a voltage pulse, large enough to turn on the alamethicin conductance, the asymmetry of the nonactin-$K^+$ current voltage curve decreases, in some cases, nearly to zero. During the pulse, the alamethicin conductance activates if a decrease in surface potential favors turn-on of the alamethicin conductance or inactivates if a decrease in surface potential favors turn-off of the alamethicin conductance. After the pulse, the nonactin-$K^+$ asymmetry returns to its original value if the alamethicin conductance is not turned on. The time-course of this return allows an estimate of the diffusion constant of lipid in the planar bilayer. The value obtained is $5.1 \times 10^{-8}$ cm$^2$/s.

Lipid redistribution between the leaflets of a bilayer is a very slow process for membranes containing no protein, only lipid (1–4). In living cells, however, lipids are initially synthesized and inserted into the inner leaflet of the cell membrane. In some cellular systems, phosphatidyl ethanolamine (PE) is methylated on the inner leaflet to *N*-mono methyl phosphatidyl ethanolamine. This compound then flips across the membrane to the outside, where it is again methylated twice to phosphatidyl choline (5, 6). This results in an asymmetric distribution of lipid with phosphatidyl ethanolamine inside and phosphatidyl choline inside.

Lipid asymmetry may be an accident of membrane assembly, but it is more likely a significant determinant of membrane properties through differences in fluidity, alteration of membrane protein environment, or changes in transmembrane electric field. Lipid asymmetry may be a signal for controlling membrane-bound enzymes and a result of various processes. In this paper, I will describe a model system in which lipid asymmetry has been shown to alter the conductance of planar lipid membranes for several different conductance mechanisms, including both carriers and pore-formers (7, 8).

Such changes in conductance properties result in some cases from changes in transmembrane electric field induced by lipid asymmetry, and lipid asymmetry can alter transmembrane electric field without *per se* affecting the membrane potential. Fig. 1 shows how the asymmetric distribution of negatively charged lipid changes intramembrane electric field. This sort of lipid asymmetry may play an important role in adjusting conductance mechanisms in living cells, especially for conductance mechanisms in excitable membranes

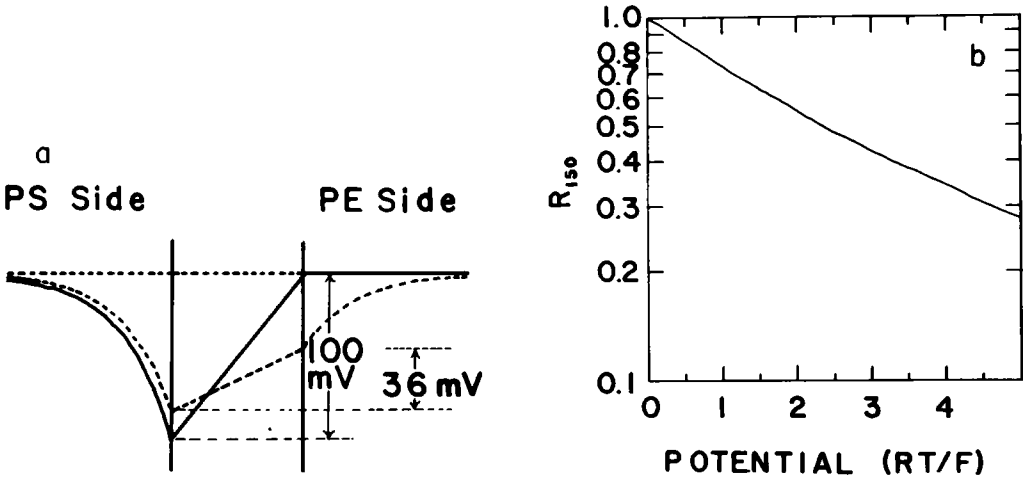


FIGURE 1 (*a*) Electrical potential profile of a membrane with one leaflet formed from PS and one from PE. The solid line shows the potential profile for a membrane with a 100-mV surface potential on the PS side. The dashed line shows how the potential drop across the membrane would change if one quarter of the charged lipid exchanged for neutral lipid (PE for example). Note that the membrane potential (the potential which would be measured by electrodes in solution far from the membrane) is zero in both cases, but the potential-drop across the membrane itself is substantial and depends on lipid asymmetry. The change in potential-drop across the membrane as membrane asymmetry decreased would tend to turn on conductance of alamethicin added to the PS side (activation) and turn off conductance of alamethicin added to the PE side (inactivation). (*b*) Calibration curve used to determine the potential drop across the membrane from the shape of the nonactin $K^+$ current-voltage curve. The ratio of current at a given positive voltage to that at a given negative voltage is plotted against the potential drop across the membrane. The PS side is taken as ground. The unit of potential is $RT/F$ or ~25 mV at 20°C. (This calibration curve is derived from a similar curve obtained experimentally by Hall et al. (16) under conditions where the membrane potential and the potential drop across the membrane were equal.)

which depend strongly on voltage. The establishment, maintenance, and consequences of lipid asymmetry are thus subjects of considerable general interest.

I show here that alamethicin, a voltage-controlled pore-former, can increase the rate of phospholipid flip-flop in asymmetrical planar lipid films from an essentially undetectable level to a value on the order of $10^{13}$ molecules/s cm$^2$. The alamethicin-induced flip-flop only occurs if the alamethicin channels are open and can thus be controlled by the applied voltage. The flip-flop partially destroys the initial membrane asymmetry, which returns once the alamethicin conductance is turned off. The rate of asymmetry return provides an estimate of the rate of lateral diffusion of phospholipids in the membrane.

These experiments use nearly the same technique of membrane formation described by Hall and Latorre (8). Membranes were formed from paired monolayers on a circular hole, usually 0.030 cm Diam, punched in an 18-$\mu$m thick sheet of Teflon. One monolayer always contained only bacterial phosphatidyl ethanolamine (Supelco, Inc., Bellefonte, Pa.). The other contained bovine phosphatidyl serine (PS, from either Supelco, Inc., or Sigma Chemical Co., St. Louis, Mo.). Lipids from either source gave indistinguishable results. In some experiments, the second monolayer contained a mixture of PS and PE in the ratio 10:1 by weight, a mixture found to increase membrane stability somewhat. Lipids were stored under argon at −5°C in

chloroform. Pentane solutions of 12.5 mg/ml of lipid were prepared immediately before use. The pentane (Mallinckrodt Inc., St. Louis, Mo.) was passed through alumina to remove surface active impurities. Instead of using vaseline to coat the Teflon partition as Hall and Latorre (8) did, a 2-$\mu$l drop of squalene (Albany International, Chemicals Division, Albany, N.Y.) passed through an alumina column was applied to the hole. The capacitance of membranes formed in this way was ~0.7 $\mu F/cm^2$, but the membrane area was taken to be the area of the hole punched in the Teflon and is probably not accurate to better than 5%.

Alamethicin was a gift from Dr. G. B. Whitfield of the Upjohn Company, Kalamazoo, Michigan and was used without further purification. Nonactin, purchased from Sigma Chemical Co., was dissolved in ethanolic solution to $10^{-3}$ M stock solutions.

The asymmetry of the nonactin-$K^+$ complex current-voltage curve was used to measure the membrane surface charge asymmetry. Since PE is a neutral lipid and PS negatively charged at the experimental pH (5.5), the nonactin-$K^+$ voltage current curve asymmetry is also a measure of lipid asymmetry (8).

Fig. 1 *b* shows the calibration curve used to determine the potential drop across the membrane at zero current from the asymmetry of the nonactin current-voltage curve. The ordinate is the ratio of current at +150 mV to current at −150 mV, denoted $R_{150}$, and the abscissa is the potential drop across the membrane at zero current in units of $RT/F$ (25 mV at 20°C). The conductance induced by alamethicin is strongly voltage-dependent and turns on when a positive voltage is applied to the side of the membrane to which the alamethicin was added (9). Thus, if alamethicin were added only to the PE side, reducing lipid asymmetry would tend to turn off the alamethicin conductance (or require a higher voltage to turn on the alamethicin conductance) because the initial surface potential tends to turn on the alamethicin conductance. But if alamethicin were added only to the PS side, reducing lipid asymmetry would tend to turn on the alamethicin conductance because the initial tends to turn off the alamethicin conductance. Thus, lipid flip-flop in the case of an asymmetric membrane with PS in one leaflet and PE in the other would produce inactivation of the alamethicin conductance if alamethicin were added to the PE side and activation of the alamethicin conductance if alamethicin were added to the PS side. Alamethicin-induced lipid flip-flop could then activate or inactivate alamethicin conductance, depending on whether alamethicin on the PS or PE side of the membrane was turned on. Alamethicin might go through its own channel, as Heyer et al. (10) have found monazomycin does. Alamethicin going through its own channel might alter the alamethicin conductance after a voltage pulse. If that happened, turning on the alamethicin on the low concentration side would result in activation, and turning on the alamethicin on the high concentration side would result in inactivation. I have, in fact, observed each of these possibilities under appropriate gradients of alamethicin concentration. It is thus vital to be able to distinguish whether or not conductance changes arise from flip-flop or from alamethicin redistribution. The asymmetry of the nonactin-$K^+$ current-voltage curve is not sensitive to alamethicin distribution but is to lipid flip-flop.

The simplest case to analyze is equal alamethicin concentration on the two sides of the membrane. In this case, once the equilibrium is established, there will be no gradient in the electrochemical potential of alamethicin across the membrane, and any inactivation or activation of the alamethicin conductance must arise from some other cause. Fig. 2 shows an experiment performed under these conditions. Curve 1 in Fig. 2 *a* shows the nonactin-$K^+$

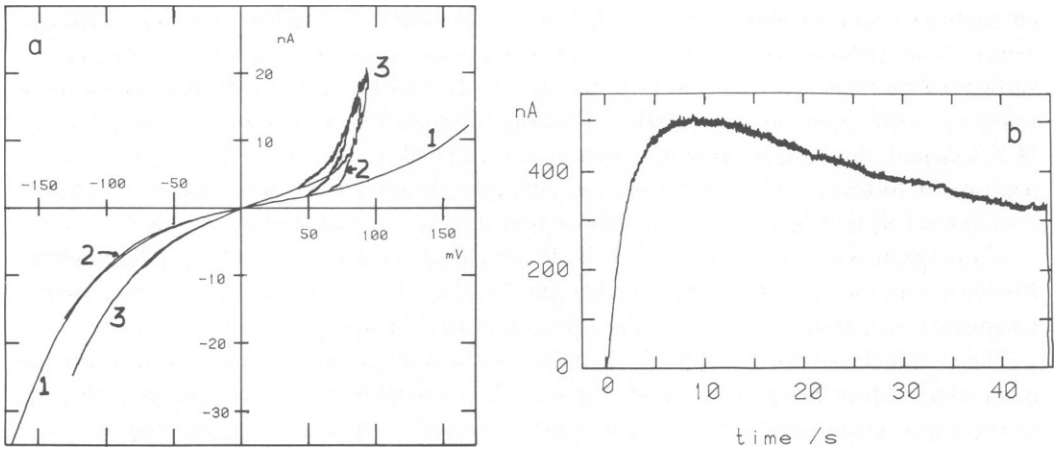


FIGURE 2 (*a*) Membrane was formed from PE on one side and PS:PE/10:1 by weight on the other. Nonactin concentration was $10^{-6}$M. Salt solution was 0.100 M NaCl unbuffered; membrane diameter was 0.30 mm, +0, −0.03 mm. Curve 1 shows the nonactin-$K^+$ current-voltage curve taken before adding alamethicin (sweep rate: 50 mV/s). Curve 2 shows the current-voltage curve 10 min after adding alamethicin to a concentration of $10^{-7}$ g/ml on both sides (sweep rate: 20 mV/s). Curve 3 shows the current-voltage curve after the treatment of Fig. 2 *b* (sweep rate: 20 mV/s). The surface potential differences as deduced from the nonactin rectification ratio were 80 mV for curves 1 and 2 and 32 mV for curve 3. Voltage unit (abcissa) is 50 mV. Current unit (ordinate) is $10^{-8}$A. (*b*) Current treatment between curves 2 and 3 consisted of this response to a voltage pulse and others not shown. The total treatment consisted of voltage pulses with the following magnitudes and durations: 89 mV, 22 s; 96 mV, 27 s; 105 mV, 45 s; 97 mV, 22 s; 105 mV, 45 s; 113 mV, and 40 s. This figure shows the first 105-mV pulse as an example. The time unit is 5 s (abcissa) and the current unit (ordinate) is $10^{-7}$A.

current-voltage curve before the addition of alamethicin. As previously shown, the asymmetry exhibited under these conditions persists for hours (3). Curve 2 shows the current-voltage curve 10 min after the addition of alamethicin to equal concentrations in the aqueous compartments on both sides of the membrane. The onset of the alamethicin conductance at a positive voltage of ~75 mV is seen. Because of the surface potential on the PS side, a large negative voltage did not turn on the alamethicin, and the current in the negative branch of the curve is entirely due to nonactin-$K^+$. The portion of curve 2 due to nonactin conductance superimposes on curve 1, indicating no change in lipid asymmetry. For both of these curves, the surface potential difference between the two sides of the membrane, determined by the $R_{150}$ value using Fig. 2 *b*, is 80 mV.

The asymmetry persists with no detectable change for 30 min in the presence of alamethicin, with no voltage applied. Thus, alamethicin does not induce rapid lipid flip-flop when it does not contribute to the conductance.

After curve 2 was taken, the membrane was subjected to a series of voltage pulses in the following order and of the magnitude and duration indicated: 89 mV, 22 s; 96 mV, 27 s; 105 mV, 45 s; 97 mV, 22 s; 105 mV, 45 s; and 113 mV, 40 s. Fig. 2 *b* shows the current response of the first 105-mV pulse. All of the pulses except the 89-mV pulse and the 97-mV pulse showed inactivation. Since the alamethicin concentrations are equal in the aqueous compartments on the two sides of the membrane, the electrochemical potentials of alamethicin in bulk are equal

on the two sides of the membrane, and since each interface is in equilibrium with its own bulk phase, the electrochemical potential of alamethicin is everywhere the same, even though the alamethicin concentration is lower at the PS interface by virtue of alamethicin's negative charge and the repulsive effect of the PS surface charge. This means that redistribution of alamethicin cannot occur, because there is no driving force for it. Thus, the inactivation observed cannot be the result solely of alamethicin redistribution.

Curve 3 in Fig. 2 *a* was taken after the pulse treatment described above. Note that the nonactin portion of the curve has shifted considerably from that of curves 1 and 2. Extrapolating the positive current to 150 mV gives a potential difference between the two sides of the membrane at zero applied voltage of 32 mV, a change from the control case of ~50 mV. The extrapolation is done using the initial shape of the nonactin current voltage curve. This method tends to underestimate the magnitude of the surface potential change, because the steepness of the current-voltage curve increases in the positive quadrant and decreases in the negative quadrant.

The asymmetry of the nonactin current-voltage curve in an asymmetric membrane is thus decreased by the presence of open alamethicin channels. The decrease in nonactin current-voltage curve asymmetry occurs regardless of the sense and magnitude of the alamethicin concentration gradient, but the alamethicin conductance either inactivates or activates, depending on the gradient of the alamethicin across the membrane. Eisenberg et al. (9) found that alamethicin added to one side of a PE membrane produced an increased conductance only when the voltage in the compartment to which the alamethicin is added is positive with respect to the opposite compartment. I have found that the same holds true for asymmetric membranes when one monolayer is PS and the other is PE. For all types of membranes studied to date, the alamethicin conductance at a given voltage, and thus the probability of an alamethicin channel opening, depends strongly on alamethicin concentration. In these experiments, by convention, the PS side is ground. Thus, a positive voltage turns on conductance due to alamethicin on the PE side and a negative voltage turns on conductance due to alamethicin on the PS side. For each sign of voltage, the expected effects of flip-flop and movement of alamethicin can be either synergistic or antagonistic depending on the alamethicin concentration gradient. But turning on the alamethicin conductance for tens of seconds and to a conductance on the order of $10^{-3}$ mho/cm$^2$ always resulted in the expected change in the nonactin current-voltage curve expected from flip-flop, regardless of the direction or magnitude of the alamethicin concentration gradient. The observed kinetics of the alamethicin current, however, depended on the concentration gradient of alamethicin across the membrane. For alamethicin only on the PS side of the membrane, a negative voltage pulse results in activation of the alamethicin current. The flip-flop mechanism is thus dominant. A positive voltage-pulse with alamethicin only on the PS side shows activation consistent with movement of alamethicin from the PS to the PE side of the membrane. With equal concentrations of alamethicin on both sides, the observed results are consistent with the flip-flop mechanism. For alamethicin on the PE side only, alamethicin conductance can be observed only with positive voltage. The observed inactivation is consistent with either mechanism.

If the alamethicin concentration on the PS side is increased to twice that on the PE side, a positive voltage pulse produces activation as expected from the diffusion of alamethicin

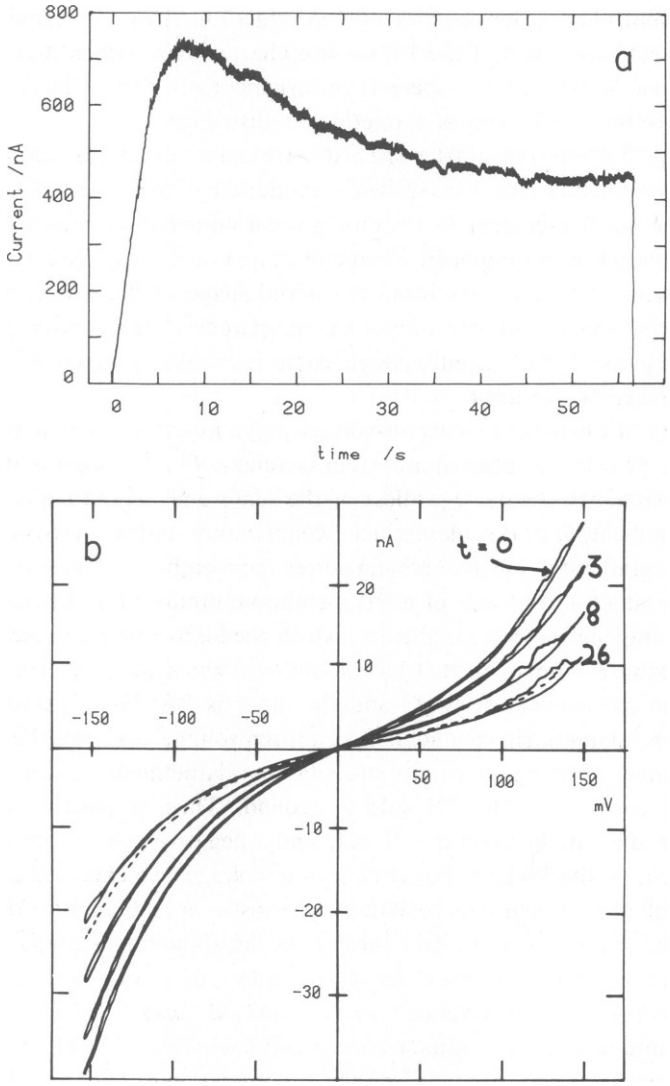


FIGURE 3 (*a*) Experimental conditions as in Fig. 2. This single current record shows the response of the membrane to a pulse of voltage to 158 mV. This was the only pulse applied between the dashed I–V curve in Fig. 3 *b* and the I–V curve taken immediately after the pulse, marked $t = 0$. Current unit (ordinate) is $10^{-8}$A. Voltage unit (abcissa) is 50 mV. (*b*) Recovery of lipid asymmetry as a function of time. The dashed curve shows the membrane I–V curve before the treatment shown in Fig. 3 *a*. The solid curves are I–V curves taken at the indicated times (in minutes) after the first curve. Note that the recovery is essentially complete after ~25 min. Current unit (ordinate) is $10^{-7}$A. Time unit (abscissa) is 5 s. (Sweep rate: 50 mV/s. This fast rate was chosen to suppress the time-dependent alamethicin conductance, which responds slowly on this time scale and shows up as small waves on the ends of the positive branches of the I–V curves. The same results for the negative branch of the nonactin-$K^+$ I–V curve are obtained at sweep rates down to 20 mV/s.)

through its own channels. It is thus possible to find experimental cases where the two effects are antagonistic; in one case flip-flop is larger, and in the other diffusion of alamethicin is larger. Neither effect can explain the experimental results alone.

The asymmetry of the nonactin current-voltage curve recovers with time as would be expected if the flip-flop rate is smaller than the lateral diffusion rate (11). Fig. 3 *a* shows the current treatment given to the membrane, the voltage current curves of which are shown in Fig. 3 *b*. The dashed curve shows the pretreatment I–V curve, and the solid curves are I–V curves taken at the indicated intervals after the first curve (taken 2 min after the end of the treatment). A curve at 18 min is not shown for clarity of reproduction. The Gouy equation was then used to calculate the remaining surface charge of that which moved from the PS side to the PE side during recovery. The recovery is essentially complete after 25 min.

From these data, it is possible to obtain a measure of the diffusion coefficient of lipid in the plane of the membrane. Table I shows the surface potential difference calculated from the curves in Fig. 3 *b* and the calibration curve in Fig. 1.

Assuming that each lipid monolayer composing the bilayer is in contact with an infinite monolayer of either PE or PS, depending on which side we consider, we can solve the diffusion equation for the redistribution of lipids once flip-flop is turned off. The result is expressed in terms of cylindrical Bessel functions (11), the slowest decaying term of which has the exponential time dependence shown in the footnote to Table I.

The rate of return of asymmetry of surface charge is accurately exponential with a time constant of 12.7 min. Comparison of this time constant with that of the slowest decaying term in the diffusion equation solution gives a diffusion coefficient of $5.1 \times 10^{-8}$ cm$^2$/s, a value which agrees well with that obtained by several other methods (12–14) for diffusion of lipids in cell membranes, but is a little faster than diffusion recorded in lipid bilayers. This paper

TABLE I
TIME-COURSE OF ASYMMETRY RECOVERY

| $\Delta V$ | $F\Delta V/2RT$ | $\sinh(F\Delta V/2RT)$ | $\sigma_{eff}^{(t)}$ | $\sigma(\infty) - \sigma(t)$ | Time |
|---|---|---|---|---|---|
| *(mV)* | | | *(e/A²)* | *(e/A²)* | *(min)* |
| 22 | 0.45 | 0.47 | 1.09 | 2.17 | 0 |
| 32 | 0.64 | 0.68 | 1.58 | 1.68 | 3 |
| 40 | 0.80 | 0.89 | 2.07 | 1.19 | 7 |
| 50 | 1.0 | 1.18 | 2.74 | 0.54 | 18 |
| 58 | 1.14 | 1.40 | 3.26 | 0 | 25 and + |

Regression gives $\Delta\sigma = 2.13 \times 10^{-3}/e/A^{02}$
$t_0 = 12.7$ min
$[\sigma(\infty) - \sigma(t)] = \Delta\sigma e^{-t/t_0}$, $r^2 = 0.999$.

Since the diffusion equation has the solution

$$\rho(r, t) = \frac{N(0)}{\pi r_0^2} \sum_{i=0}^{\infty} \frac{2}{Z_i J_1(Z_i)} J_0(Z_i r/r_0) \exp\left(-\frac{Z_i^2 Dt}{r_0^2}\right),$$

the diffusion coefficient can be approximately obtained as

$$D = \frac{r_0^2}{Z_1^2 t_0} = \frac{(0.015)^2}{(2.405)^2\, 12.7 \times 60} = 5.1 \times 10^{-8} \frac{\text{cm}^2}{\text{s}}.$$

thus contains three new results and a new technique: it demonstrates that alamethicin can induce a voltage-dependent lipid exchange between leaflets of a bilayer, it shows that alamethicin probably goes through its own channel, and it suggests that each half of a planar bilayer is in contact with a monolayer of lipid with which it can exchange lipids with a diffusion constant of $5 \times 10^{-8}$ cm$^2$/s. Finally, it provides a new technique for estimating the lateral diffusion of membrane components.

The first result demonstrates control of flip-flip rate according to the potential drop across the membrane. The potential drop across the membrane depends on both the lipid (in this case, because of the electrostatic potential at the membrane-water interface) asymmetry and the membrane potential, which is determined by the ionic composition of the solutions and the membrane permeabilities to various ions. Lipid asymmetry may thus alter the electric field across the membrane which is present for a given membrane potential, thereby controlling both voltage-dependent conductances and lipid flip-flop rate. This suggests both a possible reason for lipid asymmetry and a means for its control, not by a stoichiometric mechanism, but by a feedback mechanism which stops flip-flop when the appropriate electric field across the membrane is reached. That alamethicin goes through its own channel shows that the structural alterations induced by alamethicin are massive and complex and cannot be considered in terms of simple holes in the membrane, a view consistent with that of Donovan and Latorre (15), who found that large hydrophobic molecules can go through the alamethicin channel.

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