NONACTIN-K^+ COMPLEX AS A PROBE FOR MEMBRANE ASYMMETRY

JAMES E. HALL *and* RAMON LATORRE

From the Department of Physiology and Pharmacology, Duke University, Durham, North Carolina 27710. Dr. Latorre's present address is the Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637.

Since the introduction of artificial lipid bilayers (Mueller et al., 1962), several molecules have been used as probes of membrane structure. In particular, the nonactin-K^+ complex has been used to probe membrane surface charge (McLaughlin et al., 1970) and the shape of the electrochemical barrier to transport inside the membrane (Hall et al., 1973). More recently phospholipid bilayer membranes have been made with negative lipids forming one monolayer and neutral lipids forming the other (Montal, 1973). Such membranes are of considerable interest because similar asymmetry may exist in biological membranes (McLaughlin and Harary, 1974). The barrier shape for a membrane with a neutral lipid on one side and a negative lipid on the other should be skewed by the surface potential of the negative lipid. We ask first if this is reflected in the current-voltage curve. We find that the nonactin-K^+ current-voltage curve shows a pronounced asymmetry in the expected manner and consequently provides a direct measure of the difference in the surface potential of a neutral and a negatively charged lipid. We ask next how the observed membrane asymmetry changes with time.

We formed asymmetric membranes in a Teflon chamber with an 18 μm thick Teflon partition using the technique described by Montal (1973). The surface area of each compartment was 4 cm^2 and the volume 3 ml. Monolayers were spread on the surface of the electrolyte solution using 10 μl of lipid in pentane at a concentration of 12.5 mg/ml. One monolayer was formed from bacterial phosphatidyl ethanolamine (PE, Supelco, Inc., Bellefonte, Pa.) and the other from bovine phosphatidyl serine (PS, Supelco). The aqueous solutions were symmetric and consisted of 0.05 M KCl at pH 5.5 (unbuffered). Electrical measurements were made using Ag/AgCl electrodes in a four electrode system (Eisenberg et al., 1973). Current-voltage curves were recorded directly on an *X-Y* recorder at a voltage-sweep rate low enough to introduce negligible hysteresis (less than 20 mV/s). Measurements were made at 22°C unless stated. Nonactin (E. R. Squibb & Sons, New York) was added to both compartments in ethanolic solutions of various dilutions (control experiments showed that an equivalent amount of ethanol had no effect on membrane conductance).

Fig. 1 is a current-voltage curve of an asymmetric membrane in 0.05 M KCl. The voltage is positive when current flows from the PE side across the membrane to the PS

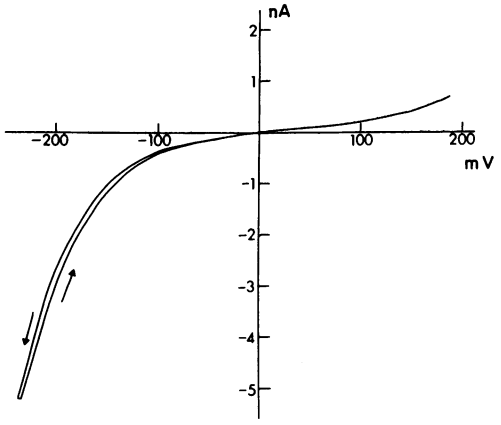

FIGURE 1 Current-voltage relationship for an asymmetric membrane (PS-PE) doped with nonactin. Salt concentration 0.05 M KCl. Nonactin concentration 10^{-6} M. Membrane area 4 × 10^{-4} cm^2. Temperature 22°C. Positive potential is defined by current flowing from the neutral side (PE) to the negatively charged side (PS). Small hysteresis in the third quadrant is due to the limited band width of the *X-Y* recorder.

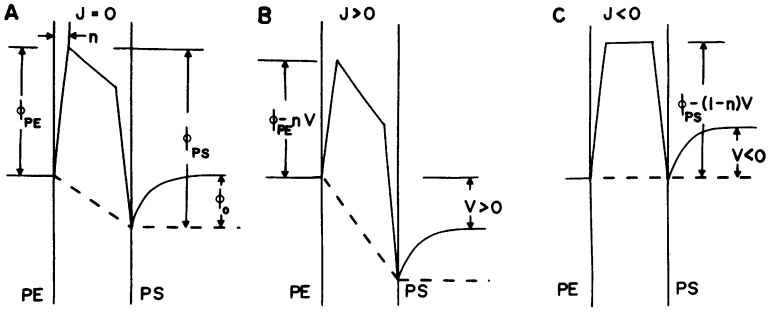

FIGURE 2 Trapezoidal approximation to the actual barrier under various conditions of applied voltage. J is current, ϕ_{PE} the barrier seen by a nonactin-K^+ complex at the phosphatidyl ethanolamine (PE) surface, at zero voltage, ϕ_{PS} the barrier seen by a nonactin-K^+ complex at the phosphatidyl serine surface, ϕ_o the surface potential arising from the negative charge of the PS, n the fractional distance from the edge of the membrane to the corner of the barrier ($n \sim 0.3$ for the membranes used in these experiments), and V is the applied voltage.

FIGURE 2*A* Shows the barrier profile obtained at zero applied voltage. The dashed line shows the electric potential across the membrane. The barrier profile results from the addition of this potential which arises from the surface potential and the barrier for translocation of nonactin-K^+ complex. The concentration of complex is greater on the PS side than on the PE side, but the barrier for translocation (ϕ_{PS}) is greater than from the PE side (ϕ_{PE}), so no net current flows.

FIGURE 2*B* Shows the barrier for a *positive applied voltage*. The barrier for translocation from the PE side is reduced by nV and the current flows from left to right. For large voltage ($V >$ 50 mV) $J \sim \exp(enV/kT)$.

FIGURE 2*C* Shows the barrier for a *negative applied voltage* equal to the surface potential, ϕ_o. The barrier for the translocation from the PS side has been reduced by $(1 - n)V$ and for $|\phi_o| < V < 0$ $|J| \sim \exp(e(1 - n)V/kT)$. If the voltage is further reduced, the right-hand corner of the barrier will become rate limiting and J will be proportional to $\exp(enV/kT)$.

side. The current-voltage curve is clearly asymmetric and shows marked rectification. The sense of the rectification is consistent with a negative surface potential on the PS side and can be quantitatively calculated from the expected barrier shape.

The steady-state current-voltage curves in bacterial PE-decane membranes[1] can be explained over a wide range of salt concentrations by a simple barrier profile not very different from that expected from an image force calculation. This barrier can be approximated reasonably well by a trapezoidal barrier of a height greater than 500 mV and a width about 0.6 the thickness of the membrane (Hall et al., 1973). Rectification is a consequence of the actual barrier, but it is easier to get a feel for how the rectification arises from the trapezoidal approximation. Fig. 2 A shows the approximate barrier profile expected for an asymmetric membrane with a negative lipid on one side. For current in the positive direction (PE to PS) the rate limiting energy is the difference between the height of the left-hand corner of the barrier and the solution to the left (ϕ_{PE} in Fig. 2 A). This distance is reduced by a positive voltage an amount nV (Fig. 2 B), where n is the fractional distance to the corner, and the current will thus be proportional to $\exp(neV/kT)$. For current in the negative direction the rate-limiting energy is the difference between the height of the same left-hand corner and the right-hand solution (ϕ_{PE} in Fig. 2 A). This difference is reduced by a factor of $(1 - n)V$ (Fig. 2 C), and the current in the negative direction is proportional to $\exp[(1 - n)eV/kT]$. These approximations are valid only at large voltages, and the current depends linearly on voltage for small applied voltages. Nevertheless, we expect a difference in the functional dependence of the current on voltage for different signs of the applied voltage. Plotting the data of Fig. 1 as logarithm of current vs. voltage for both signs of voltage shows such a difference. This result is shown in Fig. 3. Two slopes are clearly apparent, and the steeper slope occurs when current flows from PS to PE, as expected. For large negative voltages, the right-hand corner of the barrier would become rate limiting and the current would be proportional to $\exp(neV/kT)$ again.

The surface potential calculated from the observed rectification is 86 mV. This compares very favorably with the value estimated from the Gouy equation:

$$\sinh(e\phi_o/2kT) = 136\sigma/\sqrt{C},$$

where σ is in electronic charges per square angstrom, C is the ionic strength, ϕ_o is the surface potential, and k and T have their usual meanings. σ is assumed to be about 0.02 charges/Å^2. (McLaughlin et al., 1970).

This method of measuring membrane asymmetry has several advantages. First, it measures an effect *directly* related to the asymmetry of the membrane at any time and can therefore be used to monitor changes in asymmetry of a given membrane as a function of time. Second, it measures a difference in *shape*, not in magnitude, and it therefore is insensitive to changes in nonactin concentration as a function of time. This method is thus ideal for investigating the time dependence of the asymmetry.

[1] Bilayers made only of PE with the monolayer technique and doped with nonactin gave essentially the same barrier profile.

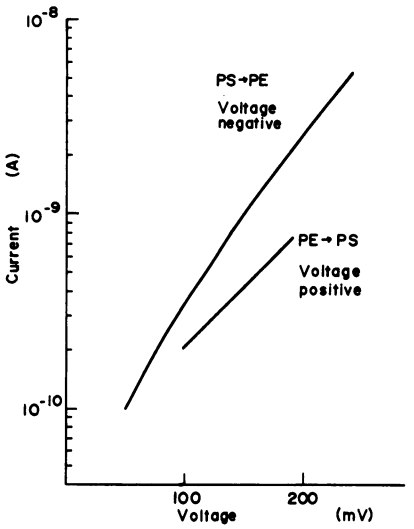

FIGURE 3 Plot of the log of the current vs. voltage, taken from the data of Fig. 1. The lower curve is for positive voltages with current flowing from the PE to the PS side, and the upper curve is for negative voltages with current flowing from the PS to the PE side. The slope of the lower curve is less steep than the slope of the upper curve, as expected. The decrease in slope of the upper curve with increasing voltage results from a shift of the rate limiting point from left to right. The upper curve corresponds to the barrier of Fig. 2 *C* and the lower to that of Fig. 2 *B*. (The lower voltage points are omitted because of limited experimental accuracy).

We have attempted to measure phospholipid flip-flop with this technique, and we have found no detectable change in asymmetry in a membrane lasting 10 h. In addition we have found no evidence of flip-flop in a membrane exposed to a positive potential of 100 mV on the serine side for 2 h. Increasing temperature to 33°C gave essentially the same results in a membrane lasting 3 h. We therefore conclude that phospholipid flip-flop in our membranes must be a very slow process, with a half time considerably greater than ten hours. We could have detected a change in surface potential difference less than 5 mV corresponding to a change in surface charge of 0.0004 charges/Å^2 on either side. Such a change was not observed.

The fact that this method reveals no flip-flop is not surprising. Recently, Rothman and Dawidowicz (1975) have reported that in their vesicular preparation the half time for the flip-flop process must take at least several days or longer. We are aware that lateral diffusion in planar membranes can dominate flip-flop, as pointed out by Sherwood and Montal (1975). They reported flip-flop occurring much more rapidly than we observed, using a different detection method. The difference between their results and ours may arise in part from use of different lipids.

This work was supported by grant HL-17961-01 from the National Institutes of Health to J. E. Hall and by grant HL-12157-07.

Received for publication 26 September 1975.

REFERENCES

EISENBERG, M., J. E. HALL, and C. A. MEAD. 1973. The nature of the voltage-dependent conductance induced by alamethicin in black lipid film membrane. *J. Membrane Biol.* **14**:143.

HALL, J. E., C. A. MEAD, and G. SZABO. 1973. A barrier model for current flow in lipid bilayer membranes. *J. Membrane. Biol.* **11**:75.

MCLAUGHLIN, S., and H. HARARY. 1974. Phospholipid flip-flop and the distribution of surface charges in excitable membranes. *Biophys. J.* **14**:200.

MCLAUGHLIN, S. G. A., G. SZABO, G. EISENMAN, and S. M. CIANI. 1970. Surface charge and the conductance of phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **48**:1177.

MONTAL, M. 1973. Asymmetric lipid bilayers: response to multivalent ions. *Biochim. Biophys. Acta.* **298**: 750.

MUELLER, P., D. O. RUDIN, H. T. TIEN, and W. C. WESCOTT. 1962. Reconstitution of excitable cell membrane structure in vitro. *Circulation.* **26**:1167.

ROTHMAN, J. E., and E. A. DAWIDOWICZ. 1975. Asymmetric exchange of vesicles phospholipids catalyzed by the phosphatidylcholine exchange protein. Measurement of inside-outside transitions. *Biochemistry.* **14**:2809.

SHERWOOD, D., and M. MONTAL. 1975. Transmembrane lipid migration in planar asymmetric bilayer membranes. *Biophys. J.* **15**:417.