LETTERS TO THE EDITOR

Does Phospholipid Flip-Flop Affect Axon Potassium Channels?

Dear Sir:

In a recent paper, McLaughlin and Harary (1974) advanced the hypothesis that the asymmetrical distribution of lipids on the two sides of a biological membrane could be directly attributed to the voltage difference across the membrane. This is based on the mechanism of phospholipid flip-flop, according to which lipid molecules can move from one side of a bilayer to the other and still maintain the required bilayer alignment. The characteristic time of such a flip-flop for phospholipids in excitable membrane vesicles prepared from the electroplax of *Electrophorus electricus* is between 4 and 7 min at 15°C (McNamee and McConnell, 1973). If the turnover rate of lipids is much slower than the rate of flip-flop, charged lipids would be expected to reach a steady-state distribution between the inside and outside surfaces in accordance with the Boltzmann distribution appropriate to the membrane potential difference. Combining the Boltzmann relation with the Guoy equation for a diffuse double layer and an estimate of the fraction of membrane lipids which are charged, McLaughlin and Harary have calculated the phospholipid charge densities at both the inner and outer membrane surfaces. Their values are in good agreement with the surface charge densities measured for squid axon membranes in the region of sodium and potassium channels (Gilbert and Ehrenstein, 1969; Chandler et al., 1965).

This agreement is consistent with the hypothesis that charged phospholipids make up the membrane surface charge in the region of the ionic channels. We have tested this hypothesis by changing the electric field across the squid axon membrane and measuring the external surface charge density at various times thereafter.

To do this, we obtained a large membrane depolarization for a relatively long time ($\frac{1}{2}$ h or more) by replacing the normal artificial seawater bathing the axon with a high potassium solution. The region of the membrane near the potassium channels was chosen for surface charge measurement because with these channels inactivation is not a serious problem and because two independent methods of determining changes in surface charge density can be employed (Ehrenstein and Gilbert, 1973). These two methods are the measurement of shifts in the conductance vs. voltage curve along the voltage axis and the measurement of shifts in the relaxation time vs. voltage curve along the voltage axis. Each of these shifts is an independent measure of changes in the component of electric field within the membrane produced by changes in the surface charge.

If the relevant surface charge is composed of phospholipids that can undergo rapid flip-flop, then a depolarization to zero membrane potential should cause about half of the negative charge on the external membrane surface to flip to the internal surface. From the Guoy equation and the measured external surface charge (Gilbert and Ehrenstein, 1969), this would correspond to a shift of about 27 mV to the right along the voltage axis for both conductance and relaxation time. If a correction is applied to take into account the estimated surface charge already on the internal membrane surface (Chandler et al., 1965; McLaughlin and Harary, 1974), the expected shift is about 21 mV. By comparison, our resolution is about 5 mV for a single experiment. Thus, we should be able to see processes with time constants almost four times slower than our experimental waiting times.

Typical experimental current-voltage curves after 5 min in the depolarizing solution and after 35 min at 10°C are shown in Fig. 1. There is no evidence of a voltage shift. In four other

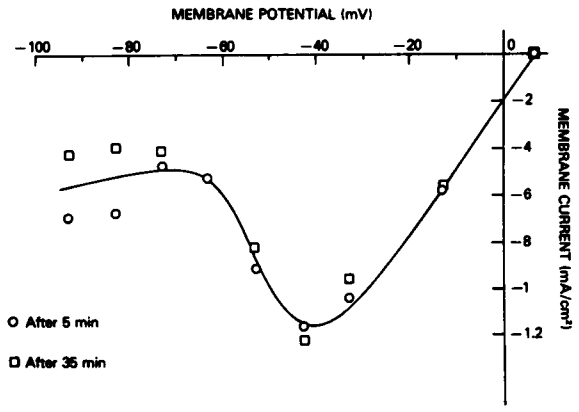

FIGURE 1

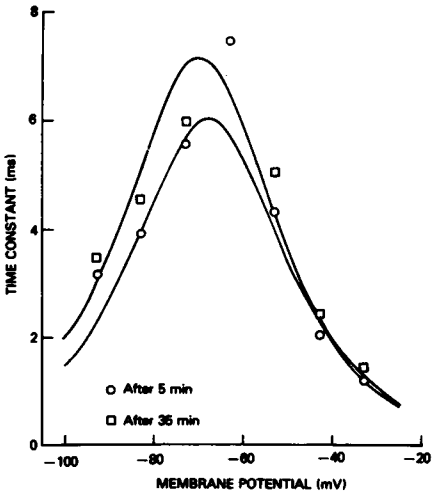

FIGURE 2

FIGURE 1 Steady state current-voltage curve after depolarization. Times refer to time after axon immersion into the high potassium solution, which was composed of 440 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, and 10 mM Tris buffer.

FIGURE 2 Relaxation time (τ_n) vs. membrane potential after depolarization. The same current-time records were used as in Fig. 1. The upper curve corresponds to 5 min and the lower curve to 35 min after immersion into the high potassium solution.

experiments at 20°C (three with a half-hour between measurements and one with a full hour), the average voltage shift was 2 mV to the left.

Experimental values of the relaxation time of the potassium channel as a function of membrane potential after 5 min and after 35 min in the depolarizing solution are shown in Fig. 2 for the same axon used for Fig. 1. The solid curves in Fig. 2 are least-squares fits (Knott and Reece, 1972) of the experimental points to hyperbolic secant functions. The voltage shift is about 2 mV to the right.

Thus, both the conductance and relaxation time measurements indicate that, within experimental error, there is no voltage shift after ½ h of depolarization.

In all experiments, we waited 5 min after changing solutions to start the initial measurements in order to insure that solutions and potentials had equilibrated. The possibility that there is a very fast flip-flop giving rise to a voltage shift which reaches steady state in less than 5 min can be ruled out by comparing the results of previous experiments. The voltage shifts of squid axon potassium conductance obtained for changes in external calcium concentrations by Gilbert and Ehrenstein (1969) in high potassium solutions were quite similar to those obtained by Frankenhaeuser and Hodgkin (1957) in sea water.

Our failure to observe depolarization-induced shifts along the voltage axis for both the current and relaxation time of the potassium channels requires us to conclude at least one of the following:

(*1*) The surface charge in the region of axon potassium channels is not composed of phospholipids.

(*2*) The surface charge in the region of axon potassium channels is composed of phospholipids, but these phospholipids are bound so tightly to the channel that they cannot undergo phospholipid flip-flop even 20 times more slowly than ordinary phospholipid molecules.

(*3*) The phospholipids of the axon membrane do not flip-flop on the time scale of a few hours

or less, and the observations of flip-flop in excitable membranes are either an artifact of the spin label method or else do not apply to squid axons. Recently, Dawidowicz and Rothman (1975) have reported their measurements for phospholipid flip-flop rate in a vesicular preparation. Using radioactivity measurements in the presence of an exchange protein, they found that flip-flop is very much slower than expected on the basis of spin label experiments.

Received for publication 17 March 1975.

REFERENCES

CHANDLER, W. K., A. L. HODGKIN, and H. MEVES. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol.* **180**:821.

DAWIDOWICZ, E. A., and J. E. ROTHMAN. 1975. Asymmetric exchange of vesicle phospholipids by the phosphatidyl choline exchange protein: measurement of flip-flop rate. *Biophys. J.* **15**:27a.

EHRENSTEIN, G., and D. L. GILBERT. 1973. Evidence for membrane surface charge from measurement of potassium kinetics as a function of external divalent cation concentration. *Biophys. J.* **13**:495.

FRANKENHAEUSER, B., and A. L. HODGKIN. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**:218.

GILBERT, D. L., and G. EHRENSTEIN. 1969. Effect of divalent cations on potassium conductance of squid axons: determination of surface charge. *Biophys. J.* **9**:447.

KNOTT, G. D., and D. K. REECE. 1972. MLAB: a civilized curve-fitting system. *In* Proceedings of the ON-LINE '72 International Conference, Brunel University, England. **1**:497.

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

MCNAMEE, M. G., and H. M. MCCONNELL. 1973. Transmembrane potentials and phospholipid flip-flop in excitable membrane vesicles. *Biochemistry.* **12**:2951.

GERALD EHRENSTEIN
DANIEL L. GILBERT
Laboratory of Biophysics
National Institute of Neurological Diseases and Stroke
National Institutes of Health
Bethesda, Maryland 20014
and
Marine Biological Laboratory
Woods Hole, Massachusetts 02543
RAYMOND J. LIPICKY
Department of Pharmacology
College of Medicine
University of Cincinnati
Cincinnati, Ohio 45267
and
Marine Biological Laboratory
Woods Hole, Massachusetts 02543