The Nature of the Negative Resistance in Bimolecular Lipid Membranes Containing Excitability-Inducing Material

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ABSTRACT When sufficiently small amounts of excitability-inducing material (EIM) are added to a bimolecular lipid membrane, the conductance is limited to a few discrete levels and changes abruptly from one level to another. From our study of these fluctuations, we have concluded that the EIM-doped bilayer contains ion-conducting channels capable of undergoing transitions between two states of different conductance. The difference in current between the "open" and "closed" states is directly proportional to the applied membrane potential, and corresponds to a conductance of about 3×10^{-10} ohm^{-1}. The fraction of the total number of channels that is open varies from unity to zero as a function of potential. The voltage-dependent opening and closing of channels explains the negative resistance observed for bimolecular lipid membranes treated with greater amounts of EIM.

INTRODUCTION

Addition of "excitability-inducing material" (EIM) (Mueller and Rudin, 1963) causes a dramatic change in the electrical properties of a bimolecular lipid membrane. Before EIM[1] is added, the current-voltage curve for a membrane with 0.1 M KCl on both sides is linear and has a conductance of about 10^{-8} mho cm^{-2}. After EIM is added, the conductance increases by as much as four orders of magnitude. This EIM-induced conductance is sufficiently voltage-dependent to give a negative resistance that is quite similar to that found for excitable cells (Mueller and Rudin, 1963). Bean, Shepherd, Chan, and Eichner (1969) have recently shown that during the addition of EIM the increase in conductance occurs in discrete steps of about 4×10^{-10} mho, suggesting the presence of individual conducting channels. Mueller and Rudin (1968) had previously observed resistance fluctuations, which they assumed

[1] Abbreviation used in paper: EIM, excitability-inducing material.

were caused by opening and closing of individual channels. From their records, they estimated that the channel conductance is between 10^{-11} and 10^{-9} mho.

In order to measure electrical properties of individual channels, we have investigated spontaneous discrete current jumps that occur in membranes with very few conducting channels. We wished to determine whether the negative resistance observed in membranes with many channels arises from the voltage dependence of the conductance of individual channels or from the opening and closing of channels of fixed conductance.

MATERIALS AND METHODS

Preparation of Materials EIM was prepared from a medium in which *Aerobacter cloacae* ATCC 961 had been grown by the method of Kushnir (1968). A synthetic medium (500 ml) containing, per liter, sodium citrate, 6 g; ammonium sulfate, 3.5 g; calcium chloride, 1 g; glucose, 1 g; 1 M potassium phosphate buffer, pH 7.0, 8 ml; and 10% Tween 80, 2.5 ml, was inoculated with 0.25 ml of a culture grown overnight in nutrient broth. The bacteria were grown for 24 hr at 37°C with vigorous aeration and then harvested by centrifugation for 20 min at 9000 *g*. The supernatant fluid, which contained the EIM, was stored at 6°C.

Extracts of total brain lipids were prepared by a modification of the procedure of Folch and Lees (1951) outlined by Mueller, Rudin, Tien, and Wescott (1964). The crude lipid extract was flushed with nitrogen and stored in the dark at −90°C. Oxidized cholesterol was prepared by the method of Tien et al. (1966) and stored at room temperature.

The lipids used to form membranes were either (*a*) oxidized cholesterol mixed with *n*-decane (40 mg cholesterol/ml decane) or (*b*) brain phospholipids mixed with α-tocopherol (Distillation Products Industries, Rochester, N.Y.) and cholesterol (Sigma Chemical Co., St. Louis, Mo., chromatography grade, 99% pure) in the ratio 1 ml split lipid extract:400 mg α-tocopherol:30 mg cholesterol.

Experimental Procedures The experimental arrangement is shown in Fig. 1. The method of membrane formation is essentially that of Mueller, Rudin, Tien, and Wescott (1964). Membranes are formed across a 1 mm diameter hole in a 5 ml Teflon cup, which is immersed in a solution of 0.1 M KCl buffered with 0.005 M histidine chloride (pH 7.0). The outer container is transparent, so that membrane formation can be observed. To form a bilayer membrane, a lipid solution is brushed over the hole with a small sable brush, forming a thick membrane. This membrane thins spontaneously in about 1–15 min.

For oxidized cholesterol membranes, formation and addition of EIM are both performed at room temperature. For brain phospholipid membranes, formation is done at 33°C and the EIM is added at 38°C. These temperatures were obtained by heating from above with a light bulb. We found no essential differences between the two types of membrane. Oxidized cholesterol was used for most of the experiments reported in this paper—all except those referred to in Fig. 9—because membranes

made of this material are stable at room temperature. If the EIM-induced conductance does not exceed 10^{-8} mho, the membranes sometimes last up to 8 hr. In the experiments reported in this paper, they lasted 1–2 hr. The evaporation of water from the solution is less than 1 %/hr at room temperature.

In the experiments designed to obtain few channels, about 10 μl of a 1:10 aqueous dilution of the EIM solution was added to the interior of the membrane cup, as close as possible to the membrane. The solutions were not stirred.

The electrical properties of the membrane were measured with the circuit shown in Fig. 1. The membrane was placed in series with a variable "ammeter" resistance and a voltage source. The ammeter resistance can be varied from 0.1 to 10.0 megohms.

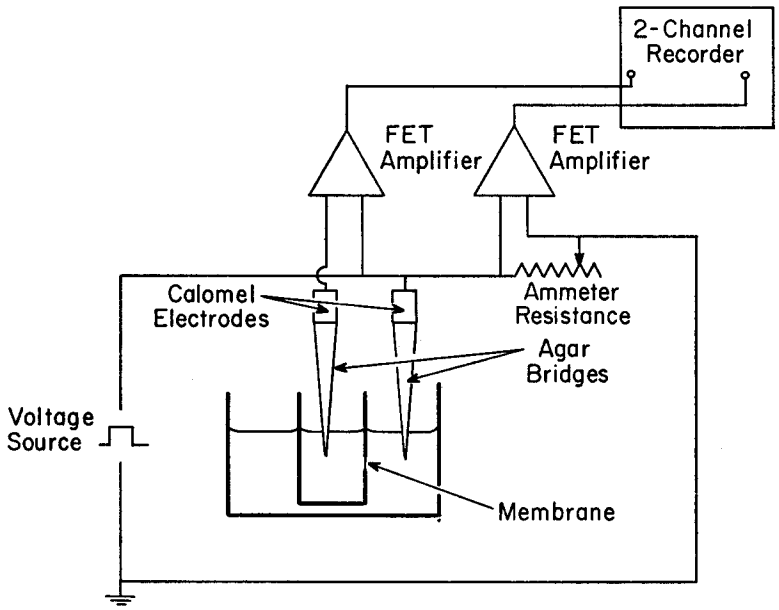

FIGURE 1. Schematic diagram of equipment used to determine electrical properties of artificial membranes.

Positive potential and current are defined to correspond to cation flow into the compartment containing EIM. The membrane apparatus and electrodes were enclosed in a Faraday cage to minimize extraneous electrical noise. The amplifiers across the membrane and across the ammeter have FET inputs to provide input impedance of at least 2×10^{11} ohms. The amplifier outputs are fed into a two-channel recorder. To make a measurement, the voltage source is adjusted to provide a desired voltage across the membrane. The voltage simultaneously recorded across the ammeter determines the corresponding current through the membrane. Because of the high membrane resistance (approximately 10^9 ohms), this circuit acts as a voltage clamp, except for transition times of 10 msec or less. In these experiments, only long pulses were used.

RESULTS

Fig. 2 a shows a typical steady-state current-voltage curve for an oxidized cholesterol membrane to which a moderate amount of EIM has been added.

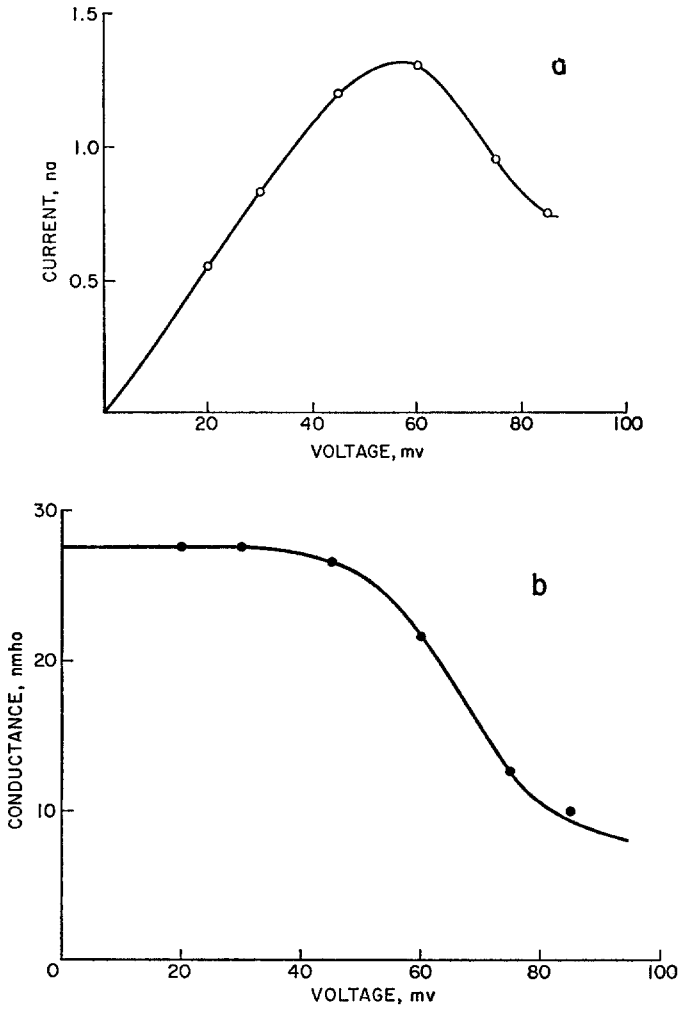

FIGURE 2. Electrical properties of a lipid bilayer membrane with many EIM channels. a. Current-voltage relation. b. Conductance-voltage relation. Points were obtained by experiment. The solid curve was determined from equations 5 and 6.

Although the exact shape of the curve depends upon the lipid used, and even varies from membrane to membrane with the same lipid, a negative resistance in the positive quadrant is always clearly observed. There is usually another, less pronounced, negative resistance region in the negative quadrant, but we have restricted our analysis to the positive quadrant.

When sufficiently small amounts of EIM are added to an oxidized cholesterol membrane, the membrane current no longer attains a steady value in response to an applied potential. Rather, the membrane current continually fluctuates. Fig. 3 shows typical patterns of discrete current jumps between a

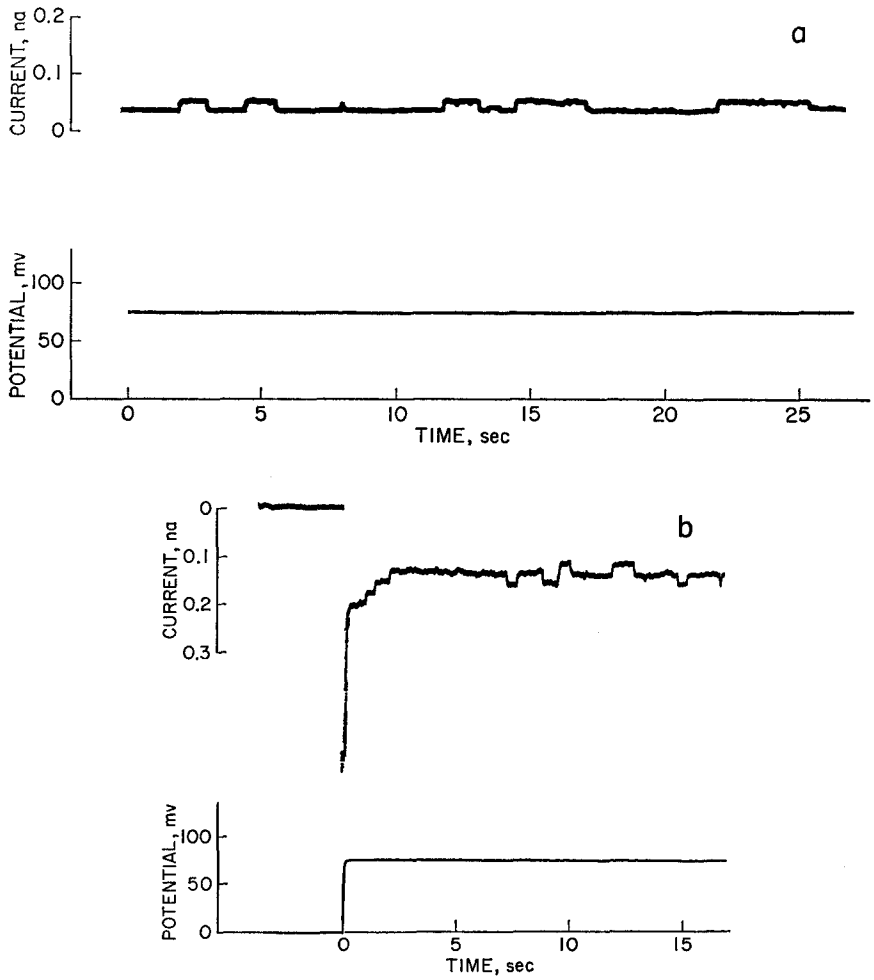

FIGURE 3. Current vs. time records, showing discrete current jumps for a few EIM channels. Membrane potential, 75 mv. The top trace shows current; the bottom trace, applied voltage. a. One-channel record. b. Four-channel record.

small number of levels. In this paper, we report on the properties of membranes with two, three, and five levels. If the membranes have individual channels that can be open or closed, the corresponding numbers of channels are one, two, and four.

In Table I the amplitudes of the discrete current jumps are tabulated for

each membrane. For the single-channel membrane, there is a unique bump height for each applied voltage. This is consistent with the notion of a stable channel. Furthermore, the observation of only two levels is consistent with the notion that the channel must be open or closed.

TABLE I

MAGNITUDE OF CURRENT JUMPS AT FIXED VOLTAGE

No. of channels	Voltage	Average current	Standard deviation	Range	No. of jumps measured
1	30	5.0	0.0	5.0–5.0	2
	35	7.4	0.8	6.7–8.3	3
	50	10.8	1.4	8.5–12.5	10
	61	15.1	1.2	13.9–16.3	3
	74	17.3	0.7	16.5–18.3	5
	75	17.5	0.7	16.5–18.8	28
2	20	6.3	1.4	5.5–8.0	3
	31	13.0	1.5	11.5–15.0	5
	40	11.1	1.2	9.8–12.8	5
	51	18.0	2.6	13.0–21.0	11
	65	27.4	1.4	25.5–29.0	5
	75	27.5	0.7	27.0–28.0	2
4	33	9.0			1
	44	11.8	0.4	11.5–12.0	2
	57	15.8	1.5	14.0–18.0	5
	60	16.9	0.9	16.0–18.5	10
	71	20.0	1.6	18.0–23.5	13
	75	22.4	2.5	17.0–25.5	31

In treating channels that can be either open or closed, it is convenient to define the following quantities:

$g_c \equiv$ conductance of a closed channel
$g_o \equiv$ conductance of an open channel
$g_\Delta \equiv g_o - g_c$
$G_B \equiv$ conductance of the membrane without channels
$N \equiv$ total number of channels
$n \equiv$ number of open channels
$\bar{n} \equiv$ average number of open channels

In terms of these quantities, the total steady-state conductance of a membrane is

$$G = G_B + Ng_c + \bar{n}g_\Delta \quad (1)$$

Fig. 2 b illustrates the voltage dependence of the steady-state conductance,

G, obtained from the data of Fig. 2 a. The main question we wish to answer is wherein this voltage dependence resides. Equation 1 shows that G can vary with potential if either the conductance of the individual channels or the number of open channels is a function of potential.

In the single-channel experiment, the smaller current level for each potential corresponds to $\bar{n} = 0$ in equation 1. In Fig. 4, this current is plotted as a function of potential. The linearity of this plot demonstrates that the conductance, $G_B + Ng_c$, is not voltage-dependent. The voltage dependence of g_Δ can be determined from a plot of bump height vs. voltage. Fig. 5 shows that the bump height varies linearly with voltage. Therefore g_Δ is also voltage-independent. We are left with the conclusion that $\bar{n}$ must contain all of the voltage dependence of the membrane conductance.

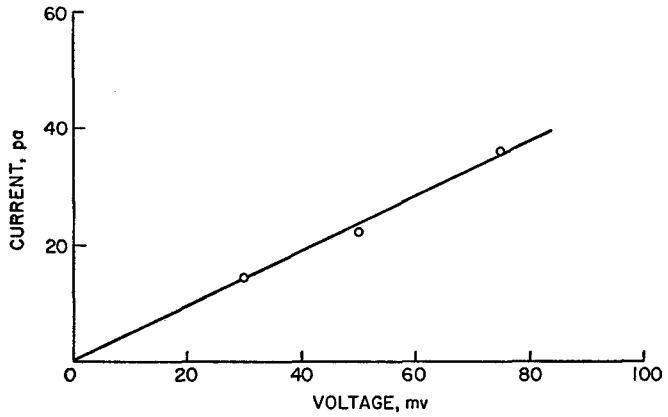

FIGURE 4. Current-voltage curve for a membrane with a single closed channel.

Next, we determined the explicit voltage dependence of $\bar{n}$ from the statistics of openings and closings of channels in membranes with small numbers of channels. For this purpose it is convenient to consider the average value of the fraction of channels open:

$$f(V) \equiv \frac{\bar{n}(V)}{N} \tag{2}$$

When there are many channels, the fraction of channels open at any instant of time will not depart appreciably from the average value $f(V)$. For small numbers of channels, however, there are relatively large deviations from the average. In particular, for a membrane with a single channel, the instantaneous number of open channels can only be zero or one. However, the average fraction of time that the channel stays open at a given potential should be equal to $f(V)$.

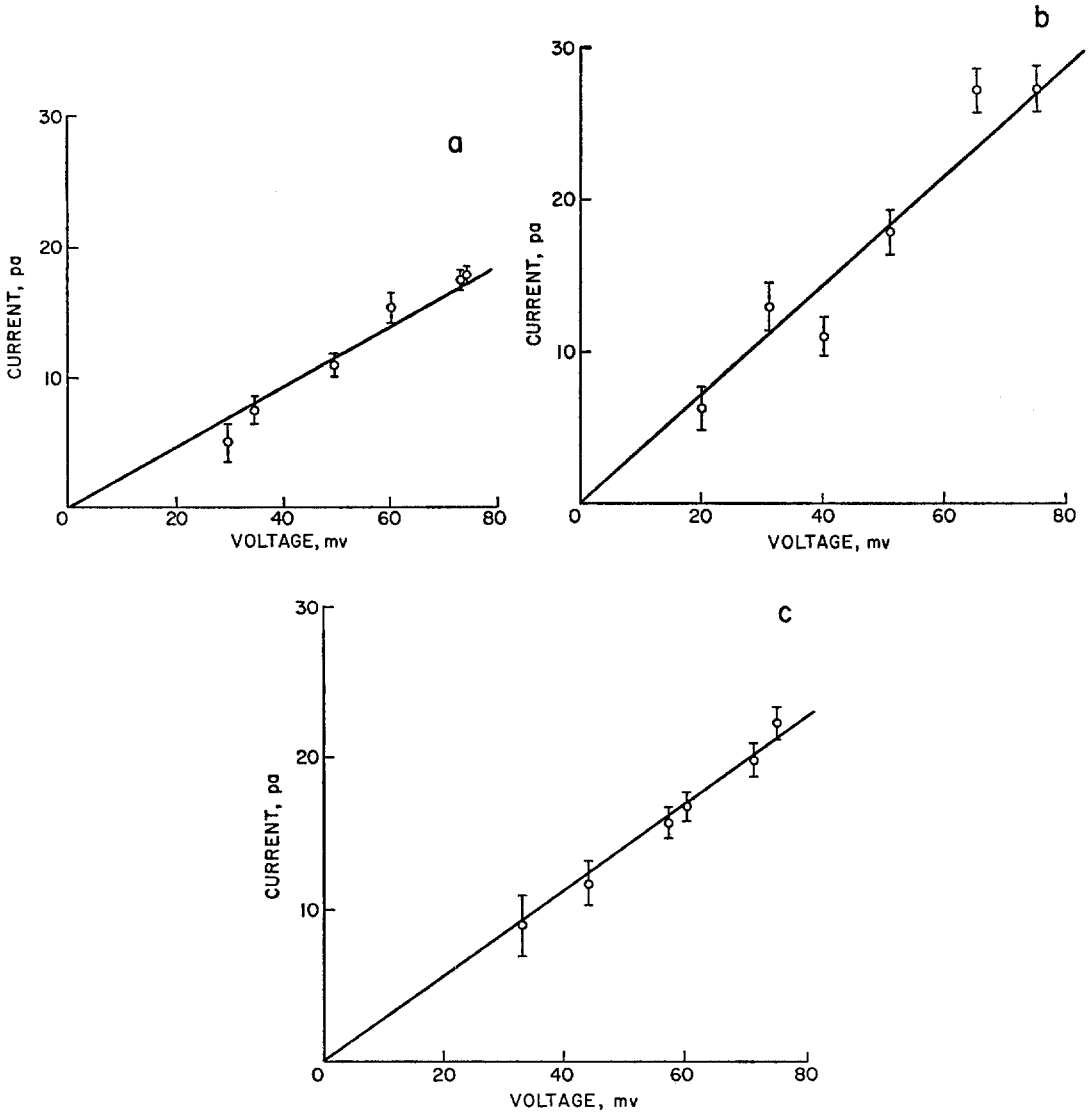

FIGURE 5. Current-voltage curves for an EIM channel. a. One-channel experiment. b. Two-channel experiment. c. Four-channel experiment.

The circles in Fig. 6 show $f(V)$ determined from the fraction of time that the channel was open in the one-channel experiment. For each potential, the time of observation was between 1 and 5 min, and the number of changes from off-to-on or on-to-off was between 0 and 30. The first 5 sec of each pulse were not counted, in order to allow time for the initial transient to die away. The necessity for this procedure is illustrated by Fig. 3 b. The staircase pattern at the beginning of the pulse shows the change from a state with

channels mostly open to a state with channels mostly closed. The rest of the record shows the random opening and closing of channels in the steady state. We also determined $f(V)$ from the two- and four-channel data. These values are shown as triangles and squares in Fig. 6.

The experimental points in Fig. 6 demonstrate that $f(V)$ varies between zero and one. Thus, the minimum conductance in Fig. 2 b corresponds to *all* of the channels being in the closed state, and the maximum conductance corresponds to *all* of the channels being in the open state. Therefore, from equation 1, the minimum conductance in Fig. 2 b is equal to $G_B + Ng_e$. If this residual conductance is subtracted from the curve in Fig. 2 b, the resultant, $\bar{n}g_A$, should exhibit the same voltage dependence as $f(V)$. This comparison is made in Fig. 6. The points determined from the statistical

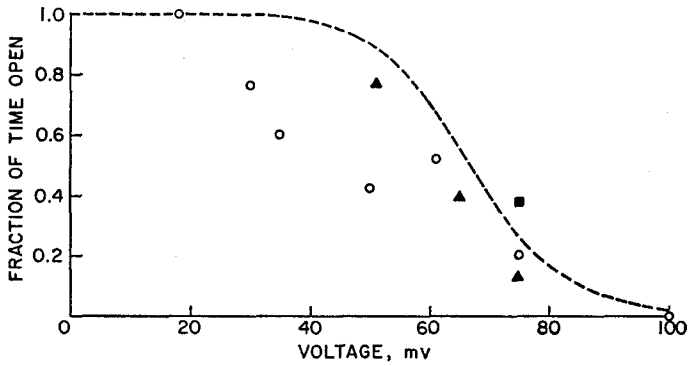

FIGURE 6. Voltage dependence of the fraction of time a channel is open. The dashed curve is relative conductance of many-channel membrane replotted from Fig. 2 b. ○, determined from one-channel experiment; ▲, determined from two-channel experiment; ■, determined from four-channel experiment.

measurements of $f(V)$ and the curve determined from conductance measurements are in approximate agreement.

For a few cases, in which the current records of the two- and four-channel membranes are sufficiently long, it is possible to determine the statistical distribution of the number of open channels. This may be compared with the theoretical distribution for independent channels. As indicated above, the fraction of time that a single channel is open is $f(V)$. The fraction of time during which n particular channels are open and the other $(N - n)$ channels are closed is $f^n(1 - f)^{N-n}$. In order to determine the fraction of time during which any n channels are open, this must be multiplied by the number of ways n channels can be chosen from a total of N. Thus, the fraction of time that exactly n channels are open is

$$P(n) = \frac{N!}{n!(N - n)!} f^n(1 - f)^{N-n} \tag{3}$$

Fig. 7 shows the comparison between theoretical and experimental distributions for several cases. In each case, the value of f was determined from the average number of open channels by use of equation 2.

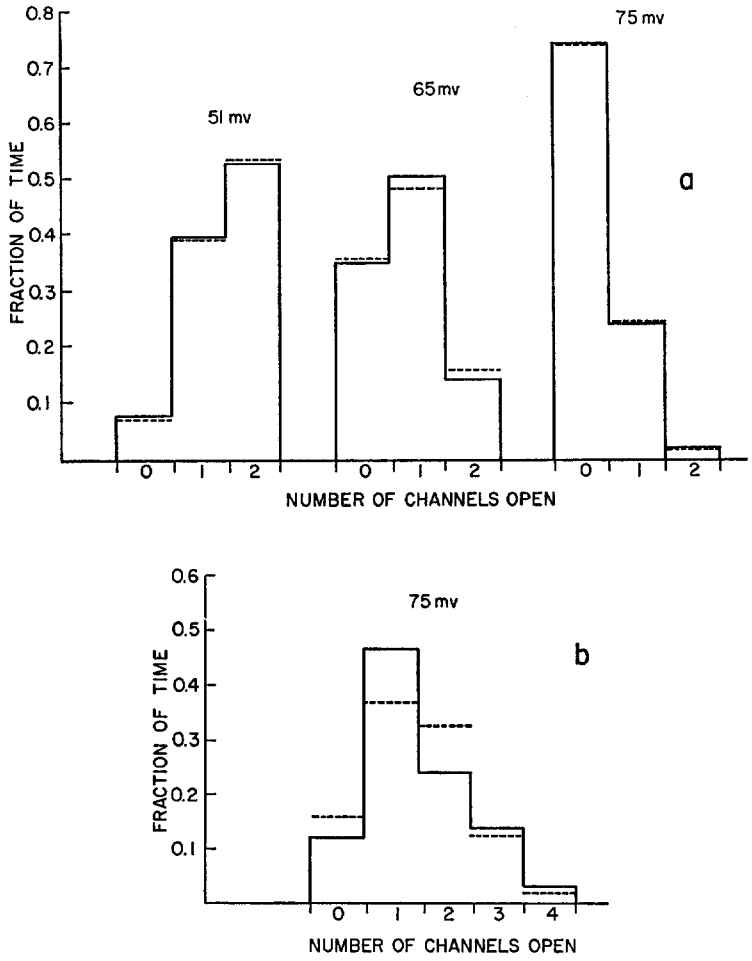

FIGURE 7. Probability histograms for number of open channels. Solid lines represent experimental observations. Dashed lines were calculated from equations 2 and 3. a. Two-channel experiment. For membrane potentials of 51, 65, and 75 mv, the observation times were 230, 105, and 142 sec, during which the number of jumps were 52, 12, and 19, respectively. b. Four-channel experiment. For a membrane potential of 75 mv, the observation time was 81 sec, during which there were 15 jumps.

A further consistency check was afforded by applying a sawtooth potential across a membrane with EIM. If the individual channels are ohmic, and the discrete jumps of current shown in Fig. 3 are caused by changes in the number of open channels, the current response for a membrane with few channels

should be a series of straight lines with abrupt changes of slope occurring whenever there is a change of current. This is, in fact, observed, as shown in Fig. 8. The current response of a membrane with a larger number of channels is shown in Fig. 9. Individual conductance jumps cannot be resolved, but

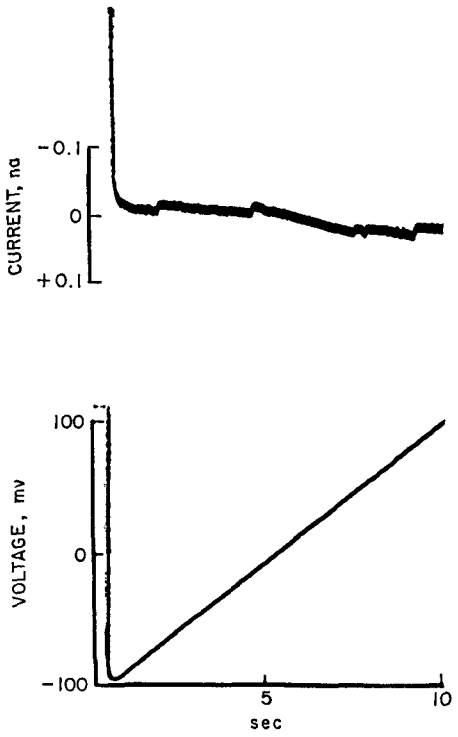

FIGURE 8. Current response to a sawtooth potential by a membrane with few channels. Discontinuities in the slope of the current show the switching of individual channels.

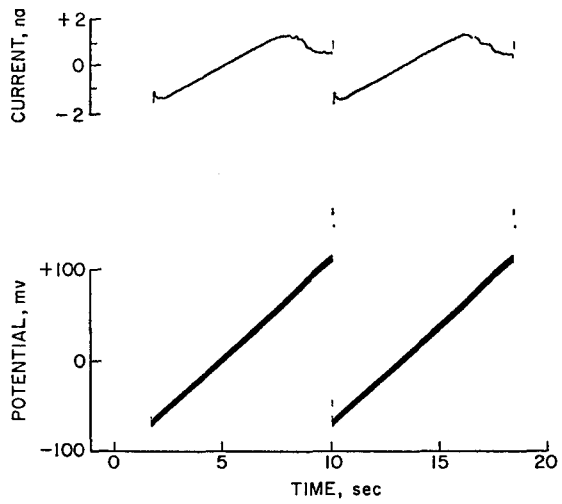

FIGURE 9. Current response to a sawtooth potential by a membrane with about 70 channels.

the noise level is seen to be greatest in the negative resistance region, where conductance jumps are expected to be most frequent.

The values of unit conductance bump, g_Δ, determined from Fig. 5 are:

One-channel experiment	$2.3 \pm 0.2 \times 10^{-10}$ ohm^{-1}
Two-channel experiment	$3.6 \pm 0.4 \times 10^{-10}$ ohm^{-1}
Four-channel experiment	$2.8 \pm 0.2 \times 10^{-10}$ ohm^{-1}

If these conductances are weighted by the number of channels they represent, the average value is $3.0 \pm 0.5 \times 10^{-10}$ ohm^{-1}, where the error estimate is compounded of the error in a single experiment and the variability from membrane to membrane.

DISCUSSION

In the experiments of Bean et al. (1969), discrete conductance steps were observed when EIM was added to the membrane. The "formation bumps" they observed provide strong evidence for individual channels. In our experiments, discrete conductance steps were observed even after the formation process had been completed. These "transition bumps" provide additional evidence for channels. More importantly, the transition bumps provide information about the nature of the conducting channel.

The existence of only two conductance levels over a period of many minutes in one of our experiments argues strongly that the channel has only two conductance states. Furthermore, in the experiments with three or five levels, there are two reasons to conclude that the multiple number of levels arises from several channels each with two states, rather than from one channel with three or five states. First, at any given voltage the individual bump heights in the five-level experiment are approximately equal to the bump height for the two-level experiment. Second, the distribution of open channels in the multiple-level experiments is in reasonable agreement with the distribution predicted from a model assuming several independent two-state channels (see Fig. 7). Since the number of current jumps in these records is small, the qualitative nature of the distribution is more significant than the detailed fit to equation 3. In every case, the distribution shows a peak at the number of channels predicted by the two-state assumption. Attempts to fit the data to distributions appropriate to multistate conductances did not give even a qualitative fit.

Our conclusion that the channel is a two-state system disagrees with the suggestion of Bean et al. (1969) that the channels are multistep systems for which each conductance step is about one-fifth of the total channel conductance. Their conclusion is based on the current response at fixed voltage

for a membrane with about 20 channels. Their current records for this case are rather noisy, and it is difficult to resolve individual discrete conductance changes. We have experienced similar difficulty for a comparable number of channels.

Fig. 6 shows that the fraction of time an individual channel stays open is voltage-dependent, and that this voltage dependence does indeed mirror the shape of the conductance curve. The precision of the individual-channel data which we present is limited by the relatively short times of our current records. If all EIM channels are identical and act independently of each other, then the $f(V)$ curve for an individual channel and the conductance curve for many channels should exactly coincide. A systematic discrepancy between the two might indicate cooperativity. However, in the present experiment the statistical inaccuracies in the $f(V)$ determination do not allow such a detailed comparison.

We have also demonstrated that the current-voltage curve for the transition between the two conductance states of an individual channel is linear (see Fig. 5). This means that all of the voltage dependence resides in the opening and closing of the channels. Although the experimental results for each membrane shown in Fig. 5 fit a straight line rather well, the two-channel membrane exhibits considerable scatter. A likely explanation is that the two channels have somewhat different conductances. Further evidence that the conductance varies from channel to channel is the difference in slopes of the three current-voltage curves, which seem to be beyond experimental error. Thus, our data tend to confirm the observations of Bean et al. that there is not a unique channel conductance.

A theoretical model of two-state channels was previously proposed by Mueller and Rudin (1963) to account for the EIM negative resistance. The present work has demonstrated that such channels do in fact exist and have the proper behavior to give the observed negative resistance. Although discrete conductance fluctuations have not yet been observed for biological membranes, there is evidence that biological membranes contain channels with a conductance comparable to that for EIM (del Castillo and Suckling, 1957; Lüttgau, 1958; Cole, 1968; Hille, 1968; Verveen and Derksen, 1968).

It is premature to speculate on the details of the molecular events involved in the opening of the channels. However, from the form of the observed conductance we can estimate the activation energy required for the switching process. If we assume the channels to be in equilibrium at voltage V, then

$$\frac{\bar{n}}{N - \bar{n}} = \exp\left[\frac{-W(V)}{kT}\right] \tag{4}$$

where $W(V)$ is the voltage-dependent activation energy of a single channel.

Combining equations 4 and 2, we obtain

$$f(V) = \left(1 + \exp\left[\frac{W(V)}{kT}\right]\right)^{-1} \tag{5}$$

In trying to fit $W(V)$ to the data in Fig. 6, we first note that at sufficiently low voltage $f(V) \to 1$, and at sufficiently high voltage $f(V) \to 0$. As can be seen from equation 5, this requires that $W(V)$ change sign at a value of V corresponding to $f(V) = \frac{1}{2}$. Thus, if we define the switching voltage V_0 by the relation $f(V_0) = \frac{1}{2}$, we can represent the activation energy in the negative resistance region by the simple form

$$W(V) \cong a(V - V_0) \tag{6}$$

From the conductance data of Fig. 2 b, we can determine two parameters: an activation barrier aV_0 and a parameter a, which is a measure of the strength of the interaction between the electric field and an open channel. The activation barrier is 0.22 ev and the interaction strength is 3.3 electron charges. The curve shown in Fig. 2 b and Fig. 6 is, in fact, the theoretical curve of equations 5 and 6 with these parameters.

Finally, the model of random opening and closing of conducting channels explains the transition noise in the negative resistance region. By a statistical mechanics argument for calculating energy fluctuations, one can show that the mean square excess current noise in a two-state system is given by the following formula, which we state here without derivation:

$$\overline{(\delta I)^2} = Ng_\Delta{}^2V^2f(V)[1 - f(V)] \tag{7}$$

The current response of a many-channel membrane to a sawtooth voltage, shown in Fig. 9, gives a qualitative indication of the prediction of equation 7 that there should be little noise when $f(V)$ is approximately unity, and a rather sharp increase in noise as $f(V)$ departs from unity in the negative resistance region.

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REFERENCES

BEAN, R. C., W. C. SHEPHERD, H. CHAN, and J. T. EICHNER. 1969. Discrete conductance fluctuations in lipid bilayer protein membranes. *J. Gen. Physiol.* **53**:741.

COLE, K. S. 1968. Membranes, Ions, and Impulses. University of California Press, Berkeley.

DEL CASTILLO, J., and E. E. SUCKLING. 1957. Possible quantal nature of subthreshold responses at single nodes of Ranvier. *Fed. Proc.* **16**:29.

FOLCH, J., and M. LEES. 1951. Proteolipids, a new type of tissue protein. *J. Biol. Chem.* **191**:807.

HILLE, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *J. Gen. Physiol.* **51**:199.

KUSHNIR, L. D. 1968. Studies on a material which produces excitability in bimolecular lipid membranes. I. Production, isolation, gross identification and assay. *Biochim. Biophys. Acta.* **150**:285.

Lüttgau, H. C. 1958. Sprunghafte Schwankungen unterschwelliger Potentiale an markhaltigen Nervenfasern. *Z. Naturforsch.* **13b**:692.

MUELLER, P., and D. O. RUDIN. 1963. Induced excitability in reconstituted cell membrane structure. *J. Theor. Biol.* **4**:268.

MUELLER, P., and D. O. RUDIN. 1968. Resting and action potentials in experimental bimolecular lipid membranes. *J. Theor. Biol.* **18**:222.

MUELLER, P., D. O. RUDIN, H. T. TIEN, and W. C. WESCOTT. 1964. Formation and properties of bimolecular lipid membranes. *Recent Progr. Surface Sci.* **1**:379.

TIEN, H. T., S. CARBONE, and E. A. DAWIDOWICZ. 1966. Formation of "black" lipid membranes by oxidation products of cholesterol. *Nature* (*London*). **212**:718.

VERVEEN, A. A., AND H. E. DERKSEN. 1968. Fluctuation phenomena in nerve membrane. *Proc. IEEE* **56**:906.