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Multiple Conductance States in Single Channels of Variable Resistance Lipid Bilayer Membranes*

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Summary. The properties of the cation-permeable excitability-inducing material (EIM) channels in lipid bilayer membranes have been investigated using membranes penetrated by only one to four active channel units. Under these conditions, individual channels may be observed to undergo on-off conductance transitions that may be correlated with the negative resistance characteristics of membranes having a large number of channels.

In many lipid membrane systems, EIM channels have several conductance levels for each channel. Such channels may open or close with either a single, large conductance step or in several smaller jumps having a sum equivalent to the large jump. The conductance of the fully closed state in such channels is generally less than 2% of the open-state conductance, too low to be differentiated from membrane conductance in the absence of an EIM channel. This ratio is reflected in the current-voltage characteristics of membranes containing large numbers of channels, where ratios of maximum conductance to minimum conductance may range from 50 to 200.

EIM channels in oxidized cholesterol bilayers are exceptional in showing only two conductance levels normally. The closed state in this system corresponds to a low intermediate state in the general channel.

The conductance of each state appears to be linearly related to the activity of the electrolyte.

Lipid bilayer membranes treated with the macromolecular substance "excitability-inducing material" (EIM) (Mueller & Rudin, 1963) may increase conductance (at low polarizing potentials) by several orders of magnitude and develop a strong negative resistance characteristic. Results of investigations by Bean, Shepherd, Chan, and Eichner (1969) and by Ehrenstein, Lecar, and Nossal (1970) have demonstrated that the EIM-stimulated conductance develops in a series of discrete, small jumps, of relatively uniform size; switching between open and closed conductance states in the negative resistance transitions also occurs in steps. These

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factors, together with other evidence, suggest that the EIM conductance and negative resistance is due to cation-conductive macromolecular bridges that may exist in either open or closed conductance states. The potential-dependent conductance transitions, such as the negative resistance, then reflect voltage-induced changes in relative populations of open and closed states of a large number of channels.

This evidence is generally compatible with the two-state channel model originally advanced by Mueller and Rudin (1963) to account for the EIM negative resistance. However, some of the evidence in the studies on discrete current jumps by Bean *et al.* (1969) seemed to suggest that each EIM channel might actually have stable conductance states intermediate between the fully open and fully closed conditions. This relation was not observed in the subsequent study by Ehrenstein *et al.* (1970) in which the channels appeared to follow a purely two-state behavior. Since the original multi-level evidence was obtained in membranes containing a relatively large number of active channels (10 to 20) where on-off switching of two or more channels of slightly different conductance, occurring simultaneously within the time resolution of the monitoring system, might produce the observed smaller conductance jumps, this question has been re-examined under conditions similar to those utilized by Ehrenstein *et al.* (1970) in which the possibility of simultaneous switching of two or more channels is minimized by utilizing membranes containing only one to three active channels.

The experimental results of these studies, presented below, appear to demonstrate that discrete conductance fluctuations of several different sizes do occur regularly in most membranes penetrated by only one or a few EIM units. The sequences of channel formation and subsequent conductance fluctuation suggest that the multiple conductance values may represent different, stable conductance levels of a single-channel unit. However, it might also be possible to consider the multiple values due to independent channels of varying size. Evaluation of the data seems to favor the first alternative, i.e., existence of multiple conductance levels in individual channel units.

Materials and Methods

Techniques and procedures have been modified only slightly from those previously described (Bean *et al.*, 1969). Membranes were formed on an aperture in a polyethylene vial supported in a thermostatted glass cell. The polyethylene vial will be referred to as the inner (electrolyte) compartment and the glass cell as the outer (electrolyte) compartment. Membranes were spread by the brush technique. Conductance measurements and EIM reactions were initiated after thinning to the black stage. EIM was added to the inner compartment only, while both compartments were continuously stirred with

magnetic fleas. Stirring was usually halted, after allowing time for uniform distribution of EIM, to minimize electrical noise during conductance observations.

To increase the probability for maintaining only a few channels in the membrane over long periods of time, relatively small apertures (0.4 to 0.8 mm^2) were used. Temperatures were maintained as low as feasible to obtain reactions, and EIM concentrations were greatly restricted.

EIM was used in one of two forms; the dried egg-white preparation originally described by Mueller, Rudin, Tien, and Wescott (1963), or a high specific activity bacterial extract previously described (Bean *et al.*, 1969).

Most lipids used in the membranes were prepared or obtained as previously described. Oxidized cholesterol, a mixture of several different substances, was prepared as described by Tien, Carbone, and Dawidowicz (1966). Tocopherol succinate and tocopherol phosphate were purchased from Mann Research Laboratories (New York) and used as received. We thank the Roche Division of Hoffmann-LaRoche Laboratories (Nutley, N.J.) for their gift of alpha-tocopherol used in some of the membranes.

Various lipid formulations were used to form membranes as described for specific experiments.

Membrane conductance was monitored through salt-agar bridges to calomel electrodes to two Keithley electrometers (model 610 B). One of these was used in the high-input impedance mode (10^{14} Ω) for potential measurement and the other in the low-input impedance mode for current measurement. Electrometer outputs were recorded on an X-Y recorder (Moseley, model 135 A) with current applied to the Y-axis and either time or potential to the X-axis.

Except where otherwise indicated, the active electrode was placed in the inner compartment and the reference (ground) electrode in the outer compartment. A positive current, produced by a positive potential, thus indicates a cationic current moving from the EIM (active) side of the membrane outward into the reference or outer compartment. A negative current indicates cationic migration into the EIM-containing compartment. It should be noted that this convention is opposite to that used in the previous report on discrete conduction fluctuation with EIM (Bean *et al.*, 1969).

Specific conditions for certain experiments are indicated in Results and Discussion.

An X-Y recorder in time mode was used for current-time curves in these experiments. Frequently, several sequential current curves were made on single chart sheet by repetitive scans across the sheet, resulting occasionally in some overlap of curves. Consequently, some of the traces presented in the report have been hand-copied (as noted) from the original chart to eliminate confusing overlapping traces.

Results and Discussion

In our original studies on discrete current steps in EIM kinetics (Bean *et al.*, 1969), two kinds of current jumps were observed. Those called "formation" steps were found during initial interaction of the EIM with the lipid bilayer, rising in relatively uniform jumps above the base conductance of the unmodified membrane, as shown in Fig. 1. Each of these uniform steps was attributed to the formation and opening of a single transmembrane bridge and cation-conducting unit. Ehrenstein *et al.* (1970) later concurred with this interpretation. After formation, the EIM conductance also decreased stepwise either spontaneously or when the polarizing potential was

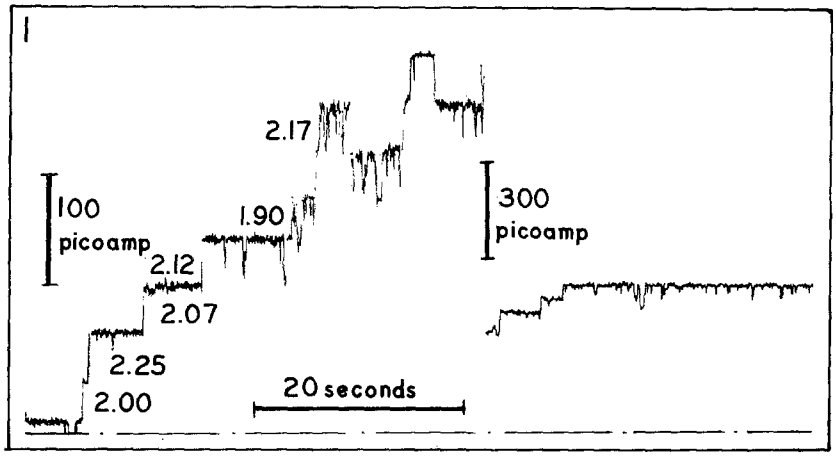

Fig. 1. Conductance jumps during early reaction of EIM with a lipid bilayer membrane. A sphingomyelin-tocopherol (5%:40% in chloroform-methanol) film was spread across an 0.7 mm aperture in a polyethylene septum and allowed to thin and develop constant resistance. The electrolyte was 1 M KCl, and temperature was maintained at 36 °C. Diluted, high-specific-activity EIM was added to the compartment containing the active electrode (positive potential indicates flow of cations outward from the EIM side of the membrane) about 30 sec before the section of the recording shown here. The membrane was polarized at +20 mV. Penetration of EIM produced 11 active channels before slowing to apparent equilibrium with little further reaction. Conductances of the first seven channels were: 2.0, 2.25, 2.07, 2.12, 1.9, 2.17 and 2.1 n-mho per channel. Upward jumps of 2.17, 3.5, 2.1 and 2.1 n-mho are observed subsequently, but some of these may be repetition due to return to open state of channels temporarily closed. Dashed line indicates zero current level

changed to favor the high-resistance state of the EIM membrane. A few reversals can be seen in Fig. 1. These may be called "switching" steps or "on-off" jumps. Ehrenstein *et al.* (1970) found that the action unit corresponding to a single formation step also continued to act as an individual unit in continued on-off fluctuations. Consequently, the action units identified by the formation steps have been designated as EIM channels or channel units, and this term will be used in the present discussion without intent to designate by this term any specific configuration or mechanism of action.

Under selected conditions (as demonstrated by Ehrenstein *et al.*, 1970), a membrane may be maintained over a long period of time with only one or a few active channels operating so that the on-off fluctuations of a single channel may be studied under varying conditions. An example of a short record for the formation and on-off fluctuations of two EIM channels in an oxidized cholesterol membrane is given in Fig. 2. This shows formation

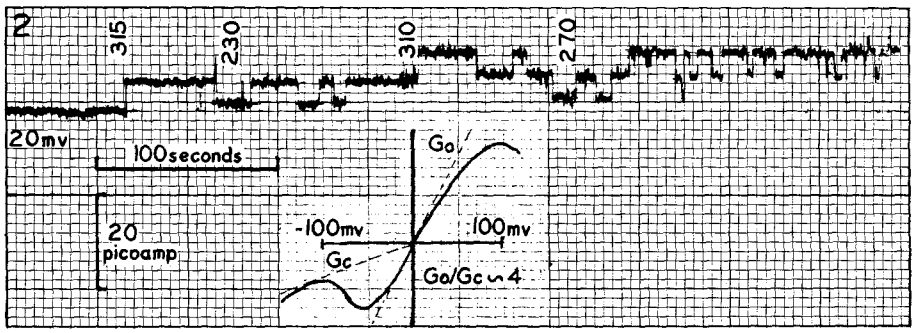

Fig. 2. On-and-off conductance jumps in an oxidized cholesterol membrane with two EIM channels. EIM was added prior to the trace reproduced here. The membrane (1 % oxidized cholesterol in nonane) was formed in 0.1 M NaCl, at 24 °C. One formation step of 315 p-mho is followed by several on-off jumps of 230 p-mho. A second channel is then introduced with a 310 p-mho step following which a 270 p-mho on-off jump is apparent in addition to the first, smaller jump. The broken line indicates the base membrane conductance. It should be noted that the recordings of channel fluctuations reproduced in this report were all made with cation flow outward from the EIM side of the membrane in order to utilize the region of maximum conductance for some of the more asymmetric membrane systems. This is opposite to the current polarities used by Ehrenstein *et al.* (1970) in their examination of EIM channel fluctuations in oxidized cholesterol membranes. However, varying the magnitude or polarity of the polarizing potentials only affected the time distributions between open and closed, or intermediate states, as outlined elsewhere in this report. *Inset:* A typical $I(V)$ curve for similar membranes with many EIM channels. Slope G_0 would be the normal open-state conductance. Slope G_c approximates the apparent closed state conductance. However, the basic bilayer membrane $I(V)$ curve shows an increasing conductance with increasing polarization and this non-linearity may be accentuated by penetration with EIM (particularly in oxidized cholesterol membranes). This factor is responsible for the increasing conductance beyond the minimum where, presumably, all EIM channels would be in the closed state

steps of 315 and 310 p-mho (reciprocal ohms $\times 10^{-12}$), and on-off jumps 230 and 270 p-mho, leaving a residual conductance of 75 and 40 p-mho in the off state for these channels. This behavior is consistent with the observations of Ehrenstein *et al.* (1970) for the same type of membrane, where they found that individual channels demonstrated a pure two-state behavior in which the off conductance was, on the average, about one-fifth the open-state conductance. This is also compatible with the current-voltage relations for an oxidized cholesterol membrane having a great many EIM channels (inset of Fig. 2) for a two-state theory in which the negative resistance behavior is attributed to a potential-dependent change in population of channels in the on or off state. For a large number of EIM channels in which the fraction of the population in the open state may be varied from

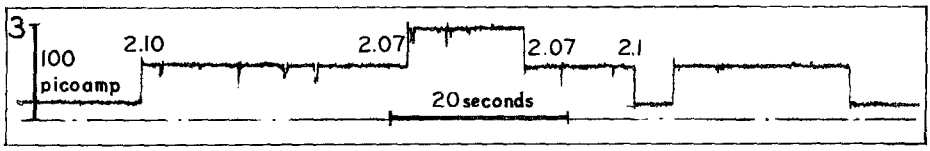

Fig. 3. Formation and on-off conductance jumps in a sphingomyelin membrane with two EIM channels. The sphingomyelin-tocopherol (5%:50% in chloroform methanol) membrane was formed in 1 M KCl at 40 °C. EIM was added about 80 sec before observation of the first jump recorded here. Recordings of current were made with constant +20 mV polarization. Two channels, with 2.10 and 2.07 n-mho conductance, were formed and then turned off with equal jumps to return to the original base conductance. This full return to near zero conductance is one form of typical transition for EIM channels in membranes of this composition. The original recorder trace is reproduced here, but with overtracing to augment contrast with background. The broken line indicates zero current level

zero to unity as a function of potential, one would expect the ratio of maximum (open) conductance to minimum (closed) conductance to be about five if the average on-to-off conductance ratio per channel averages five.

In many other membranes, however, current-voltage relations have demonstrated open-to-closed state ratios above 100 (Mueller & Rudin, 1963, 1968; Bean *et al.*, 1969). Such ratios can be obtained, in the two-state channel mechanism, with an EIM channel having a very small off-state conductance. This capability is indicated in the two-channel experiment in Fig. 3, using a sphingomyelin-tocopherol membrane in place of the oxidized cholesterol-decane system of Fig. 2. The sphingomyelin membrane with many EIM channels frequently has an open-to-closed state conductance ratio of greater than 100. In the recording traces shown here, each of the two channels, after forming, turns off to a conductance level indistinguishable from the base conductance before its formation. It should be noted that 1 M KCl was used in these experiments to increase the single-channel conductance and reduce effective noise level, but similar relations are observed at lower electrolyte concentrations. Thus, it becomes immediately obvious that the lipid milieu has sufficient effect upon the switching characteristics of individual channels to account for the changes in the observed relations for membranes having many EIM channels.

But even in the sphingomyelin membrane, a conductance level similar to that of the "closed" state of Fig. 2 (oxidized cholesterol membrane) exists, as demonstrated in Fig. 4. After the formation step, indicating a full channel conductance of 1,320 p-mho (in 1 M NaCl), conductance drops again to a level 250 p-mho above the original base level. This corresponds

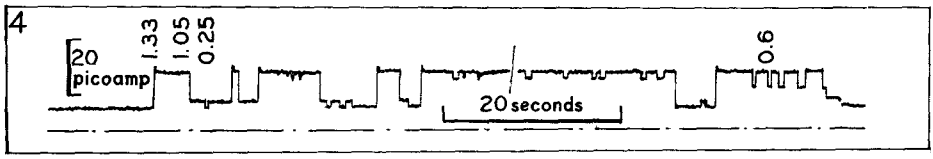

Fig. 4. Multi-level conductance transitions in a single EIM channel. Membrane was formed with sphingomyelin-tocopherol in 1 M NaCl at 40 °C. A constant +10 mV polarization was used during current recording. A single formation step of 1.33 n-mho is followed by on-off jumps of 1.05 and 0.25 n-mho. In the initial portion of the recording, the smaller jumps occur independently of one another as well as simultaneously. In a later portion of the recording of the same channel, a different set of transitions, especially one of 0.6 n-mho, may be seen, apparently due to division of the original 1.05 n-mho jump into two. Tracings are hand copies of original recordings to eliminate confusing overlap of other traces

to about one-fifth the full conductance, or just about the same level as the closed state for the channel in the oxidized cholesterol membrane. In this channel, though, it is apparent that this state is no longer the lowest state possible, since on-off fluctuations also occur between this level and the ground level for the membrane. Furthermore, it is evident in the later section of this recording that the small fluctuation can occur just below the full conductance level as well as above base level. Thus, it becomes apparent that the channel represented here acts essentially as if it were composed of two separate fluctuation units, one very small and the other much larger. Each is more or less independent of the other in its on-off fluctuations.

This behavior is not only characteristic of the EIM channel in sphingomyelin membranes, but also in most other membrane compositions (oxidized cholesterol being exceptional). Most frequently, the sequence is similar to that observed here: an initial, standard-size formation step followed by on-off fluctuations to a lower level and to the base level. The size of the intermediate, stable conduction state may vary widely, however, with values ranging from about one-tenth to one-half of the full channel conductance. Examples of extremes in the intermediate level are shown in Fig. 5. With a channel with 2,200 p-mho conductance, 780 p-mho fluctuations occur above base level.

It seems substantially clear that an intermediate conductance state, of rather variable character from one channel to another, may exist in most EIM channels and that the closed-state conductance is very small compared with that of the open-state. It would be pleasant if the situation were no more complex than this, but occasional channels are found with more than one intermediate level. Such a multi-level situation is quite apparent in the examples of Fig. 6. In sequence *A*, the initial formation jump is

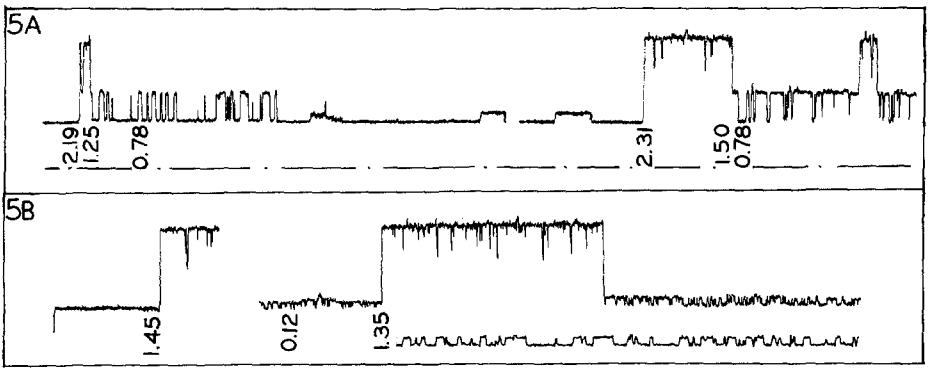

Fig. 5. Further examples of two conductance transitions in single EIM channels. (*A*) A membrane was formed from a mixture of dipalmityl lecithin and oxidized cholesterol in nonane at 48 °C in 1 M KCl. Recording was carried out under constant +40 mV polarization. An initial formation jump of 2.19 n-mho is followed by on-off transitions of 1.25 and 0.78 n-mho. A second sequence, occurring later, with a 2.31 n-mho initial jump and subsequent 1.50 and 0.78 n-mho on-off jumps may be due to a second, independent channel since it appears to differ slightly in open-state conductance, and the fully closed-state conductance, of about 0.05 n-mho above base membrane level, disappears in between the two sequences. (*B*) Very small on-off fluctuations in an EIM channel in an egg lecithin-cholesterol-tocopherol (1 % : 2 % : 20 % in chloroform methanol) membrane. The channel was formed, as shown, with a 1.45 n-mho jump (1 M NaCl, 25 °C, +20 mV polarization) and then subjected to several potential changes before the portion of the recording reproduced here. In all situations, the small, rapid fluctuation of 0.12 n-mho with a major on-off jump of 1.35 n-mho was observed. The lower inset curve shows a record of the small jumps with doubled time scale and slightly expanded current scale

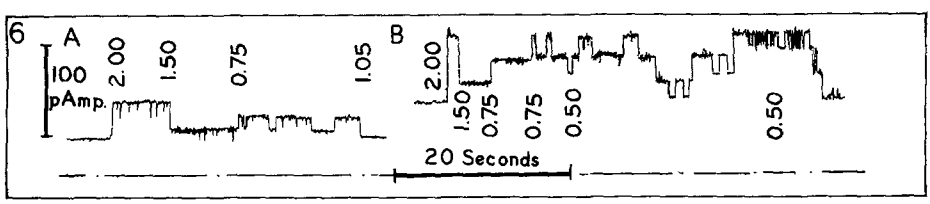

Fig. 6. Triple-level conductance transitions in a single EIM channel. (*A*) A sphingomyelin-tocopherol membrane in 1 M KCl at 40 °C. The formation jump of 2.00 n-mho is followed by a sequence showing stable levels at 0.50 and 1.2 n-mho above base conductance. Polarization was at constant +20 mV during recording. Direct reproduction of original trace. (*B*) Same channel under 30 mV constant polarization, after negative polarization caused switching-off. The same intermediate levels and transitions as in (*A*) may be observed, but a different sequence of transitions also gives rise to new levels above base, indicating that all three transitions may occur more or less independently. The rapid on-off sequence at the end of this trace is frequently found in a given channel and may last for several minutes. The original recording trace was hand-copied to eliminate an overlapping trace

divided, upon subsequent on-off fluctuations, into a small section of about one-fourth the maximum conductance, with two approximately equal larger states of three-eighths the fully open state. The same set of divisions is found in sequence *B*, for the same channel at a higher polarizing potential, but with some inversions of order of fluctuation that suggest that all three transitions occur independently, as well as in unison.

The demonstration of multiple levels in EIM channels poses some questions about the roles that the separate states might play in the transitional kinetics of membranes having many EIM channels. Do the various states differ in mechanisms or selectivity of ion transport? Is there any difference in the potential dependency of conductance in the different states? How are transitional time constants affected by additional states?

An examination of variation in conductance with ion concentration for EIM channels with intermediate conductance levels (Fig. 7) demonstrated that the conductance of each state within limits of experimental variability is linear with the activity of the electrolyte. There was substantial scatter of data for the intermediate levels since a different membrane was required for each concentration, but the variations were generally related to the individual channel characteristics (as indicated above) rather than to specific concentration effects. The linearity suggests that conductance is not limited by space charge in any state.

The ionic selectivity relations are considerably more difficult to obtain and will be the concern of a later paper. Preliminary measurements with ion concentration gradients or biionic gradients (KCl, NaCl) seem to indicate that the potential difference across the channel remains constant in all states, but some discrepancies in time of development of the EIM channel and observation of an effective diffusion potential make evaluation of the responses difficult.

We have attempted to determine, through adaptation of the time statistics method of Ehrenstein *et al.* (1970), if the on-off fluctuations of the different conductance levels show different responses to polarizing potentials. A sphingomyelin-tocopherol membrane was used in these studies. Since the EIM channels have some tendency to turn off irreversibly at negative potentials with the sphingomyelin preparation presently in use, a small amount of glyceryl mono-oleate was added to improve reversibility. After development of one or two channels in a membrane, the membrane was polarized at different potentials and the relative time spent in each conductance state was observed over a period of 3 to 10 min at each potential. Observations for channels that turned off completely at negative potentials (zero time in open state) were not included in the statistical evaluations unless

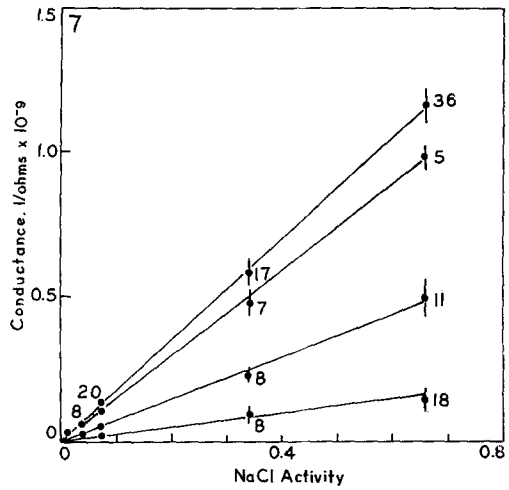

Fig. 7. Variation in conductance of EIM channels and sub-levels with electrolyte concentration. Observations were made with sphingomyelin-tocopherol membranes. Membranes were formed in NaCl solutions of indicated activity. At least two membranes were tested at each concentration used. Recordings were made at constant potential as long as only a single active channel was apparent. A +40 mV polarization was generally used, but +60 or even +80 mV was used with 0.02 and 0.05 M NaCl to increase jump size and facilitate measurement. The plots of step measurements showed clusters of points at four different levels at the three higher concentrations, corresponding to expected levels for a "four-state" channel. These clusters are represented as single points with the vertical bar showing standard deviation. At lower concentrations, certain clusters overlapped and could not be distinguished as different transitions statistically, even though the differentiation might be clear in the original recording sequence. Numbers adjacent to the points indicate the number of transitions observed and included in calculations for that point

there was a rapid reappearance of on-off fluctuations upon return to a positive polarizing potential. Five membranes, each with no more than two channels, were used.

Results, shown in Fig. 8, are compared with voltage-conductance relations for a membrane with many EIM channels. It appears, from these curves, that there is a significant difference in the effect of potential on the fluctuations of the two states of the channel. Although there is a marked tendency for the channels to turn off both levels at the more negative potentials, the small state seems prone to stay on much longer than the larger state at the positive potentials.

It is notable that even at the potentials for optimal conductance in the many-channel membrane, the individual channel appears to be in fully open state only slightly more than half the time in these experiments. This

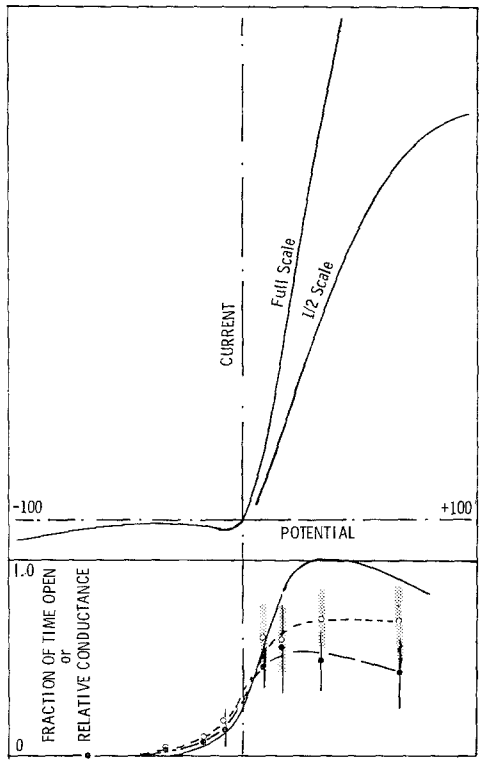

Fig. 8. Effect of polarizing potential on fraction of time in open condition for each state of an EIM channel and comparison with conductance-potential relations for a membrane with many EIM channels. (*A*) Steady-state current-voltage curve for a sphingomyelin-tocopherol membrane with more than 500 EIM channels. Electrolyte is 1 M KCl, at 42 °C. (*B*) Conductance vs. potential for many-channel membrane (solid line) and fraction of time open vs. potential for small conductance state (open circles) and large conductance state (filled circles) for individual EIM channels. The membranes were generated in 1 M KCl from sphingomyelin-tocopherol solutions containing a small amount of glyceryl-mono-oleate. The latter constituent was added to counteract the tendency of EIM channels in the sphingomelin membranes to close irreversibly when subjected to negative potentials over a long period of time. KCl tends to inhibit the division of the large conductance state (ca. 4/5 of full channel conductance) into two smaller states so that only the two transitions, ca. 1/5 and 4/5 of full channel, are usually observed in addition to the full channel jump.

Membranes and EIM channels were formed under +40 mV polarization. After one or two channels were formed, varying polarizing potentials were applied and observations at each potential carried out over a period of 3 to 10 min. The fraction of time in the open state for each of the two states at each potential was determined from analysis of the curves and plotted here. Five membranes with eight channels were used. Statistics obtained at negative potentials where channels closed completely (zero time in open state) were not used unless the channels showed a rapid return to open state upon return to positive potentials. Standard deviations are indicated by the hatched bars and vertical lines on the points

might have been expected from earlier studies on the effect of temperature on EIM current-voltage relations (Bean & Chan, 1969). The EIM membrane under constant potential polarization may show a steep negative temperature coefficient for conductance, even in regions for optimal conductance. This was attributed, then, to a shift in equilibrium between open and closed states due to the effect of temperature on a reaction with a small, positive free energy (considering the forward reaction as the transition from open to closed state). Thus, at elevated temperatures, even at potentials for optimal conductance one might expect that the equilibrium should be shifted well in favor of the closed state. These studies were carried out at 42 °C, at which a substantial shift toward the closed state might be expected for the sphingomyelin system.

Conclusions

There can be little doubt, if the original assumption (that the large formation jump defines the EIM channel) is maintained, that the EIM channel can exist in several (more than two) different conductance states. It can be argued that the formation jumps actually represent the development of an assembly of different channels of rather variable characteristics, but this argument seems more relevant to a debate about the mechanisms responsible for the multilevel conductance—whether multiple path, multiple conformation, or variable association—than to a decision about the definition of the channel. The observations, in general, are entirely consistent with a unit concept in which the macromolecular complex inserts into and through the lipid barrier to form ion-conducting units that may have several discrete conductance levels, with a high degree of interaction and cooperativity in the processes that cause transitions between conductance levels.

As a consequence of the conclusion that the EIM channel does exist in multiple states, certain questions arise. How does the three-or four-state condition affect the two-state theory for EIM kinetics developed by Mueller and Rudin (1963) and extended by Ehrenstein *et al.* (1970), and how may one mechanistically account for several stable conductance levels within a single-channel unit?

It does not appear that the multiple levels should have a profound effect on qualitative aspects of EIM switching kinetics under the two-state theory. The major assumptions of the two-state concept remain valid. The potential-dependent variation in membrane resistance is still due to the effect of a polarizing potential on the equilibrium between two (or more) different conducting states of a large number of channels rather than to a

graded change in resistance within each channel as a function of potential. However, some perturbation must arise from the apparent difference in potential response of the different states (e.g., Fig. 8) and in the failure of the first conductive state to turn off at all in oxidized cholesterol membranes. It seems reasonable to assume that the difference in energetics of the different conductive states might eventually be used to account for a number of rather interesting behavioral aspects of the EIM membranes, such as variable hysteresis (Bean, Shepherd & Eichner, 1971), multiple negative resistance regions (Bean *et al.*, 1971), and tri-stable states (Mueller & Rudin, 1968). However, such correlations must await further developments on rate constants, potential-dependence relations, temperature coefficients, and specific effects of lipids in the membrane and of solutes in the aqueous phase.

On the question of mechanisms for developing several conductance states in an EIM channel unit, information is still inadequate to allow realistic evaluations of many models. It may be reasonable, however, to suggest some guidelines.

Any model must be based largely on transport via a macromolecular bridge in which protein is essential for maximum conductance and the ion mobility is minimally affected by the specific nature or charge of the lipids of the membrane; i.e., the channel is essentially contained within the macromolecular complex (Mueller & Rudin, 1968; Bean *et al.*, 1969). On the other hand, as established here, the lipid of the membrane has a significant effect on the size of intermediate conductance states and the on-off transitions. In addition, the lipids have a profound effect on switching kinetics and equilibria between open and closed states (Mueller & Rudin, 1968; Bean & Chan, 1969; Bean *et al.*, 1971). Models based on either a multi-path or multi-configurational process should be compatible with existing information. However, since each of the sub-channels in a multi-path system must be subject to some configurational transition (where "configuration" may encompass the total arrangement of a macromolecular complex and closely associated membrane lipids) to develop open and closed states, this may be treated as one of the many potential forms of multi-configurational mechanisms. Within these limits, many different arrangements could be conceived that should provide within the complex several interchangeable states of minimal energy with small energy barriers between states. The different configurations may greatly alter the energy barrier at some point in the channel for ions traversing the channel. Selection between the diverse choices must await development of further information.

The full significance of the multi-level conductances in kinetic functions still remains to be determined. However, recent studies of the quantal

fluctuations in univalent salt media with small amounts of multivalent cations and/or protamine indicate that the slight divergence of the low conductance level from the remainder of the channel in its potential dependent switching (*cf.* Fig. 8) may be strongly accentuated under the influence of these additives under certain circumstances (*report in preparation*). Initial evaluation of these phenomena suggests that the intermediate levels could be responsible for some of the multi-stable states found by Mueller and Rudin (1968) in EIM membranes treated with protamine.

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