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VOLTAGE-CLAMP EXPERIMENTS ON OXIDIZED CHOLESTEROL MEMBRANES MODIFIED WITH EXCITABILITY-INDUCING MATERIAL AND COMPARISON WITH A MODEL

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(Received April 21st, 1976)

SUMMARY

The conductance of oxidized cholesterol membranes modified with excitability-inducing material was observed in membranes containing either single conductance channels or 100–1000 channels. Membranes containing single channels have several conductance states for each voltage polarity, and the current through membranes containing many channels decays with at least two, and probably three, time constants following a step change in voltage (voltage-clamp). The time constants differ by about an order of magnitude. The multi-state behavior seems more pronounced in membranes made from highly oxidized cholesterol. Although a given conductance state could occur at either positive or negative voltages, each state was much more frequent at one polarity or the other. The most frequently observed single-channel conductance states in 0.1 M NaCl are about 0.3, 0.1, 0.03, and 0.0 $n\Omega^{-1}$ for negative voltages and 0.25, 0.05, 0.03, and 0.0 $n\Omega^{-1}$ for positive voltages. The current following a voltage clamp decays to a quasi-steady state within 1 min for positive voltages and 1–15 min for negative voltages. When the holding voltage is −20 mV, the decay constants and quasi-steady state conductances as functions of clamping voltage are reasonably well described by either a three-state model of the conductance or a two-state model applied independently at negative and positive voltages. However, for high voltages, the quasi-steady state does not appear to approach a state in which all the channels are in a low conductance state.

INTRODUCTION

The conductance of bilayer lipid membranes can be increased several orders of magnitude by modifying the membranes with "excitability-inducing material" [1]. The conductance induced by this material exhibits negative resistance regions, and, under certain conditions, action potentials similar to those found in some excitable cells have been produced [1, 2].

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Bean et al. [3] have presented convincing evidence that the conductance properties of membranes so modified are a result of the formation of discrete, proteinaceous, trans-membrane channels which have two or more well-defined conductance states. Ehrenstein et al. [4], Latorre et al. [5], and Ehrenstein et al. [6] have investigated the properties of these channels in oxidized cholesterol membranes. For a single voltage polarity, only two distinct channel conductance states were generally observed in these membranes, and the ratio of the high to low conductance was approximately 5: 1. Bean [7] observed that in other membrane preparations the channel had three or more conductance states at a given voltage polarity. One of the low conductance states corresponded approximately to the low conductance state observed in oxidized cholesterol membranes, and a nearly zero conductance state was also frequently observed.

In membranes containing many channels of excitability-inducing material the conductance change following a step change in potential (voltage clamp) has been observed by Bean [8] to follow an exponential rise or decay involving one or two decay constants. Ehrenstein et al. [6] found that for oxidized cholesterol membranes the conductance change following a step change from zero to some higher voltage was an exponential decay with a single decay constant. They also determined the voltage dependence of the decay constants for one voltage polarity and found a good correlation between the decay constants in membranes containing many channels and the average time that single channels existed in either the high or low conductance state. The transition rates for the opening and closing of the channels was found to depend exponentially on the voltage across the membrane.

In this paper we report on observations of voltage-clamped oxidized cholesterol membranes modified with excitability-inducing material which have several rather than two conductance states at a given voltage polarity. We first present evidence of the multistate conductance behavior of the membranes containing either a single channel or several hundred channels. Next, we consider the kinetics for the conductance transition following a step change in voltage. The decay constants for the transition to a quasi-steady state and the relative conductance of this state were determined for a range of negative and positive clamping voltages, and the experimental results are compared with models. Finally, we speculate on the types of models for the channel which are consistent with the experimental data.

MATERIALS AND METHODS

Oxidized cholesterol was prepared according to a procedure given by Tien [9]. A sample of the oxidized cholesterol was further oxidized for 8 h and will be referred to as reoxidized cholesterol. Membranes were formed from oxidized or reoxidized cholesterol dissolved in decane which was applied to a 1.0 mm or 1.6 mm diameter aperture in the septum of a chamber filled with 0.1 M NaCl buffered to pH 7.0 with 5 mM histidine. Excitability-inducing material was prepared according to Bean et al. [3] and Ehrenstein et al. [4]. The general procedures for making and modifying the membranes and for making electrical measurements were similar to those used by other investigators [1, 3, 4, 9, 12] with the following exceptions pertaining to most experiments. The salt solution in the chamber was first brought to 32 °C. A membrane was then formed and preconditioned by applying trans-membrane voltage of 80 mV for 3–15 min. Membranes that were electrically leaky or noisy at this stage were eliminated. Next, an aliquot of excitability-inducing material was added to the solution bathing one side of the membrane and was allowed to react until the membrane conductance reached the desired value. The temperature was then lowered to the working temperature, which was controlled to ±0.1 °C. Excitability-inducing material that still remained in solution was removed by perfusion.

Current transients following a step change in membrane voltage were recorded on a transient recorder of our own design [10]. Positive current and voltage are defined by cation flow into the compartment containing excitability-inducing material. Additional experimental details are available elsewhere [11].

RESULTS AND DISCUSSION

A. Multi-state behavior of EIM-modified membranes

In our initial experiments with oxidized cholesterol membranes modified with excitability-inducing material, we attempted to duplicate some of the results obtained by Ehrenstein et al. [4, 6] and Bean [7, 8] in their studies of oxidized cholesterol membranes containing many channels. Our observations were in general agreement, with two exceptions. First, the conductance did not attain a steady value with constant applied voltages in the range 50–80 mV but continued to decrease slowly. Second, the current produced by a slow (0.1–0.5 mV/s) triangular wave voltage showed more hysteresis than that observed by Bean [8]. Observation of the slow decrease in conductance under constant voltage conditions was complicated by the fact that the membranes always broke in less than 10 min if the applied potential was 60 mV or greater, and observations in general were complicated by the fact that in these early experiments the excitability-inducing material was not removed from solution and continued to react with the membrane during an experiment.

A statement by Bean [8] that very low conductance states in oxidized cholesterol membranes are more likely if the ratio of cholesterol to oxidized

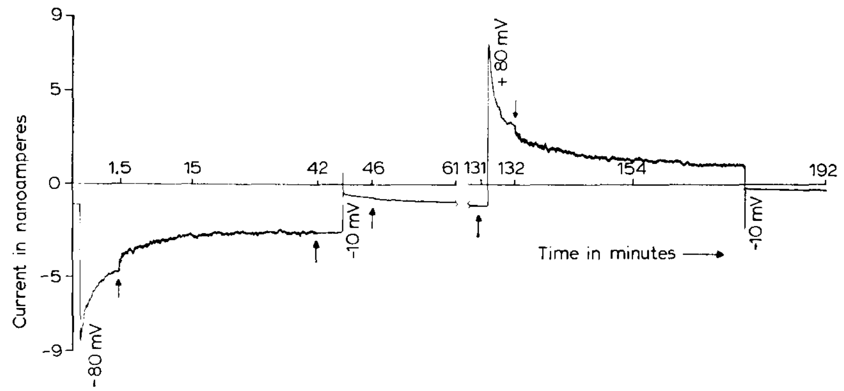

Fig. 1. Conductance behavior during long-duration voltage-clamp. Vertical arrows denote points at which the recorder speed was changed. The membrane was formed from a saturated solution of reoxidized cholesterol in decane. Temperature was 22 °C.

cholesterol is high, suggested that the oxidized cholesterol initially prepared required further oxidation in order to produce the "two-state" channel observed extensively by Ehrenstein et al. However, membranes made from the reoxidized cholesterol did not reproduce the Ehrenstein observations. Furthermore, reoxidized cholesterol membranes modified with excitability-inducing material showed the same hysteresis effects as did oxidized cholesterol membranes, and in single-channel experiments several low conductance states were observed, the lowest of which is indistinguishable within experimental error from the conductance of the unmodified membrane. The modified reoxidized cholesterol membranes were generally much more stable at high voltages than modified oxidized cholesterol membranes. However, we noted an aging effect on reoxidized cholesterol similar to that reported by Yafuso et al. [12].

Fig. 1 is an example of the conductance behavior of modified reoxidized cholesterol membranes subjected to prolonged high voltage. Following a step change in voltage from -10 to -80 mV the current appeared to have nearly reached a steady value after approximately 1.5 min. The chart recorder speed was then reduced by a factor of six (arrows in the figure indicate points at which the chart speed was changed), and the current was observed to continue decreasing with a time constant about 10 times that of the initial change. Upon returning the voltage to -10 mV the current increased, apparently with two time constants, to its initial value and beyond. A part of this current increase was due to continued slow reaction of excitability-inducing material with the membrane. Similar behavior is observed at $+80$ mV, but in this case the conductance decrease is essentially irreversible. Similar records were observed in many other membranes which lasted up to 11 h. It was also demonstrated on several membranes that switching to -80 mV after a long polarization at $+80$ mV caused almost no recovery from the conductance decrease produced at $+80$ mV. Following the transition to -80 mV the current immediately began to decrease. Thus the $+80$ mV potential produced a seemingly irreversible change in conductance. Results similar to those shown in Fig. 1 were also observed for membranes made from various mixtures of oxidized and reoxidized cholesterol.

The multi-state behavior of reoxidized cholesterol membranes was conclusively demonstrated in single-channel experiments. Many levels were observed for both positive and negative polarizations, although the actual conductance of various states varied slightly among different channels. Although a given conductance state could occur at either positive or negative voltages, each state was much more frequent at one polarity or the other. The conductance states most frequently observed at negative clamping voltages were approximately 0.3, 0.1, 0.03, and 0 $n\Omega^{-1}$, and at positive voltages approximately 0.25, 0.05, 0.03, and 0 $n\Omega^{-1}$. A zero conductance state means that the channel conductance was not distinguishable within experimental error from the conductance of the unmodified membrane. Fig. 2a shows that for small negative voltages a channel is nearly always in a state with conductance 0.3 $n\Omega^{-1}$, while at small positive voltages the normal state has a conductance of about 0.25 $n\Omega^{-1}$. Fig. 2b shows several conductance levels at positive polarization. The sudden absence of fluctuation in the zero conductance state is a common phenomenon in these membranes, but this probably does not indicate removal of the channel because a return to a higher conductance state is frequently observed upon changing to a lower voltage or a voltage of opposite polarity. However, the duration of polarization at large voltages seems to affect the ability of a channel in the zero

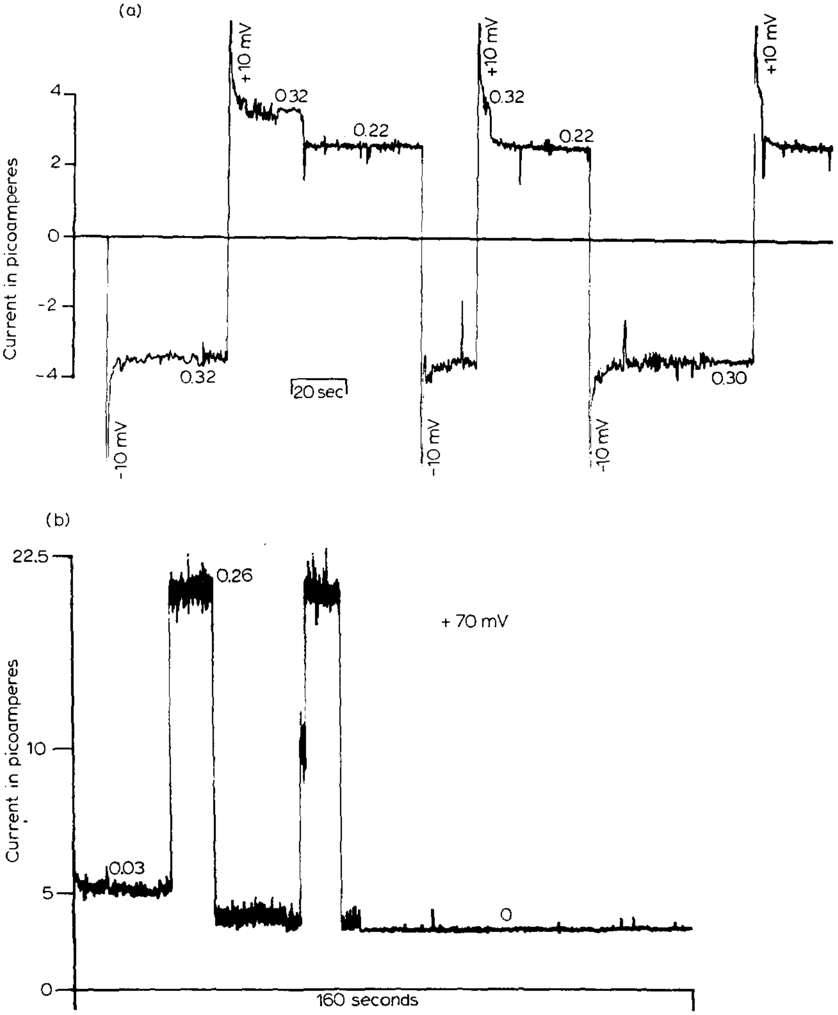

Fig. 2. Single channel conductance states. In (a) and (b) the channel conductance (measured conductance minus the conductance of the unmodified membrane) at selected levels is given in units of nΩ^{-1}. The noisy level of 10 pA in (b) is unusual and may represent a rapid fluctuation between high and low levels that was too fast to be followed by the recorder. Both (a) and (b) are records of a single membrane formed from reoxidized cholesterol in decane 10 mg/ml. Temperature was 25 °C.

conductance state to return to a higher conductance state. Channels have been observed which, when forced into the zero conductance state by a large positive voltage lasting several minutes, could not be brought to a higher conductance state even after prolonged polarization at negative voltage up to the membrane's breakdown voltage. This would seem to explain the irreversible decrease in conductance observed in membranes containing many channels. Multiple conductance states as well as a zero conductance state are also observed at negative voltages, as shown in Fig. 3.

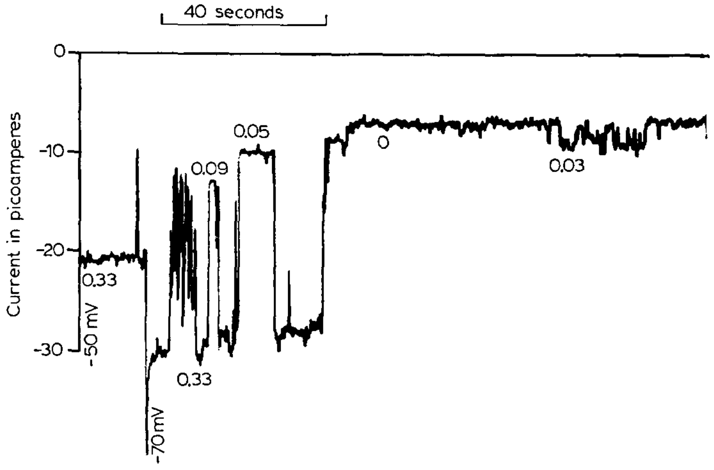

Fig. 3. Single channel conductance states. This is a record of the current of a single channel at −70 mV. Several conductance states are apparent although the channel is in the zero conductance state most of the time. The membrane was formed from a saturated solution of reoxidized cholesterol in decane, and the temperature was 24 °C.

The quantity of our data on single channels is not sufficient to allow a quantitative comparison between the statistical behavior of individual channels and the behavior of membranes containing many channels. The data are sufficient, however, to demonstrate that, for a given voltage polarity, the channels are multi-state rather than two-state systems and that the irreversible transition to low conductance states in many-channel membranes is probably due to channels going to near-zero conductance states, from which a return to higher conductance states is improbable.

B. Determination of decay constants and relative conductance

Because of the essentially irreversible state induced by prolonged polarization at large positive and (to a smaller degree) large negative voltages, an initial attempt to make a systematic study of both the short and long time constants of modified, reoxidized cholesterol membranes was abandoned. However, since the time constants involved differ by about an order of magnitude, it was thought that if only short-duration voltage pulses were used one could reasonably assume that a quasi-steady state would be reached at both positive and negative voltages. And it was thought that the time dependence for the transition to the quasi-steady state as well as the quasi-steady-state conductance might be described by a three-state model such as developed by Arndt and Roper [13].

To test these assumptions, a series of voltage-clamp experiments on modified reoxidized cholesterol membranes was performed using a temperature of 25 °C. The holding voltage (membrane voltage prior to a step change in voltage) was −20 mV, approximately the voltage at which these membranes had the maximum conductance. For each voltage-clamp experiment the time-dependent current data were first fitted to the function

$$I(t) = I_\infty + I_d \exp(-\alpha t), \tag{1}$$

where $I(t)$ is the current as a function of time, I_∞ is a constant equal to the current at infinite time, I_d is the difference between I_∞ and the current at $t = 0$, and α is a decay constant. The fitting was done by the standard method of least squares [14], i.e. minimizing χ^2 by varying the parameters in the function, e.g. I_∞, I_d, and α in Eqn. 1. The experimental errors on the data were primarily due to apparently random fluctuations about a smooth average behavior; this error was estimated by observing the transient current signal on a storage oscilloscope and assigning the error a value of 2/3 the maximum fluctuation from the average.

These fits to the data were generally good. However, for many of the voltage-clamp experiments at voltages of 40 mV or greater the best fits to Eqn. 1 consistently gave values for the current at short times that were smaller than the corresponding experimental currents. This seemed to indicate that a better fit to the data could be obtained by using two exponential factors, one of which had a decay constant approximately equal to that obtained from the fit to Eqn. 1 and the other of which had a larger decay constant. In attempting to fit the data using two exponential factors we applied the constraint that the current at $t = 0$, $I_0(V)$, was held at a constant value given by

$$I_0(V) = I(V_h) \cdot \frac{V}{V_h},$$

where $I(V_h)$ is the current at the holding voltage V_h and V is the clamping voltage. The function used for this fit was

$$I(t) = a\,[\exp(-\alpha_1 t) - 1] + b\,[\exp(-\alpha_2 t) - 1] + I_0, \tag{2}$$

where a, b, α_1, and α_2 were the variable parameters. There was general improvement in the fits when Eqn. 2 was used rather than Eqn. 1, but at a given voltage there was considerable scatter and often large uncertainties in the best-fit parameters from different experiments. This has made it difficult to extract physically meaningful information by comparing the parameters with a model. Fig. 4 compares the fits obtained using Eqns. 1 and 2 to fit the data from a particular experiment.

In order to compare all the data simultaneously with a model, all parameters having the unit of current were divided by $I_0(V)$ to give dimensionless, normalized parameters. For the case of $I_\infty(V)$, the normalized parameter is the relative conductance, g, given by

$$g = \frac{I_\infty(V)}{I_0(V)} = \frac{I_\infty(V)}{I(V_h)} \cdot \frac{V_h}{V} = \frac{g_\infty(V)}{g(V_h)} \tag{3}$$

The uncertainty in g, Δg, comes from an uncertainty in both $I_\infty(V)$ and $I_0(V)$. The uncertainty in I_∞, and in all other parameters which are varied in fitting a function using the least squares method, has the meaning that, if the parameter is varied by the uncertainty while varying all the other parameters to minimize χ^2, then χ^2 increases by 1. The uncertainty in $I_0(V)$ is due to experimental uncertainty in determining $I(V_h)$, V, and V_h; the resultant uncertainty in $I_0(V)$ was estimated as 1 %.

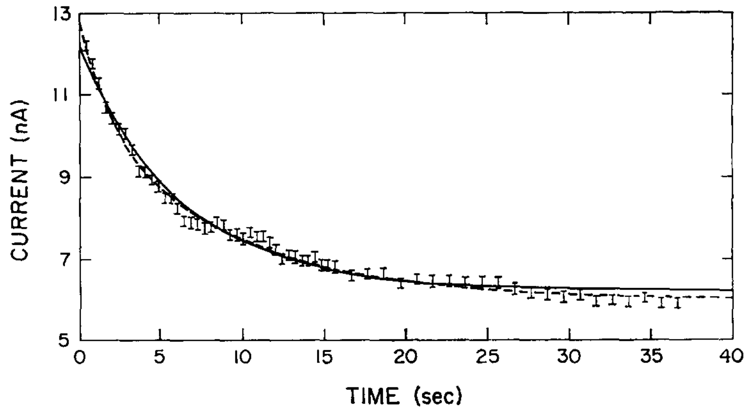

Fig. 4. Comparison of one and two decay constant fits. The solid curve is the best fit to the transient current using Eqn. 1, and the dashed curve is the best fit to the same data using Eqn. 2. The experiment was performed with a clamping voltage of +90 mV.

The uncertainty in g resulting from the uncertainties in $I_\infty(V)$ and $I_0(V)$ was calculated by the standard formulas for propagation of errors.

Thus for each voltage-clamp experiment, parameters α, g, etc. and their errors were determined. As described in the next section, the values of these parameters for all of the experiments at each particular clamping voltage were averaged for comparison with theoretical models.

Table I lists the weighted averages of α and g for the one-exponential fits, and α_1, α_2, g, $A = a/I_0$, and $B = b/I_0$ for the two-exponential fits. Note that for the two-exponential fits we have, as required, $g+A+B = 1$.

C. Comparison with models

The first model with which we will compare the experimental data is the three-state model of Arndt and Roper [13], henceforth referred to as the Arndt and Roper model. In this theory, the three conductance states considered are a high conductance state and two low conductance states; one of the low states is associated with negative voltages and the other is associated with positive voltages. The energies of and barriers between states are assumed to be of the form $U_i = \varepsilon_i - \vec{p}_i \cdot \vec{E}$, where U_i is the energy of the ith energy level or barrier measured relative to an arbitrary reference, ε_i is an intrinsic energy, $\vec{p}_i$ is a dipole moment, and $\vec{E}$ is the electric field.

The expression for the average steady-state mobility at voltage V resulting from the Arndt and Roper theory is [13]

$$u(V) = \frac{u_+ \exp(-\varepsilon_+ + p_+ V/w) + u_0 + u_- \exp(-\varepsilon_- - p_- V/w)}{\exp(-\varepsilon_+ + p_+ V/w) + 1 + \exp(-\varepsilon_- - p_- V/w)} \tag{4}$$

where u_+ is the mobility when all channels are in a low conductance state at large positive voltage (+state), u_0 is the mobility when all channels are in a high conductance state (0 state), u_- is the mobility when all channels are in a low conductance state at large negative voltages (−state), ε_+ and ε_- are the intrinsic energies of the

TABLE I

WEIGHTED AVERAGES OF PARAMETERS FROM EXPONENTIAL FITS

The relative conductance, g, has been calculated from the fits using Eqn. 3, and A and B are the coefficients a and b, respectively, of Eqn. 2 divided by I_0. The first column gives the clamping voltage and the second column the number of experiments whose parameters are averaged at each voltage.

V (mV)	No. of expts.	One decay constant		Two decay constants				
		g	α (s^{-1})	g	A	α_1 (s^{-1})	B	α_2 (s^{-1})
+10	8	0.9529±0.0067	0.094 ±0.011	0.9456±0.0048	0.0132±0.0013	0.128 ±0.017	0.0402±0.0047	0.0507±0.0096
+20	9	0.9129±0.0033	0.168 ±0.025	0.9062±0.0030	0.0349±0.0030	0.38 ±0.13	0.0581±0.0051	0.079 ±0.024
+30	10	0.8836±0.0060	0.094 ±0.015	0.8966±0.0057	0.0499±0.0014	1.92 ±0.27	0.0524±0.0051	0.128 ±0.029
+40	13	0.8730±0.0089	0.083 ±0.012	0.8633±0.0096	0.0763±0.0022	1.15 ±0.15	0.0587±0.0079	0.0618±0.0061
+50	14	0.800 ±0.022	0.0538±0.0085	0.8583±0.0091	0.0581±0.0033	0.619 ±0.064	0.0854±0.0088	0.074 ±0.010
+60	10	0.673 ±0.031	0.082 ±0.012	0.731 ±0.014	0.1226±0.0062	0.357 ±0.17	0.158 ±0.011	0.072 ±0.010
+70	7	0.550 ±0.035	0.0971±0.0082	0.515 ±0.031	0.147 ±0.028	0.218 ±0.035	0.319 ±0.031	0.0676±0.0080
+80	5	0.546 ±0.015	0.1442±0.0063	0.514 ±0.013	0.146 ±0.031	0.329 ±0.044	0.341 ±0.026	0.0986±0.0073
+90	4	0.4869±0.0051	0.181 ±0.013	0.4628±0.0040	0.1877±0.0035	0.5454±0.0060	0.3495±0.0005	0.1080±0.0040
+100	4	0.473 ±0.021	0.210 ±0.012	0.5095±0.0085	0.1923±0.0063	0.68 ±0.20	0.300 ±0.016	0.1768±0.0049
+110	2	0.4566±0.0088	0.271 ±0.026	0.4450±0.0061	0.1822±0.0079	2.331 ±0.061	0.3752±0.0055	0.212 ±0.018
+120	2	0.429 ±0.015	0.348 ±0.032	0.415 ±0.022	0.259 ±0.082	1.25 ±0.35	0.323 ±0.061	0.223 ±0.077
−40	3	0.879 ±0.012	0.0060±0.0020					
−50	3	0.6378±0.0040	0.0080±0.0018					
−60	3	0.541 ±0.018	0.0280±0.0014					
−70	5	0.480 ±0.034	0.073 ±0.011					
−80	3	0.5799±0.0048	0.0506±0.0029					
−90	2	0.566 ±0.012	0.0760±0.0050					
−100	2	0.543 ±0.018	0.076 ±0.022					
−110	2	0.5651±0.0061	0.1490±0.0041					
−120	2	0.5940±0.0091	0.136 ±0.012					

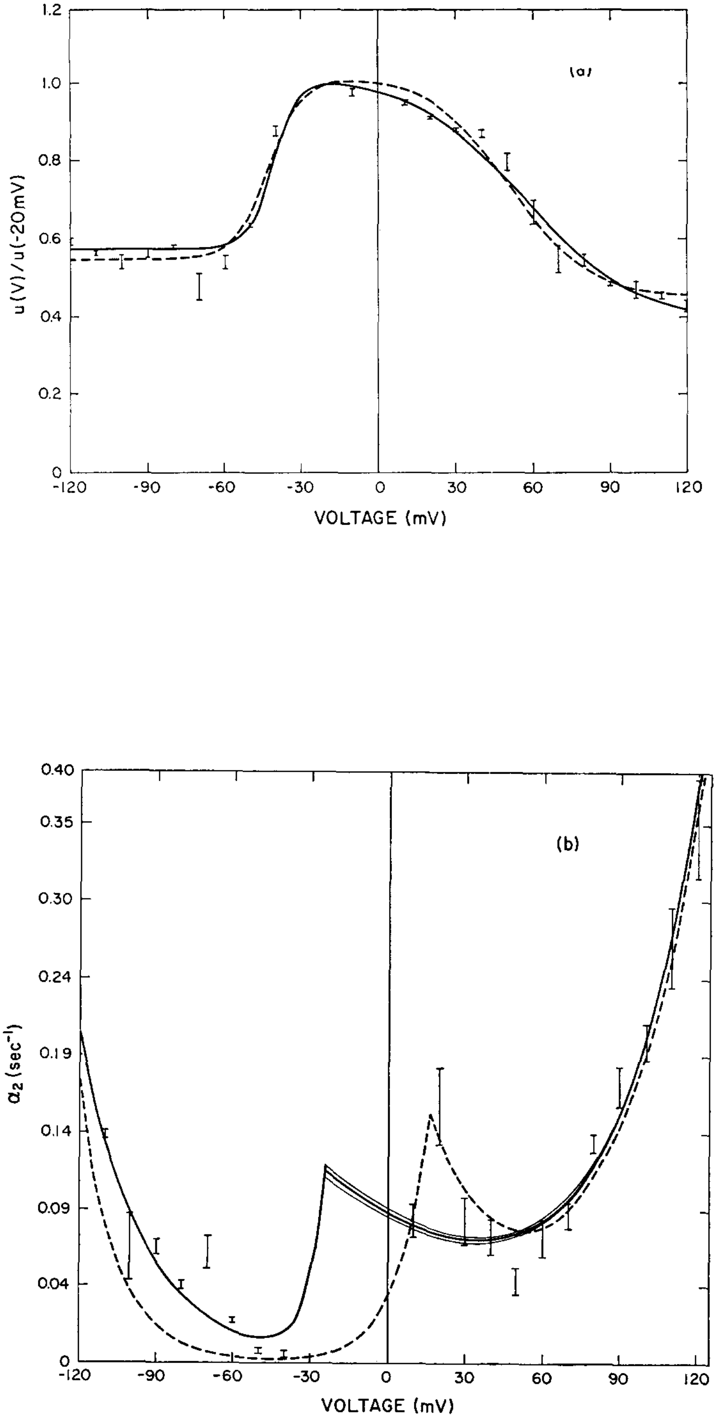

Fig. 5. For legend see page 791.

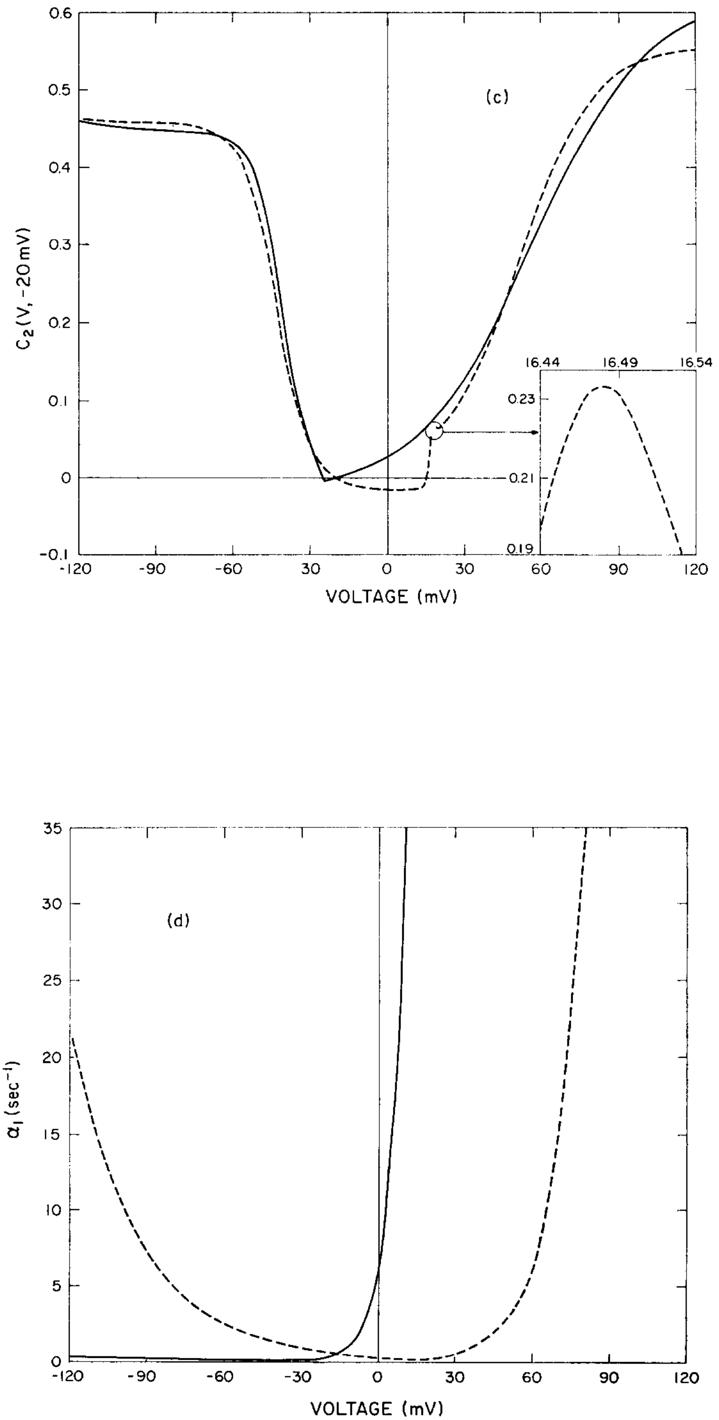

Fig. 5. For legend see opposite page.

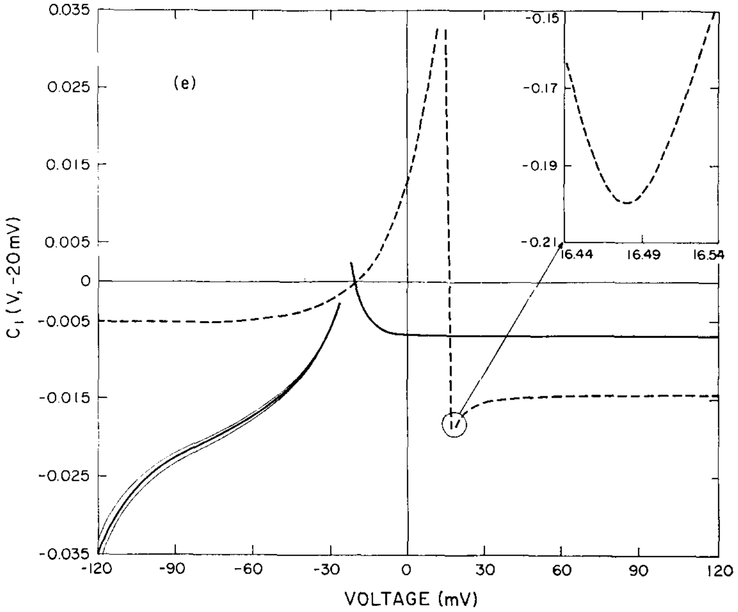

Fig. 5. The solid curve in (a) and (b) are the best fits to the experimentally determined relative conductance and decay constant (α_2). The flanking solid curves in (b) indicate the error channel as calculated from the error matrix of the fit to the model. The error channel is too small to be shown in (a), (c), and (d). The dashed curve is a less probable fit in which the model's α_2 peak is forced to occur near where the data appear to peak. (c), (d), and (e) are the predictions of the model for the other parameters in Eqn. 5. The coefficients C_1 and C_2 are normalized to the initial current, I_0. The effect of the second larger decay constant, α_1, is small for this holding voltage (−20 mV). At other holding voltages it can be important.

TABLE II

PARAMETERS FOR BEST FITS TO THEORIES

In the theories, $\varepsilon_\pm$ have units of kT, and $p_\pm$ and $\bar{p}_\pm$ have units of kT/mV. A temperature of 300 °K was assumed for expressing these units as eV and electronic charge, e, respectively.

Parameter	Arndt and Roper theory	Two-state theory applied to each polarity
u_+/u_0	0.376 ±0.016	0.39 ±0.01
ε_+ (eV)	0.064 ±0.002	0.075 ±0.010
p_+/w (e)	1.11 ±0.05	1.3 ±0.2
a_+ (s^{-1}) ★	0.0074±0.0007	0.0060±0.0005
$\bar{p}_+/w$ (e) ★	0.83 ±0.03	0.90 ±0.02
u_-/u_0	0.558 ±0.004	0.586 ±0.003
ε_- (eV)	0.21 ±0.02	0.34 ±0.01
p_-/w (e)	5.2 ±0.5	8.0 ±0.2
a_- (s^{-1}) ★	0.0019±0.0002	0.0015±0.0001
$\bar{p}_-/w$ (e) ★	1.02 ±0.02	1.10 ±0.03

★ See Arndt and Roper [13] for the meaning of these parameters.

+ and − states respectively, w is the thickness of the membrane, and p_+ and p_- are dipole moments. For a step change in voltage from the holding voltage V_h to the clamping voltage V at time $t = 0$,

$$u(V, V_h, t) = u(V) + C_1(V, V_h)\exp[-\alpha_1(V)t] + C_2(V, V_h)\exp[-\alpha_2(V)t] \qquad (5)$$

where the coefficients C_1 and C_2 are functions of both the holding and clamping voltages. The decay constants α_1 and α_2 and the steady-state mobility $u(V)$ are functions only of the clamping voltage.

We will compare the parameters α and g from Eqns. 1 and 3, obtained by fitting the original data with a single exponential factor, with the Arndt and Roper model. In fitting the relative conductance to the Arndt and Roper model it was assumed that the high conductance states for positive and negative voltages could be grouped as a single state, and the low conductance states at each polarity could also be grouped into single states. Since the relative conductance at the holding voltage (−20 mV) is by definition 1, this point was added to the data array with sufficiently small error to assure that the fitted value would also be 1. For all other voltages the weighted averages of g and α were calculated at each voltage, where the weighting is based on the errors described previously. The error ΔA assigned to the weighted average of each parameter was calculated as

$$\Delta A = f\left[\sum_i \frac{1}{e_i^2}\right]^{\frac{1}{2}},$$

where e_i is the error on the ith value of the parameter being averaged and f is a multiplicative factor which causes the χ^2 of the "best fit" weighted average to be 1. The factor f was included to take into account the possibility that the errors had originally been underestimated. Its effect is to increase the errors on each data point to take into account the scatter in the data. Table I gives the weighted averages of the parameters obtained from fitting the current transients.

The relative conductance in the Arndt and Roper theory is given by $g = u(V)/u(V_h)$. There are two decay constants in this theory, and we have chosen the smaller of the two to compare with α. The reason for this choice is that the larger decay constant is, according to the model, only important when the holding to clamping voltage change occurs across both negative-resistance regions.

The best fit obtained by fitting the weighted average g and α parameters simultaneously using the Arndt and Roper theory is shown as the solid curve in Fig. 5. The values for the various parameters in the theory are given in Table II. The dashed curve is a less probable fit in which the model's α_2 peak is forced to occur near where the data appear to peak. The fit to both g and α is reasonably good, but it must be interpreted with caution for three reasons. First, the leveling of the relative conductance curve to the values u_+/u_0 and u_-/u_0 does not occur at a relative conductance corresponding to any of the frequently observed single channel conductances. Considering the fact that the conductance reached only a quasi-steady state in our voltage-clamp experiments, the parameters u_+/u_0 and u_-/u_0 should more properly be interpreted as average conductance states of an ensemble of channels at an intermediate stage in the decay of the conductance. Second, α and g at small positive voltages may be influenced by transitions from a negative-voltage high conductance state to a positive-voltage high conductance state. This is particularly evident in the leveling of the g data between +20 and +40 mV (see Fig. 5a), which suggests an intermediate state for positive voltages. However, the fit was not significantly changed when α and g for clamping voltages of +10 to +30 mV were removed from the data set. The third factor which might be considered is the possibility that partial breakdown of the membrane at the highest voltages produced a leakage conductance in parallel with the conductance of the channel, thus giving an erroneously large conductance. This is unlikely, however, because the membranes in these experiments remained apparently stable for many hours, and the membranes for which the highest clamping voltages were used became unstable only during attempts to apply a clamping voltage of ±130 mV. An effect indistinguishable from membrane leakage could also be produced if an appreciable fraction of the channels had significantly slower rate constants for transitions to closed states. Occasional channels have been observed which only infrequently exist in low conductance states even at voltages of 50 mV or greater. The evidence, however, is not sufficient to permit an estimate of the importance of an inhomogeneity in the channel population. Another possibility is that cooperative effects among channels cause different conductances than those seen in single channels.

At our holding voltage, single channels were observed to be nearly always in a high conductance state. Thus, applying a clamping voltage of a given polarity would be expected to cause transitions mainly from a high conductance state to low conductance states for that polarity. The theoretical description of g and α for positive clamping voltages should then be independent of the theoretical parameters describing the energies of and barriers between the negative conductance states and vice versa. To check this possibility g and α were fitted using the two-state model of Ehrenstein et al. [6] applied independently for positive and negative voltages. The details for converting parameters in this two-state model to those of the Arndt and Roper theory are given elsewhere [11]. The results of the fits, are given in Table II.

As described in Section B above the data are slightly better fitted by two exponentials than by one. In later work we shall attempt to fit the two-exponential fit parameters with a three-state model that has as its three states the low negative-polarity high-conductance state, a low positive-polarity high conductance state, and a high positive-polarity low-conductance state for positive clamping voltages or a high negative-polarity low-conductance state for negative clamping voltages. We would also like to obtain data for holding to clamping voltage values that span the entire voltage range so that we can attempt to fit such data with four or five-state models.

CONCLUSIONS

The modified oxidized cholesterol membrane has been observed to be a multi-state conductance system. The prominance of near-zero conductance states seems to be greater for more highly oxidized cholesterol. The existence of several conductance states in a single channel gives rise to several decay constants in the decay of the conductance for many-channel membranes following a step change in voltage. Since some of the time constants are very large, a quasi-steady state can be reached, and the decay to this quasi-state is reasonably well described by a single decay constant.

The voltage dependence of the decay constant and quasi-steady state relative conductance were reasonably well described in terms of models in which the energy levels of and barriers between the conductance states of the channels depend linearly on the voltage across the membrane. This voltage dependence could represent either a dipole interaction with the electric field or a charge-gaining energy by moving through the electric field. Since several of the parameters in the models have physical interpretations as energies or dipole moments it would be tempting to proceed immediately to consider what types of molecular structures could have approximately the same energy states and dipole moments that the models predict for our system. One should be cautious, however, because our system does not have all the properties required in these simple two or three state models.

In particular, the channels in our membranes have many, rather than three conductance states, and it would seem advisable to first try to understand why the three-state model represents the experimental data as well as it does, or, conversely, why these membranes behave approximately like three-state systems. It must be kept in mind that only a quasi-steady state was reached in our voltage-clamp experiments, and the effect this has on a theory should be estimated. A further fact requiring justification is that u_+/u_0 and u_-/u_0 in the Arndt and Roper theory do not appear, for our experiments, to represent ratios of the conductance (or mobilities) of single-channel states. This is not surprising when one considers the quasi-steady-state approximation we have made, but it suggests that a further effort should be made to understand what this approximation means.

If we assume that the parameters from fits to the models do realistically describe energy levels and barriers in a channel, then some restrictions can be placed on the kinds of interactions which are possible between the molecular structure of the channel and the electric field. The intrinsic energy levels $\varepsilon_\pm$ are the same order of magnitude as hydrogen bonds, and they are easily explained by many possible structural models. The dipole-related terms, however, seem to be too large to be explained by models in which a small part of the total structure of the channel interacts with the electric field through a $\vec{p} \cdot \vec{E}$ interaction. As an example, p_-/w is $5e$ to $8e$. If we take the length of the channel, w, to be 50 Å, the minimum dipole moment of the structure which interacts with the electric field is about 1000 D (Debye). A typical amino acid has a dipole moment of about 2 D, and Urry [15] has calculated the maximum dipole moment per amino-acid residue of certain channel-forming polypeptides to be about 1 D. The molecular weight of excitability-inducing material has been estimated at $2 \cdot 10^4$ to $1 \cdot 10^5$ [16, 17], in which case there would be about 200–1000 amino acid residues per molecule. Thus, if one assumed an average dipole moment of about 1 D for each amino acid residue, one would also have to assume that all the dipole moments in the molecule are aligned parallel in order to explain the dipole moment of 1000 D given above. If this were the case, the structure interacting with the electric field would be the entire molecule. This requirement might be met by a model, described by Arndt and Roper, in which the channel is lined with the negative ends of dipoles. It is also possible that a channel is a complex formed from subunits. A further possibility is that the large dipole moment is indicative of some kind of cooperative behavior between many channels, but this is unlikely if one accepts the voltage dependence of rate constants obtained by Ehrenstein et al. [6].