INTERACTIONS OF VOLTAGE-SENSING DYES WITH MEMBRANES

III. Electrical Properties Induced by Merocyanine 540

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ABSTRACT The effects of merocyanine 540 on the electrical properties of lipid bilayer membranes have been investigated. The alterations this dye was found to produce in the intrinsic conductances of these membranes were minimal, but it profoundly altered the conductances produced by extrinsic permeant species. These alterations were much larger for neutral membranes than for negatively charged ones. The dye increased the conductances mediated by positively charged permeant species and decreased those by negatively charged permeant species, suggesting that it produces a negative electrostatic potential on the membrane; it also altered the kinetics and the voltage dependencies of permeation by these charge carriers. The magnitudes of dye-mediated conductance changes were much larger for positively charged permeants than for negatively charged ones; also, changes in ionic strength altered these dye effects in opposite directions from those predicted by the Stern equation, and the dependence of the conductance alteration on dye concentration was steeper than that predicted by this equation. Finally, only very small changes in liposome zeta potentials were induced by the dye. Calculations show that a large fraction of these effects can be accounted for by the dipole potential produced by merocyanine at the membrane surface, but that additional effects of the dye must be postulated as well.

MEMBRANE INTERACTIONS OF VOLTAGE-SENSING DYES

Merocyanine 540

A number of dyes have been found that change their spectral properties in response to a change in transmembrane potential (Tasaki et al., 1968; Cohen et al., 1974). The mechanisms underlying the voltage responses of these dyes have been investigated, and in some cases reasonable models have been deduced (Conti et al., 1974; Waggoner and Grinvald, 1977; Dragsten and Webb, 1978). Less well characterized have been the effects of the dyes on the membranes' electrical properties and the dependence on membrane and aqueous compositions of the dyes's responses. McLaughlin and Harary (1976) have shown that binding of ANS[1] and TNS to lipid bilayer membranes is accompanied by a change in the membrane's electrostatic potential. Previous studies in this series (Krasne, 1980*a* and *b*) have shown that the thiadicarbocyanine dyes can alter both the conductances and electrostatic potentials of lipid bilayer membranes; in addition, it was shown that the adsorption of dye to the membrane and the state of aggregation of membrane-bound dye were dependent upon the membrane's surface-charge density and the ionic strength of the aqueous phase.

One of the dyes most commonly used for reporting rapid changes in transmembrane potentials in single cells is merocyanine 540 (MC 540) (referred to as "dye I" by Cohen et al., 1974). This dye was the first one reported that was able to monitor a single action potential in squid axon (Davila et al., 1973) and has been used in a wide variety of studies, including monitoring electrical activity in nerve (Salzberg et al., 1973), heart muscle (Salama and Morad, 1976), skeletal muscle (Landowne, 1974; Nakajima et al., 1976; Vergara and Bezanilla, 1976), and differentiating between transformed and normal hemopoietic and erythropoietic cells (Schlegel et al., 1980; Williamson et al., 1981).

The mechanism by which MC 540 reports changes in transmembrane potential has been studied by Dragsten and Webb (1978). They found that an electric field causes MC 540 to rotate within the membrane thereby establishing a new equilibrium between monomers of the dye, which were either parallel or perpendicular to the plane of the membrane, and dimers, which were only parallel. By postulating different quantum efficiencies and absorption

[1] *Abbreviations used in this paper:* ANS, 1-anilinonaphthalene-8-sulfonate; TNS, 1-toluidino-naphtalene-8-sulfonate; MC 540, merocyanine 540; PE, bacterial phosphatidylethanolamine; PC, dioleoylphosphatidylcholine; diPPC, diphytanoylphosphatidylcholine; PS, bovine phosphatidylserine; TØB, tetraphenylborate.

coefficients for the monomers in each of these orientations (and recognizing that dimer fluorescence was virtually quenched), these authors were able to account for the change in fluorescence of MC 540, following a change in potential across the membrane.

Thus far, no systematic studies have been reported examining whether MC 540 alters the electrical properties of membranes. The present paper presents the results of studies on the effects of MC 540 on the electrical properties of lipid bilayer membranes.

MATERIALS AND METHODS

Materials

Nonactin was a gift from Ms. Barbara Stearns (Squibb Institute for Medical Research, New Brunswick, NJ) and tetranactin a gift from Dr. Hans Bickel (CIBA); merocyanine 540 was purchased from Eastman Chemical, Div. of United-Guardian Corp., Smithtown, NY, and sodium tetraphenyl borate (TØB) from Aldrich Chemical Co., Inc., Milwaukee, WI. Stock solutions of these compounds were made up in ethanol and stored in the refrigerator. Aliquots of these solutions were added to the aqueous solution during an experiment, the final concentration of ethanol never exceeding 1%. The lipids bacterial phosphatidylethanolamine (PE), dioleoylphosphatidylcholine (PC), diphytanoylphosphatidylcholine (diPPC), and bovine phosphatidylserine (PS) were purchased from Avanti Biochemicals, Birmingham, AL, and were used without further purification. Stock solutions of lipids for forming planar bilayer membranes consisted of 25 mg/ml lipid in decane.

Electrical Measurements

The techniques of planar bilayer membrane formation, conductance, and current-voltage measurements were as described in Krasne (1980*a*). Measurement of the kinetics of the current decay following the application of a voltage step across the membrane followed the techniques of Laprade et al. (1975) except that the current was measured using a virtual ground amplifier (Analog Devices, Inc., Norwood, MA, 48K) configuration with variable feedback resistances. The amplifier output was then fed either to a Nicolet digital oscilloscope (Nicolet Instrument Corp., Madison, WI) and thence to an *X-Y* recorder, or it was fed to a Data General (Westboro, MA) Nova 3 computer and analyzed. In the former case, the values of the recorded points were determined by hand, and time constants were calculated from the straight lines drawn by eye through the logarithms of the points; in the latter, curve fitting for time constants, amplitudes, and total charge movement was done using a Levenburg-Marquardt algorithm for the nonlinear least-squares fit program.

Microelectrophoresis

Microelectrophoresis experiments were carried out on a Rank Bros. Mark II microelectrophoresis apparatus and were as described previously (Krasne, 1980*b*). Multilamellar vesicles for these experiments were formed by the method of Bangham et al. (1974).

RESULTS

Merocyanine-induced Steady State Conductances

Because MC 540 is negatively charged and lipophilic, it is important to determine whether it introduces significant extrinsic conductance pathways into membranes. At 0.1 M KCl and 30 μM MC 540, the conductance of PE bilayers was increased by only two orders of magnitude (to $<2 \times 10^{-6}$ S/cm^2), and at lower concentrations of salt and dye, the conductance increase was lower. Thus, it is unlikely that MC 540 itself acts as a significant carrier of ionic current in most biological membranes, whose intrinsic conductances are typically much higher than that introduced by MC 540 in bilayers (although exceptions might occur in some high-resistance membranes, e.g., mitochondria).

Merocyanine-induced Electrostatic Potentials

A second perturbation that could be introduced into biological membranes by MC 540 is a change in their electrostatic potentials. This change could come about by the introduction of a net change in the double-layer potential due to adsorbed charges at the membrane surface, a change in the surface dipole potential due to either adsorbed dipoles (intrinsic to MC 540), reorientation of the membrane's polar head groups, or a boundary potential arising from the adsorption of MC 540 at a plane within the membrane phase. If any of these phenomena were to occur, they could be sensed from the effect of MC 540 on the bilayer conductances induced by positively and negatively charged lipophilic ions and/or ion carriers (McLaughlin et al., 1971, Szabo et al., 1972). Fig. 1 shows the effect of MC 540 on the nonactin-K^+–mediated conductances in neutral (PE) and negatively charged (PE/PS 1:1) membranes. These data are consistent with an alteration in electrostatic potential by MC 540, and, considering that MC 540 is negatively charged, they are what would be expected if this dye simply adsorbed to the membrane's surface and produced a more negative surface

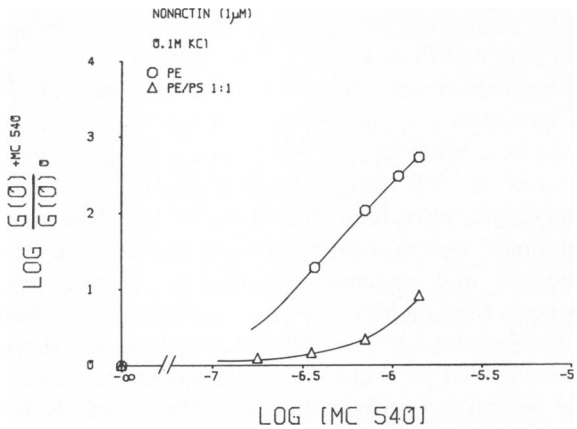

FIGURE 1 MC 540-induced conductance changes for nonactin-K^+ in neutral (PE) and negatively charged (PE/PS 1:1) bilayers. The ordinate is the logarithm of the steady state, nonactin-K^+–mediated conductance normalized to that in the absence of MC 540. The abscissa is the logarithm (base 10 in all figures) of the concentration of MC 540 added to the aqueous phases on each side of the membrane. See text for explanation. In all figures, lines are drawn to indicate the trends in the points.

potential.[2] The reduction in this effect seen in PE/PS bilayers is consistent with the reduction in surface concentration for MC 540 expected to result from this membrane's more negative surface potential.[3]

To determine that the effect of MC 540 was on the membrane's electrostatic potential and not on such parameters as fluidity, thickness, or dielectric constant, the effect of the dye on oppositely charged probes was examined. The results, plotted in Fig. 2, show that MC 540 altered the membrane conductances produced by oppositely charged probes in opposite directions, consistent with an effect of the dye on the membrane's electrostatic potential. There were, however, several anomalies associated with interpreting these data in terms of MC 540 simply altering the double-layer potential by changing the surface charge density on these bilayers. First, the apparent electrostatic potential alterations produced by MC 540 were much larger for positively charged probes than for negatively charged probes. Second, the dye-induced electrostatic potentials increased with increasing ionic strength, but they should have decreased (according to the Stern equation; see McLaughlin and Harary, 1976). Third, the changes in probe conductance with added MC 540 were steeper than those predicted by simple Gouy-Chapman diffuse double-layer theory (or the more general Stern equation; McLaughlin and Harary, 1976), as seen especially for data at 0.1 M ionic strength. That these anomalies are most likely accounted for by "discrete charge" (or possibly "discrete dipole") effects of MC 540 in the plane at which it is adsorbed to the membrane will be argued in the Discussion.

If the electrostatic potential changes induced by MC 540 are due to an alteration of the surface charge of the membrane by this dye, then one should observe zeta potentials produced by MC 540 that are comparable, at a given dye concentration and ionic strength, to the potentials inferred in Fig. 2. (Aiuche and Kobatake, 1979, observe no change in the zeta potentials in similar experiments; however, their membranes had a significant negative-charge density.) Zeta potentials produced by the

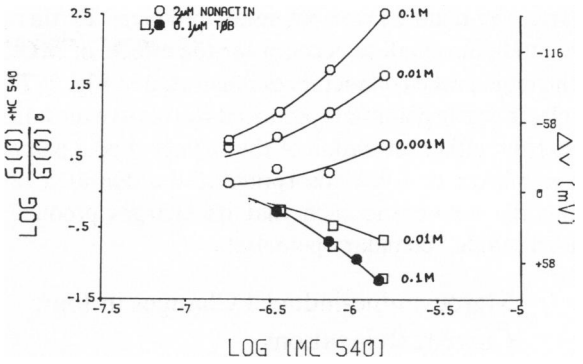

FIGURE 2 MC 540-induced conductance changes for neutral and negatively charged permeant species in bilayers. The left-hand ordinate is the logarithm of the conductance of the indicated species normalized to that in the absence of MC 540, and the abscissa is the same as in Fig. 1. The salt concentration is indicated to the right of each curve; the solutions were unbuffered KCl for the nonactin measurements and NaCl for the TØB measurements. For nonactin, the steady state conductances are plotted; for TØB, the instantaneous conductances are indicated. The right-hand ordinate is the hypothetical electrostatic potential differences between the midpoint of the membrane and the bulk aqueous phase (calculated according to the Boltzman equation as in Szabo et al., 1972) necessary to account for the MC 540 effect on conductance if the effect of this dye were solely on the membrane's electrostatic potential. ○ indicate measurements made on PE bilayers; □ denote those made on diPPC. Note the agreement for TØB for the two bilayer compositions.

adsorption of MC 540 to liposomes were measured by microelectrophoresis experiments, and the results are shown in Fig. 3. It is seen that only very small, but measurable, zeta potentials were produced by this dye; these potentials increased with decreasing ionic strength.

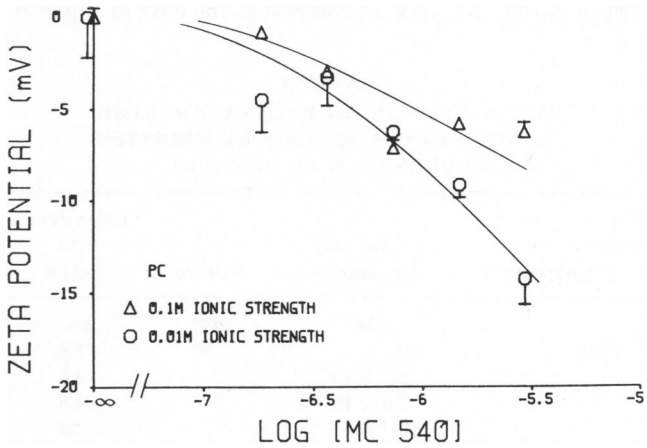

FIGURE 3 MC 540-induced zeta potentials in neutral liposomes. The ordinate is the electrostatic potential at the plane of shear (i.e., the zeta potential) of multilamellar, dioleoylphosphatidylcholine (PC) liposomes referred to the electrostatic potential in the absence of MC 540. The abscissa is the logarithm of the MC 540 concentration. The standard error for each point is indicated by the bars. Note that the zeta potential increases with decreasing ionic strength. The zeta potentials in the absence of dye were −0.075 (±0.53) mV at 0.1 M ionic strength, and −5.9 (±2.2) mV at 0.01 M ionic strength. These data could not be fit with the Stern equation (see McLaughlin, 1977), and the solid curves have been drawn simply to indicate trends.

[2]It is unlikely that MC 540 is acting to increase the conductance of cation-carrier complexes by forming ion pairs, as discussed by Stark (1980), since in that case, the steady-state current should decrease with increasing ion-carrier concentration, and the relaxation time for the ionic current should decrease with increasing MC 540; neither of these effects was observed.

[3]The shift seen is in the direction expected for adsorption of a negative molecule to the membrane, although the magnitude of the shift, of ~0.83 orders of magnitude along the concentration axis, is only half that predicted by Gouy-Chapman-Stern theory using the K^+ binding constant for PS determined by Eisenberg et al. (1979). The divergence of this data from that predicted theoretically may be due to a small negative surface charge density on the PE, but it is more likely due to whatever (unknown) factors are responsible for the same type of nonideal behavior seen in the zeta potential measurements (cf. Fig. 3).

Clearly, the double-layer potentials produced by this dye were much too small to account for the effects of MC 540 on the ionic probe conductances illustrated in Fig. 2. Thus, the electrostatic potentials produced by this dye most likely arose from either alteration of the surface dipole potential of the bilayer or from adsorption of the dye at a plane within the membrane such that its charges produce an "unscreenable" boundary potential.

Merocyanine-induced Changes in Ionic Current Relaxations

Currents mediated in PE bilayers by the ion carriers nonactin and tetranactin are time invariant after the capacitative transient associated with the charging of the membrane (which in the present case had time constants varying between 3.25 and 5 μs); but TØB, at micromolar and lower concentrations, induced an additional current relaxation having a time constant, at low voltages, of ~2 ms in PE bilayers (Andersen and Fuchs, 1975) and of 75 ms in diPPC bilayers. When the time dependences of ionic currents induced by these ionophores were examined in the presence of MC 540 and in 0.1 M ionic strength solutions, they were found to be altered. In all cases, current relaxations were slowed considerably with increasing MC 540 concentration while the capacitative transient for membrane charging remained relatively unaltered (i.e. <50% change). Typical values for time constants in PE bilayers are given in Table I. In addition, the time constants for the MC 540-induced current relaxations were decreased with increasing transmembrane voltage, as illustrated in Fig. 4 *A* for cation carriers in PE bilayers and in Fig. 4 *B* for TØB in diPPC bilayers. In contrast to the data of Fig. 4 *B*, at 0.01 M ionic strength, MC 540 causes a negligible change in the time constants for TØB current relaxations. In principle, the MC 540-induced changes in current relaxations could arise either from a change in the MC 540-induced electrostatic potential on the membrane with time (as, for example, might accompany the voltage- and time-dependent reorientation of this dye), or from an effect of the dye-induced electrostatic potential on the kinetics of the ion-carrier or lipid-soluble ion permeation process. The fact that the time constants for the current relaxation are a function of the current-carrying species argues that one is not simply observing the kinetics of a change in the MC 540-induced electrostatic potential. In addition, by calculating the integral of the current, one can show that MC 540 is actually altering the concentration of the charged,

TABLE I
MC 540 EFFECTS ON RELAXATION TIME CONSTANTS FOR IONOPHORE-MEDIATED CURRENTS IN PE BILAYERS

Ionophore*	MC 540 concentration	Voltage	Relaxation time constant
	M	*mV*	*ms*
TØB$^-$	0	50	1.9
	3.5×10^{-7}		3.0
	7.0×10^{-7}		4.8
	10^{-6}		7.4
	1.4×10^{-6}		13.0
			μs
Nonactin-NH_4^+	0	200	<5
	1.4×10^{-6}		33
	2.8×10^{-6}		49
Tetranactin-Rb^+	0	200	<5
	1.4×10^{-6}		25.5
	2.8×10^{-6}		38

*Salt concentrations were 0.1 M. Ionophore concentrations were 10^{-7} M for TØB$^-$, 10^{-6} M for nonactin, and 10^{-6} M for tetranactin.

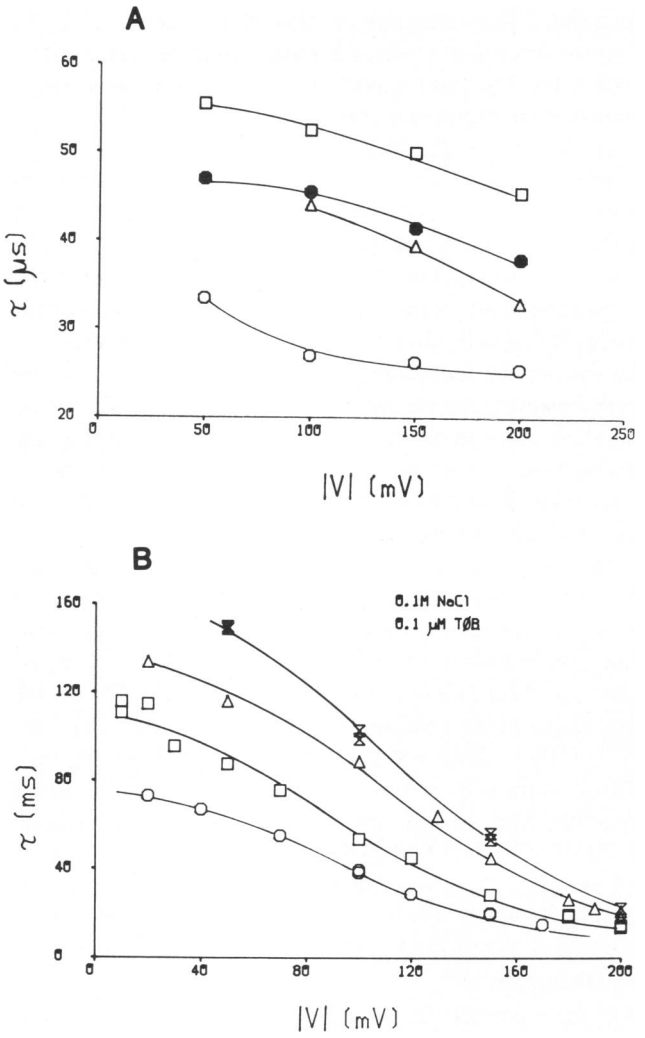

FIGURE 4 The voltage dependence of the time constants for MC 540-induced relaxations of ionophore currents in neutral bilayers. Time constants for the MC 540-induced current relaxations are plotted as the ordinate; the size of the voltage step is indicated on the abscissa. (*A*) Relaxation time constants for nonactin- and tetranactin-mediated currents in PE bilayers. Open symbols represent data collected with 1.4 μM MC 540, filled symbols for 2.8 μM MC 540. (*B*) Relaxation time constants for TØB-mediated currents in diPPC bilayers. MC 540 concentrations were 0 (○), 0.37 μM (□), 0.75 μM (△), and 1.5 μM (⧗).

permeant species near the membrane-solution interface, as is shown for TØB in Fig. 5. In this figure it is seen that the magnitude of the charge movement increased with increasing absolute voltage until a saturation value was reached above about ±150 mV. This saturation value, which represents the total charge near the membrane-solution interface that is available to move, decreased with increasing MC 540 concentration. This result is expected if MC 540 alters the electrostatic potential on the membrane. Interestingly, the data for the effects of MC 540 on TØB charge movement at 0.1 and 0.01 M (not shown) ionic strengths were virtually the same, in contrast to those for the time constants of current relaxation. An interpretation of these ionic strength effects will be proposed in the Discussion.

Alterations in Steady State Conductance-Voltage Relationships by MC 540

MC 540-induced alterations in the conductance-voltage (G-V) behaviors of cation-carrier complexes were consistent with the production of a boundary potential or alteration of the surface dipole potential. Addition of MC 540 caused a decrease in slope of the G-V relationships mediated by nonactin and tetranactin in PE bilayers, as illustrated in Figs. 6, 7, and 8. This effect was larger (or occurred at a lower MC 540 concentration) the slower the ion-carrier dissociation rate (cf. Krasne and Eisenman, 1976). For example, the effect was more pronounced for a particular ion when complexed with tetranactin than with nonactin (Fig. 6), and, for a given carrier, the effect was more pronounced for NH_4^+ and K^+ than for Rb^+ (cf. Figs. 8 and 6 *B* or 7 *A* and 6 *A*). These observations are remarkably similar to what is seen when comparing the G-V behaviors of nonactin and tetranactin in PE and glycerol

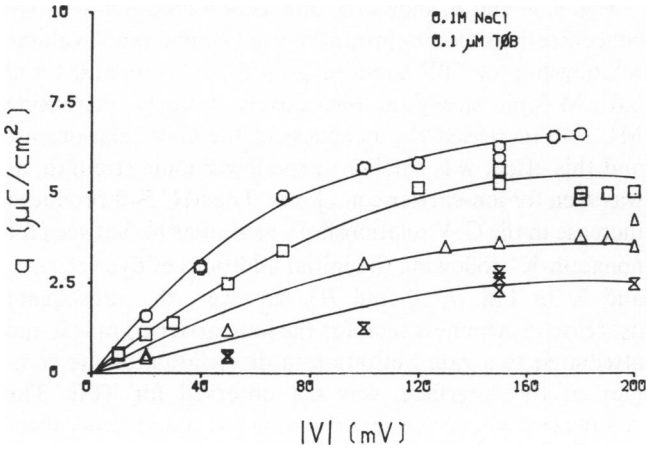

FIGURE 5 The effect of MC 540 on the adsorption of TØB to diPPC bilayers. The ordinate represents the integral of the current due to TØB, following voltage steps to the absolute values of voltages indicated on the abscissa. MC 540 concentrations were 0 (○), 0.37 μM (□), 0.75 μM (△), and 1.5 uM (⧗).

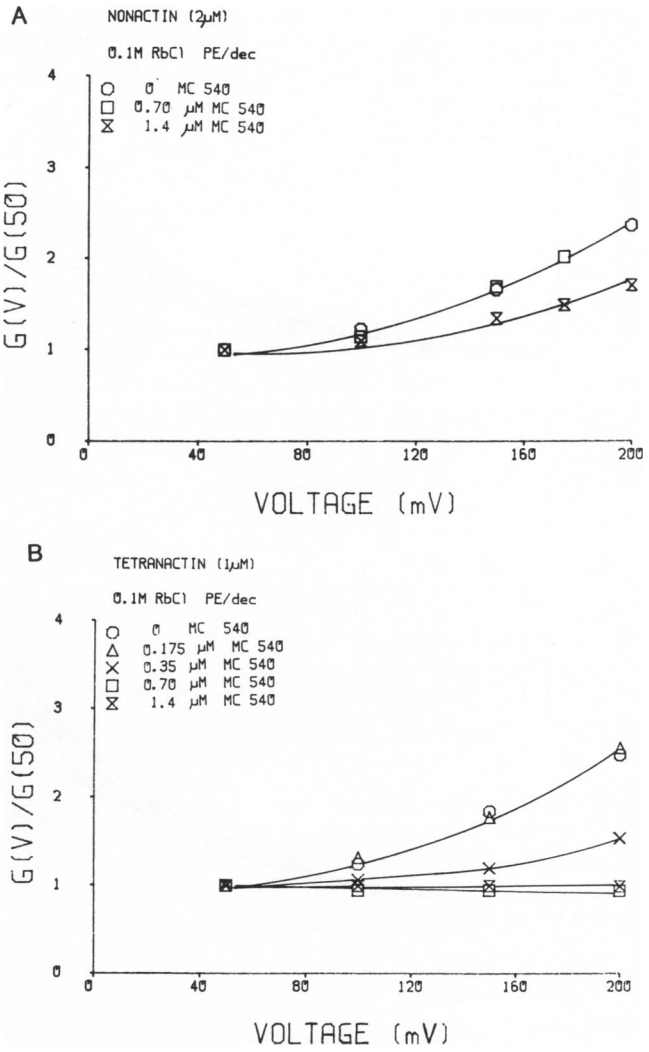

FIGURE 6 The influence of MC 540 on the conductance-voltage behaviors of PE bilayers in the presence of nonactin and tetranactin complexes with Rb^+. In all subfigures of Figs. 6, 7, and 8, the ordinate represents the steady state conductances, normalized to that at 50 mV, for the voltages indicated on the abscissa; the correspondence between the symbols and the solution conditions is also given in each subfigure. (*A*) Data for nonactin-Rb^+. (*B*) Data for tetranactin-Rb^+.

dioleate (GDO) bilayers (Krasne and Eisenman, 1976) with MC 540 making the G-V behaviors in PE membranes look like those observed for GDO. The explanation for the different lipid effects has been attributed to different surface dipole potentials for the two lipids, GDO having a more negative potential (inside relative to outside) than PE. Thus, the kinetics for ion-carrier dissociation becomes slow compared with that for crossing the membrane interior, and for the more slowly dissociating species, this dissociation step becomes rate limiting. The same explanation can be put forward for the effects of MC 540; however, the same behaviors are expected if the dye alters the boundary potential of the membrane (i.e., by producing a boundary of negative charges at a plane in the membrane interior). Two additional observations can be made on the data in Fig. 7. First, the G-V relationship was almost

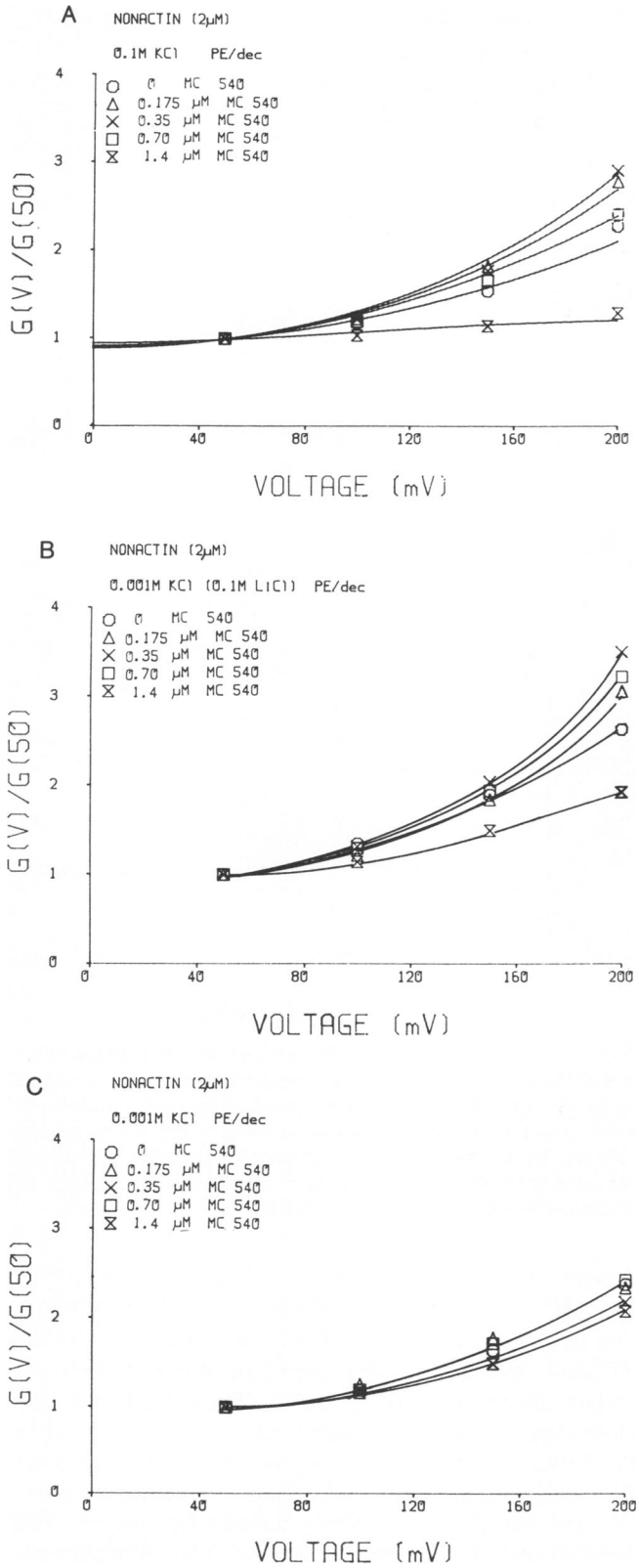

FIGURE 7 The influence of MC 540 on the conductance-voltage behaviors of PE bilayers in the presence of nonactin-K^+ complexes. (*A*) Data for 10^{-1} M KCl. (*B*) Data for 10^{-3} M KCl and 10^{-1} M LiCl (note that Li^+ is essentially not carried by nonactin and has been added to regulate the ionic strength of the solution). (*C*) Data for 10^{-3} M KCl. Symbols are defined in each subfigure.

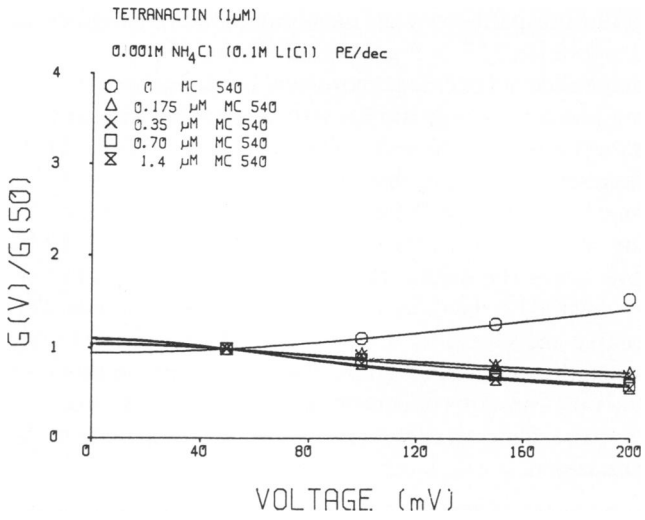

FIGURE 8 The influence of MC 540 on the conductance-voltage behaviors of PE bilayers in the presence of tetranactin-NH_4^+ complexes. Data for 10^{-3} M NH_4Cl and 10^{-1} M LiCl (note that Li^+ is essentially not carried by tetranactin and has been added to regulate the ionic strength of the solution). Symbols are defined in the figure.

independent of permeant ion concentration (cf. Fig. 7, *A* and *B*), but the effect of MC 540 decreased with decreasing ionic strength (cf. Fig. 7, *B* and *C*). This effect of ionic strength is the same as that illustrated in Fig. 2 for the zero current conductance and correlates with the fact that more MC 540 is present in neutral membranes at higher than at lower ionic strengths (Krasne, unpublished spectroscopic observations; see also Aiuche and Kobatake, 1979). The second observation is a peculiar, nonmonotonic dependence of the nonactin-K^+ G-V relationship on MC 540; at low dye concentrations the curves actually became steeper (i.e., less saturating) whereas at high dye concentrations their slopes decreased. These observations can be more readily understood after first examining the effects of MC 540 on the G-V relationships mediated by TØB.

Fig. 9, *A* and *B*, shows the effects of increasing MC 540 concentrations on the (instantaneous) conductance-voltage relationship for TØB-containing diPPC bilayers at 0.1 and 0.01 M ionic strengths, respectively. Clearly, increasing MC 540 increased the steepness of the G-V relationship, and this effect was smaller at the lower ionic strength, as was seen for ion-carrier complexes. The MC 540-produced increase in the G-V relationship was similar to that seen for nonactin-K^+ following the initial additions of dye (cf. ○, △, and X in Fig. 7, *A* and *B*); however, the subsequent decrease in steepness seen for the ion-carrier complex, and attributed to a rate limitation in dissociation of the complex at the interface, was not observed for TØB. The voltage dependence of the instantaneous conductance measurement for TØB reports the shape of the barrier for charge translocation within the membrane without the added complication of a complexation reaction at the interface. The simplest explanation for the increase in steepness observed for the G-V relationship of TØB with

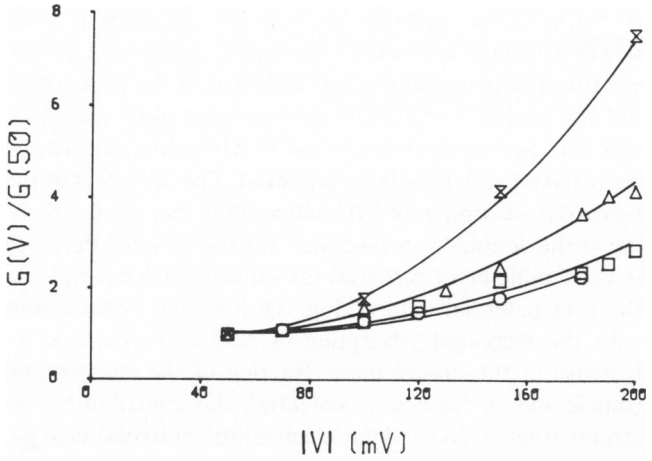

FIGURE 9 The influence of MC 540 on the conductance-voltage behaviors of diPPC bilayers in the presence of TØB. The ordinate is the instantaneous conductance, normalized to that at 50 mV, for the voltage indicated on the abscissa. For each *A* and *B*, MC 540 concentrations are 0 (○), 0.37 uM (□), 0.75 uM (△), and 1.5 uM (⧗). (*A*) For 10^{-1} M NaCl. (*B*) Data for 10^{-2} M NaCl.

increasing MC 540 and of nonactin-K^+ for small increases in this dye is that MC 540 effectively "thins" the bilayer membrane so that the breadth of the barrier for translocation is decreased. This thinning could be due either to a real decrease in membrane thickness (as might occur if the dye caused some additional solvent extrusion) or to a decrease in the thickness of the low dielectric constant region of the membrane (as might occur if the dye increased the dielectric constant of the outermost layer of the hydrocarbon region). The decrease in this effect with decreasing ionic strength appears to be due to the decreased amount of MC 540 adsorbed to the membrane and not to a consequent change in concentration of charge carriers in the membrane (due either directly to ionic strength effects or indirectly because of a change in adsorbed MC 540); this conclusion is based upon the fact that the (instantaneous) G-V curves in Fig. 9 *B* for 0.1 and 0.3 μM TØB at 1.5 μM MC 540 are virtually identical.

DISCUSSION

The results presented here illustrate that MC 540, while being a minimally conducting species in planar bilayers, produces significant alterations in the electrostatic potentials of these membranes. These potential changes are detected through the effects of MC 540 on the bilayer conductances mediated by cation carriers and lipophilic anions; this dye causes a large enhancement of cation conductances and a somewhat smaller depression of anion conductances consistent with the production of a negative electrostatic potential by the dye on the membrane. Several experimental observations argue against the interpretation that this dye simply adsorbs to membranes producing a net negative surface charge density and thus a diffuse double layer. First, the effect of MC 540 on cationic and anionic conductances is asymmetrical. Second, the dependence of the change in membrane conductance on MC 540 concentration for several of the curves in Fig. 2 is too steep to be due to the simple production of a double-layer potential by the dye (the maximum steepness for such an effect being a change of two-thirds of an order of magnitude in conductance for a one-order-of-magnitude change in dye concentration; Andersen et al., 1978). Third, only very small changes in the zeta potentials produced on membranes by MC 540 were detected in microelectrophoresis measurements. Fourth, the dye-induced electrostatic potentials sensed by charge carriers increased with increasing ionic strength (cf. Fig. 2), whereas the double-layer potential produced by adsorbed surface charges decreased with increasing ionic strength (McLaughlin and Harary, 1976).

One interpretation consistent with the data is that MC 540 binds at a plane within the membrane producing a small, screenable, double-layer potential and a much larger, unscreenable, electrostatic potential. The origin of this unscreenable electrostatic potential could be a combination of the following: the dipole potential intrinsic to MC 540, a change in the dipole potential of the lipids, or the production of a boundary potential due to adsorption of the negative charges of MC 540 to a plane that lies within the membrane. The simplest of these possibilities is that the unscreenable electrostatic potential arises from the dipole moment intrinsic to the MC 540 molecule itself, and we can examine whether the observations are consistent with this interpretation. If we assume that the charges associated with all of the bound MC 540 are at the membrane surface and are screenable (i.e., there are no boundary potentials), then the zeta potential measurements can be used to calculate the density of membrane-bound MC 540, using the Gouy equation and the estimate that the plane of shear for the microelectrophoresis experiments is actually 2 Å from the membrane surface (Eisenberg et al., 1979). For example, at 1.4 μM MC 540, the zeta potential in 0.1 M ionic solutions was −7 mV, which, using the correction in Eisenberg et al. (1979), implies a double-layer potential at the membrane surface of −10.4 mV. Introducing this latter value into the Gouy equation yields a negative surface-charge density of 1/2079 $Å^2$. Because ~90% of the membrane-bound MC 540 is in the form of monomers at this concentration (Waggoner and Grinvald, 1977), the surface concentration of the dye is approximately the same as the surface-charge density. However, Dragsten and Webb (1978) found that, for oxidized cholesterol membranes, approximately half of the monomers are oriented perpendicular to the membrane surface and the other half are parallel; therefore we might expect only half of the bound molecules to contribute to a change in the membrane's dipole potential. The dipole moment of MC 540 is quite large, being approximately 9 Debye (Dragsten, 1977; Brooker et al., 1951). Thus, at 1.4 μM MC 540, assuming only half of the bound dye contributes to the membrane's

dipole potential, the nonscreenable component of the electrostatic potential contributed by the dipole moment of the dye should be −82 mV and the screenable component should be −10.4 mV. Using the Boltzmann relation predicts that the total electrostatic potential of −92.4 mV calculated with these assumptions for 1.4 μM MC 540 should increase the conductance of cationic permeant species by 1.6 orders of magnitude and decrease that of anionic permeant species by the same amount. In fact, at 1.4 μM MC 540, the conductance of nonactin-K^+ was increased by 2.5 orders of magnitude while that of TØB$^-$ was decreased by 1.2 orders of magnitude. Although the value for the anionic permeant is similar to that predicted, that of the cationic probe is an order of magnitude too large.

One problem with the above calculation may lie in the assumption that the dipole potential generated by MC 540 is uniform in the plane of the membrane. Discrete charge effects have been previously postulated to be the source of asymmetrical alterations in conductances mediated by permeant anions and cations (Andersen et al., 1978; Tsien, 1978); so too might adsorbed, dipolar molecules, which are sufficiently dilute in the membrane, produce nonuniform potentials that affect anion and cation conductances differently. By contrast, if MC 540 simply altered the dipole potential intrinsic to the lipid moieties in the membrane, large asymmetries between the conductance changes mediated by positive and negative permeant species would not be expected, since such a change in lipid dipole potential should be reasonably uniformly distributed over the surface of the membrane. Discrete charge effects have also been shown (Andersen et al., 1978) to be able to account for steeper dependencies of the conductance change for positive and negative permeant species on the concentration of the adsorbed, charged species (in this case MC 540), and the same observations are expected for discrete dipole effects.

Even taking into account the asymmetries in conductances for permeant cations and anions expected if the electrostatic potentials due to adsorbed charges and/or dipoles are nonuniform, the calculated dipole and double-layer potentials calculated for MC 540 based upon the zeta potential measurements would not appear to be sufficiently large to account for the conductance data for permeant cations and anions in Fig. 2. Thus, it is likely that an additional effect of MC 540 is responsible for the alteration in electrostatic potentials, and the most likely source is the additional production of an unscreenable boundary potential by dye molecules adsorbed to a plane within the membrane.

Both the postulated boundary potential and alteration in dipole potential can account for the increases in MC 540-induced electrostatic potentials with increasing ionic strength seen in the data of Fig. 2. If the boundary and dipole potentials produced by MC 540 were totally unscreenable, then they should be independent of the ionic strength of the aqueous solution. In the present case, however, there is a small, screenable component to the electrostatic potential change, reflected in the zeta potential data of Fig. 3. An increase in ionic strength, therefore, will tend to increase the amount of MC 540 adsorbed, by decreasing the double-layer potential. The net effect of the increased adsorption of MC 540 and the increased screening of the double-layer potential will still be a net decrease in the double-layer potential (as reflected, for example, in the zeta potential data of Fig. 3); however, concomitant with the increased adsorption of MC 540 would be an increase in the unscreenable fraction of the electrostatic (dipole and/or boundary) potential, this contributing the largest fraction to the total electrostatic potential change. Thus, the total electrostatic potential change due to MC 540 would increase with increasing ionic strength.

An alternative hypothesis which can account for much of the data is that, upon adsorbing to membranes, MC 540 produces a relatively small change in the electrostatic potential and a somewhat larger increase (based upon the effects on permeant cations and anions) in a membrane property such as fluidity, thickness, or dielectric constant, which would enhance the conductance of both cationic and anionic permeant species. That is, one effect of MC 540 should be in opposite directions for conductances mediated by cationic and anionic species, and the other should be in the same direction (i.e., enhancement) for the conductances mediated by the two types of species. Two observations argue against this interpretation: First, factors that increase MC 540 adsorption, such as increasing ionic strength, should enhance the zero-current conductances of both permeant cationic and anionic species, although increasing ionic strength actually decreased the conductance of anionic species (cf. Fig. 2). Second, an increase in dielectric constant or fluidity or a decrease in membrane thickness should increase the rate at which both cationic and anionic species cross the membrane interior; but increasing MC 540 decreases this rate (i.e., increases the time constant for current relaxation and decreases the instantaneous current) for TØB. That some part of the observations presented for MC 540 effects might be due to changes in membrane thickness, dielectric constant, and/or fluidity cannot, however, be ruled out, especially in light of the G-V relationships discussed above.

Finally, effects of MC 540 on both the time dependences and the voltage dependences of ionic currents are consistent with the interpretation that this dye alters the electrostatic potentials of membranes. For TØB, MC 540 increased the time constant for current relaxation and decreased the total adsorbed charge (see Fig. 5, *A* and *B*). These phenomena are similar to those of Andersen et al. (1978) for the effects of negative boundary potentials on TØB. Interestingly, Andersen et al. found very little change in the time constant for TØB current relaxation between glycerol monooleate and PE bilayers (at comparable aqueous concentrations of TØB) despite the fact that the dipole

potentials of these two membranes differ by ~130 mV (PE more positive; Hladky, 1974, and Andersen et al., 1978). The decreased slope of the G-V relationships and observation of current relaxations for nonactin and tetranactin-mediated conductances in PE bilayers are consistent with MC 540 producing either a boundary potential or a more negative surface dipole potential. The decrease in these effects with decreased ionic strength is most simply explained by the decrease in MC 540 adsorption to the membrane at lower than at higher ionic strengths, as deduced from spectroscopic observations (Krasne, unpublished observations; Aiuche and Kobatake, 1979). The only exception to this observation was that the decrease in the total adsorbed TØB charge with added MC 540 was little affected by lowering the ionic strength of the aqueous medium; this observation, combined with the ionic strength effects on the relaxation time constant, suggests that the dipole or boundary potential produced by MC 540 lies more external to TØB at lower than at higher ionic strengths.

A number of molecules, most notably phloretin, have been shown (Andersen et al., 1976) to alter the dipole potential of bilayer membranes and thereby to alter the membrane's ion permeability; The observations for those molecules are remarkably similar to the ones presented here for MC 540. In terms of the consequences of the present findings for using MC 540, or similarly structured dyes, to monitor transmembrane potential changes in cell membranes, there are a couple of relevant points. First, if indeed cell membranes are accurately modeled by PE bilayers, then unilateral addition of MC 540 to the solution bathing the outer membrane surface (which is the typical way in which the dye is added) will produce a more negative electrostatic potential near the outer membrane-solution interface while leaving unaltered that near the inner membrane-solution interface; the consequences to voltage-gated channels would be comparable with that observed upon depolarizing the membrane. Thus, in voltage-clamp experiments, one might expect to see a shift of the conductance-voltage relationships for voltage-gated channels along the voltage axis; whereas, under constant current conditions, one might expect to find inactivation of channels that are normally inactivated upon depolarizing the cell. A second point is that many membranes appear to be either negatively charged or to have negative charges near the mouths of the voltage-gated channels. For such membranes, one might expect the effects of MC 540 to be lower in magnitude than those observed in neutral bilayers, based upon the decreased electrostatic potentials observed for MC 540 in PE/PS, as compared with PE bilayers (Fig. 1). Clearly, voltage-clamp experiments to determine the effect of MC 540 on the conductance-voltage relationships of voltage-gated channels would be useful in those cells in which such experiments are feasible, to determine whether effects of this dye on electrostatic potentials are observed in biological cell membranes.

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