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Dual-Wavelength Ratiometric Fluorescence Measurement of the Membrane Dipole Potential

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ABSTRACT The electrostatic potentials associated with cell membranes include the transmembrane potential ($\Delta\Psi$), the surface potential (Ψ_S), and the dipole potential (Ψ_D). Ψ_D, which originates from oriented dipoles at the surface of the membrane, rises steeply just within the membrane to ~300 mV. Here we show that the potential-sensitive fluorescent dye 1-(3-sulfonatopropyl)-4-[β[2-(di-*n*-octylamino)-6-naphthyl]vinyl]pyridinium betaine (di-8-ANEPPS) can be used to measure changes in the intramembrane dipole potential. Increasing the content of cholesterol and 6-ketocholestanol (KC), which are known to increase Ψ_D in the bilayer, results in an increase in the ratio, *R*, of the dye fluorescence excited at 440 nm to that excited at 530 nm in a lipid vesicle suspension; increasing the content of phloretin, which lowers Ψ_D, decreases *R*. Control experiments show that the ratio is insensitive to changes in the membrane's microviscosity. The lack of an isosbestic point in the fluorescence excitation and emission spectra of the dye at various concentrations of KC and phloretin argues against 1:1 chemical complexation between the dye and KC or phloretin. The macromolecular nonionic surfactant Pluronic F127 catalyzes the insertion of KC and phloretin into lipid vesicle and cell membranes, permitting convenient and controlled modulation of dipole potential. The sensitivity of *R* to Ψ_D is 10-fold larger than to $\Delta\Psi$, whereas it is insensitive to changes in Ψ_S. This can be understood in terms of the location of the dye chromophore with respect to the electric field profile associated with each of these potentials. These results suggest that the gradient in dipole potential occurs over a span ≤5 Å, a short distance below the membrane-water interface. These approaches are easily adaptable to study the influence of dipole potentials on cell membrane physiology.

INTRODUCTION

There are three distinct electrostatic potentials associated with cellular lipid bilayers (Fig. 1). The transmembrane potential ($\Delta\Psi$), the surface potential (Ψ_S), and the dipole potential (Ψ_D) (for reviews see Loew, 1993; McLaughlin, 1989; Honig et al., 1986; McLaughlin, 1977). The transmembrane potential, which results from charge separation across the membrane, can be rapidly changed through the opening of ion channels and, in turn, can modulate the activity of voltage-dependent channels in the membrane. These structural transitions can come about as a result of a coupling between the internal electric field set up by $\Delta\Psi$ and the gating charges or dipoles in the voltage sensors of the channel (Hille, 1992). Surface potential is the potential difference between the membrane surface and the bulk aqueous phase and is dependent on the density of interfacial charged molecules (for a review see McLaughlin, 1989). In biological membranes, this potential is on the order of a few tens of mV and might have an important role in affecting the conductance of channels in the membrane (Dani, 1986; Jordan, 1987; Kell and DeFelice, 1988), determining the structure of proteins (Gilson and Honig, 1988; Honig et al., 1986; Huang and Warshel, 1988; Perutz, 1978) and in the binding of charged molecules to the membrane (Green and Andersen, 1986; Green et al., 1987; Smith-Maxwell and Begenisich, 1987).

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Unlike $\Delta\Psi$ and Ψ_S, Ψ_D has been less well studied, and its impact on cell membrane biology is not well appreciated. Studies on model membranes have provided some important insights, however. The observations that hydrophobic anions bind several orders of magnitude stronger to and translocate several orders of magnitude faster across a lipid bilayer than structurally similar cations (Liberman and Topaly, 1969; Hladky and Haydon, 1973; Szabo, 1974; Flewelling and Hubbell, 1986; Honig et al., 1986; Franklin and Cafiso, 1993; Franklin et al., 1993) can be rationalized by a positive potential barrier inside the bilayer of several hundred mV (~300 mV for phosphatidyl choline) (Fig. 1). Voltage measurements with ionizing electrodes on lipid monolayers lining an air-aqueous interface resulted in a dipole potential for phosphatidyl choline of ~450 mV (Bangham and Mason, 1979; Reyes et al., 1983; Haydon and Elliot, 1986; Gabev et al., 1989). Although the discrepancy between the results with bilayers and monolayers is not fully understood, there is agreement that the dipole potential in the bilayer is positive with a magnitude of several hundred millivolts. Unlike the surface potential, this barrier potential is independent of ionic strength and is presumed to originate from oriented dipoles in the membrane/water interface. The orientation of dipoles in (a) the water molecules adjacent to the membrane, (b) the polar head groups, and (c) the ester linkages of the acyl chains to the glycerol backbone of the phospholipid, could all account for such a potential difference between the interior of the bilayer and the aqueous phase. Recent electrostatic calculations suggest that oriented water molecules are a major contributor to the dipole potential (Zheng and Vanderkooi, 1992).

The dipole potential could play an important role in modulating membrane functions. As noted, Ψ_D affects the permeability of the membrane to hydrophobic ions and the binding of such ions to the membrane. To a smaller extent, it can

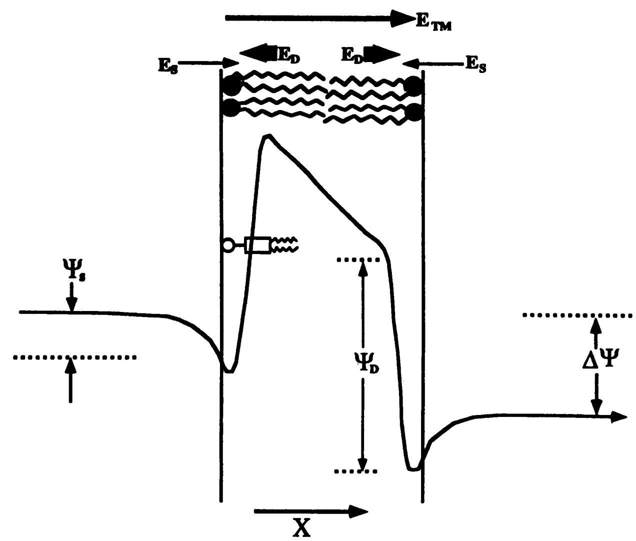

FIGURE 1 The electrostatic potential profile across phospholipid bilayers. The transmembrane potential, $\Delta\Psi$, is the potential difference between the aqueous solutions on either side of the membrane and is measurable with electrodes. It arises from gradients of selectively permeant ions and from the action of electrogenic ion pumps. The usual assumption (constant field approximation) is that $\Delta\Psi$ drops uniformly across the membrane. The surface potential, Ψ_S, is the potential difference between the membrane surface and the aqueous bulk. It arises from fixed charges at the membrane/water interface. In biological membranes, Ψ_S arises mainly from negatively charged lipids; in the above diagram, the charge density is presumed to be identical on both surfaces. The dipole potential, Ψ_D, is the potential difference between the center of the bilayer and the membrane/water interface. It arises from dipolar groups just below the interface. The electric fields arising from each of these components are represented by arrows at the top of the diagram; the location of the arrows and their length correspond to the approximate regions over which each component is effective; the thickness of the arrows reflect the approximate relative field intensities for a typical cell membrane. The putative location of one di-8-ANEPPS molecule in the membrane, with respect to the electric potential profile, is also shown. The circle represents the sulfonate head group, the rectangle represents the chromophore, and the jagged lines represent the two 8-carbon alkyl chains.

also affect the conductance properties of ion channels in the membrane. It has been suggested that the anomalous selectivity and conductivity of some potassium channels can be explained by the existence of a dipolar potential source near the mouth of the channel (Jordan, 1987; Moczydlowski et al., 1985; Vergara et al., 1984). We might also speculate that Ψ_D modulates channel kinetic and thermodynamic parameters of ion channels by interacting with the gating-charges in a channel. However, no convenient method has previously existed for measuring dipole potential in cells.

Potential-sensitive indicator dyes have been widely used for measuring transmembrane potentials of cells, cell organelles, and membrane vesicles (for reviews see Cohen and Salzberg, 1978; London et al., 1986; Waggoner, 1985; Loew, 1988; Gross and Loew, 1989). These dyes have been used to measure changes in $\Delta\Psi$ in cases where microelectrodes are impractical (e.g., large populations of cells in suspension or simultaneous multisite recording of a complex neuronal preparation). This laboratory has developed a series of potentiometric fluorescent indicators that employ a putative electrochromic mechanism (Loew et al., 1979; Loew and Simpson, 1981). The spectral shift associated with a change in $\Delta\Psi$ permitted us to develop a dual wavelength ratiometric approach for measuring membrane potential (Montana et al., 1989; Bedlack et al., 1992; Loew et al., 1992). As in the case of dual wavelength ratiometric ion indicators (Tsien and Poenie, 1986), this approach simplifies calibrations of $\Delta\Psi$ in cell suspensions because the ratio is independent of dye or cell concentration; it also obviates problems of dye bleaching and uneven staining in single cell studies with a fluorescence microscope, thus permitting the imaging of membrane potential along the surface of single cells.

It is important to appreciate that in electrochromism and other fast potentiometric mechanisms, a dye indicator responds to changes in the local electric field intensity at its binding site in the bilayer. Therefore, these dyes should be sensitive to intrinsic membrane electric fields that might be set up by differences in the surface potential between the inner and outer leaflets of the bilayer or, if it is appropriately located as in Fig. 1, to the intense electric field set up by Ψ_D. Neither of these can be measured with microelectrodes. In this paper, we show that dual wavelength ratiometric measurements of the fluorescence of a potential-sensitive dye, di-8-ANEPPS, can be used to measure changes of the local electric field associated with variation of the membrane's dipole-potential.

MATERIALS AND METHODS

Liposomes

Liposomes were prepared by mixing egg phosphatidylcholine (PC) (in chloroform:ethanol, 9:1) with di-8-ANEPPS (in ethanol). When necessary, the appropriate additive (cholesterol, 6-ketocholestanol (KC), phloretin, phosphatidyl serine (PS), or stearyl amine (SA)) in an organic solvent was added to this liposome-forming solution. The solvent was evaporated thoroughly under Argon for 1 h, and the resulting film was further dried by placing it in a vacuum desiccator for a minimum of 15 h at less than 0.5 torr. A 2 ml aqueous solution containing 20 mM HEPES, pH 7.4, was then added to the dried lipid film. A lipid suspension was formed by placing the bottom of the test tube in a round bath sonicator (Laboratory Products, Hicksville, NY) and sonicating for 30 s. A clear liposome suspension was formed by sonicating this turbid suspension for 5 min with a probe sonicator (Branson, cell disruptor 185) under an Argon atmosphere in an ice bath. The clear liposome suspension obtained this way was centrifuged at 10,000 $\times$ g to remove titanium particles. As a control, the labeled liposomes containing KC or phloretin were sonicated 3 more times for 3 min each time, and fluorescence excitation and emission spectra were taken after each sonication. No differences were found in either the excitation or emission spectra of a given sample after these sonication steps. Unless otherwise stated, the final lipid and dye concentrations in the experiment were 2.5 mg/ml and 9.0 μM, respectively. The average molecular weight of PC was taken as 700.

Cells

L1210 cells, a mouse lymphocytic leukemia line, were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI medium supplemented with 1.5% L-glutamine, 10% fetal bovine serum, and 0.5% antibiotic/antimycotic. For staining, cells were centrifuged and resuspended in Earle's balanced salt solution containing 10 mM HEPES buffer (pH 7.4) Earle's balanced salt solution (EBSS), 0.05% Pluronic F127, and 1 μM di-8-ANEPPS. The cell suspension was placed in a shaker bath at ambient temperature for 10 min, washed once in dye-free EBSS containing 0.05% Pluronic F127, and then a final time in EBSS alone. Stained cells were resuspended at a concentration of 4 $\times$ 10^6/ml for fluorescence

analysis. The staining pattern of the cells was checked with a fluorescence microscope (Nikon Diaphot, 100X oil objective, excitation 530 nm, emission 610 nm) to ascertain that the fluorescence originated from the plasma membrane and not intracellular dye.

Fluorescence ratios (*R*)

Fluorescence ratios were measured on a Spex CM dual wavelength fluorescence spectrophotometer equipped with a thermostated cell holder and a magnetic stirrer. Emission at 620 nm was excited from two excitation monochromators set at 440 and 530 nm. Excitation was rapidly alternated between the two excitation wavelengths via a 400-Hz chopper. All ratio measurements were done in a time drive mode with a 5-min scan. The ratio values (440 nm/530 nm) were averaged over the 5-min scans.

Spectra

KC and phloretin were each dissolved in a solution of dimethylsulfoxide (DMSO) + 2.5% Pluronic F127 to give 25 mM stock solutions. Aliquots from these stock solutions were added to a continuously stirring labeled liposome suspension in the cuvette, allowing 3-min incubation at room temperature, for full equilibration, before spectra were taken. The emission wavelength for the fluorescence excitation spectra was 645 nm, and the excitation wavelength for the emission spectra was 485 nm. All spectra are corrected for excitation and emission monochromator wavelength dependence using manufacturer-supplied correction factors. Spectra are also corrected for dilutions.

Titrations

Small aliquots of KC and phloretin, from an 8 mM stock solution in DMSO + 2.5% Pluronic, were added to a stirred labeled liposome suspension in the cuvette while the di-8-ANEPPS fluorescence ratio, *R*, was continuously measured. For titrations of L1210 cells, a DMSO stock solution containing 7.5% Pluronic F127 with either 2.5 mM KC or 2.5 mM phloretin was used.

Surface potential

For the surface potential experiments, the dried desiccated lipid film, containing various mole fractions of PS or SA prepared as described above, was suspended in de-ionized water instead of HEPES buffer. Liposomes were then formed as described above. KCl was added from concentrated stock solutions, in de-ionized water, to the labeled liposomes suspension. The suspension was then sonicated in a round bath sonicator for 30 s to equalize the KCl concentration in the inside and outside bulk phases of the liposomes. Final KCl concentrations ranged from 1 μM to 1 M. *R* values were measured as described above.

Chemicals

Cholesterol, Egg phosphatidylcholine (PC), HEPES, KC, KCl, phloretin, PS, and SA were purchased from Sigma Chemical Co. (St Louis, MO) and used without further purification. RPMI (no. 11875), Earle's balanced salt solution, L-glutamine, fetal bovine serum, and antimycotic/antibiotic were purchased from Gibco BRL. di-8-ANEPPS was synthesized in our laboratory similarly to di-4-ANEPPS (Hassner et al., 1984).

RESULTS

Reagents that change Ψ_D change the fluorescence ratio of di-8-ANEPPS

There are several compounds that are known to modify the bilayer's internal dipole potential. Comparing the conductances of bilayers with hydrophobic anions and cations, it was found that cholesterol, a widespread component of the plasma membrane, increases the anion conductance (tetraphenylborate and *m*-chlorophenylhydrazone) by 3000-fold as compared with lipophilic cations (tetraphenyl phosphonium and 3,3′-dipropyloxadicarbocyanine iodide) (Szabo, 1974). This effect of cholesterol was interpreted as a change in the orientation, strength, and packing density of molecular dipoles at the membrane surface leading to a net increase in the dipole potential value inside the bilayer. In Fig. 2 (*filled squares*), we show the effect of increasing the mole fraction of cholesterol in PC liposomes on the measured dual-wavelength fluorescence ratio (*R*) of di-8-ANEPPS incorporated into the bilayer. As can be seen, 50% cholesterol causes a 1.5-fold increase in *R*.

6-Ketocholestanol and phloretin are known to increase and decrease, respectively, the internal dipole potential when incorporated into bilayers (Bechinger and Seelig, 1991; Franklin and Cafiso, 1993). We thus measured the effects of these compounds, incorporated into the bilayer, on *R*. Fig. 2 shows the results of these experiments. Increasing the mole fraction of KC from 0 to 30% caused a 2.5-fold increase in *R*, whereas a threefold decrease was obtained by 15% phloretin. The effects of cholesterol, KC, and phloretin on *R* suggest that it is sensitive to changes in the intrinsic bilayer dipole potential.

R is not sensitive to membrane microviscosity

Cholesterol is known to increase the membrane microviscosity above the phase transition temperature (Shinitzky and Inbar, 1976; Gross et al., 1987; Van der Meer, 1993). It is likely that KC and phloretin also alter the fluidity of the bilayer. It was important, therefore, to determine whether

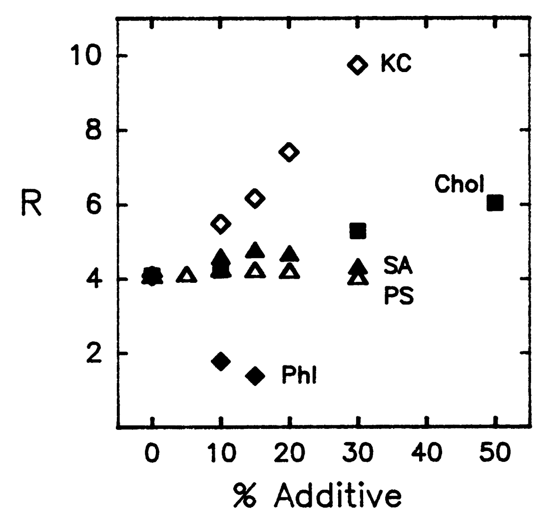

FIGURE 2 The ratio of the fluorescence intensity excited at 440 nm to that excited at 530 nm, *R*, as a function of the mole fractions of the following membrane additives: (◇) KC; (■) cholesterol; (▲) SA; (△) PS; (◆) phloretin. *R* is highly sensitive to changes in Ψ_D, but insensitive to Ψ_S. SE < 1%, n = 4. The emission wavelength was 620 nm.

R is sensitive to the membrane microviscosity. We measured the effect of the membrane microviscosity by varying the temperature of a cholesterol-free PC liposome suspension in Fig. 3. Using data from the literature on the egg PC/cholesterol system (Shinitzky and Inbar, 1976.), we varied the temperature from 6.5 to 40°C to encompass the range of microviscosities produced by our range of cholesterol mole fractions. As can be seen from Fig. 3, increasing the membrane's microviscosity by lowering the temperature had an insignificant effect on *R* as compared with the effect of cholesterol. Therefore, it is very likely that cholesterol causes an increase in *R* through a mechanism that does not involve changes in the membrane's microviscosity.

Titrations provide no evidence for dye complexation as a mechanism for the changes in *R*

Our explanation of the dye's spectral shifts (which underly the change in *R*) involves the electrostatic interaction between the intramembrane electric field, modulated by cholesterol, KC or phloretin, and the electron distributions in the ground and excited states (Platt, 1956; Liptay, 1969; Loew et al., 1978). Another possibility, which could result in a spectral shift, is the formation of chemical complexes between these compounds and the dye. If this mechanism pertains, titration of the dye with these reagents should follow a nonlinear saturable equilibrium binding curve. Over the range displayed in Fig. 2, there is no evidence of saturation.

Also, for binary complexes, the fluorescence excitation or emission spectra of the dye at different concentrations of KC or phloretin should display an isosbestic point at the wavelength where both species fluoresce with the same efficiency. Therefore, to test further for this possible source of nonpotentiometric variations in *R*, it was important to measure the full fluorescence excitation and emission spectra at different membrane concentrations of KC and phloretin. In contrast to the measurements of *R*, to properly record these spectra it was necessary to carry out the titrations as a series of additions to a preformed lipid vesicle suspension containing the dye. This avoided the problem (normalized away in *R* measurements) of uncontrollable variations in total dye and lipid concentrations when each lipid/dye/titrant composition was separately sonicated. However, binding of KC and phloretin to the membranes was slow and attained equilibrium over too long a period to make these titrations feasible. This problem was solved by the discovery that insertion of KC and phloretin into the bilayer is catalyzed by Pluronic F127. This macromolecular nonionic surfactant has been used previously to catalyze the labeling of membranes by lipohilic dyes (Davila et al., 1973), and the mechanism has been studied (Lojewska and Loew, 1986); indeed, it is used routinely to promote staining of cells with di-8-ANEPPS (see Materials and Methods section and Bedlack et al., 1992). In Fig. 4, we show the rapid changes in *R* when concentrated stock solutions of Pluronic F127 containing either KC or phloretin are added to di-8-ANEPPS-labeled PC liposomes. The equilibration times of KC and phloretin with the bilayer are shorter than the mixing time. In the absence of Pluronic F127, equilibration required hours.

Thus, Pluronic F127 permitted us to examine the effects of KC and phloretin on the excitation and emission spectra of di-8-ANEPPS-labeled liposomes. In Fig. 5, *A* and *B*, we show the fluorescence spectra at various KC concentrations. The indicated KC concentrations are the total concentrations

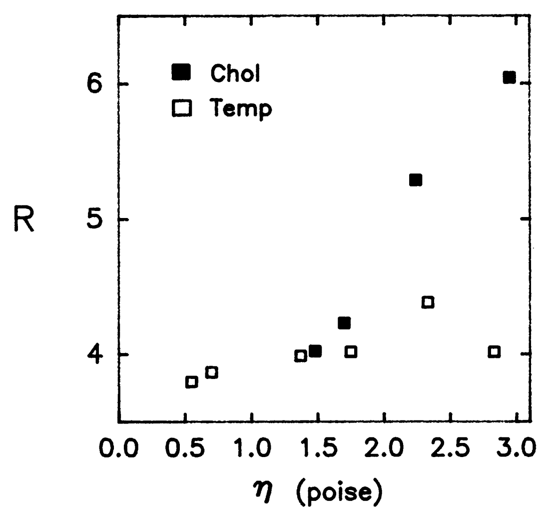

FIGURE 3 *R* is not sensitive to membrane microviscosity. Microviscosity was varied by changing the temperature (□). For comparison, the data for cholesterol (■) are also plotted with corresponding microviscosity values taken from the literature.

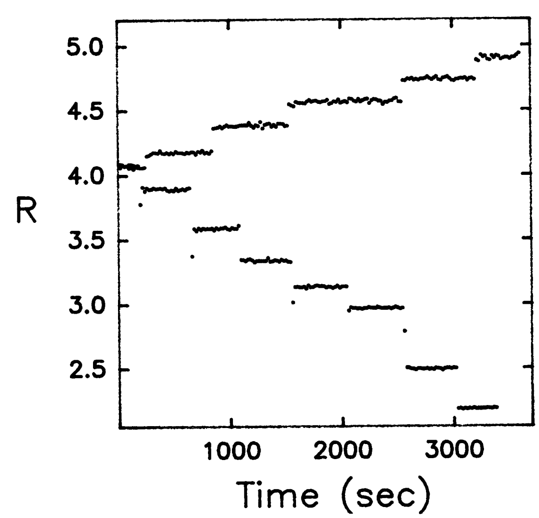

FIGURE 4 Binding of KC (*upper plot*) or phloretin (*lower plot*) to di-8-ANEPPS-labeled liposomes. The ratio was continuously monitored during additions of aliquots from DMSO stock solutions containing 2.5% Pluronic F127 and 25 mM of either KC or phloretin. The first six segments of each plot correspond to 0, 10, 30, 50, 70, and 90 μM of KC or phloretin. The last two segments in the lower plot correspond to 140 and 190 μM phloretin. The maximum concentrations of DMSO and Pluronic attained in these experiments were 2 and 0.05%, respectively; control experiments showed no effect on *R* upon addition of concentrations of DMSO + Pluronic up to 6.5 and 0.15%, respectively.

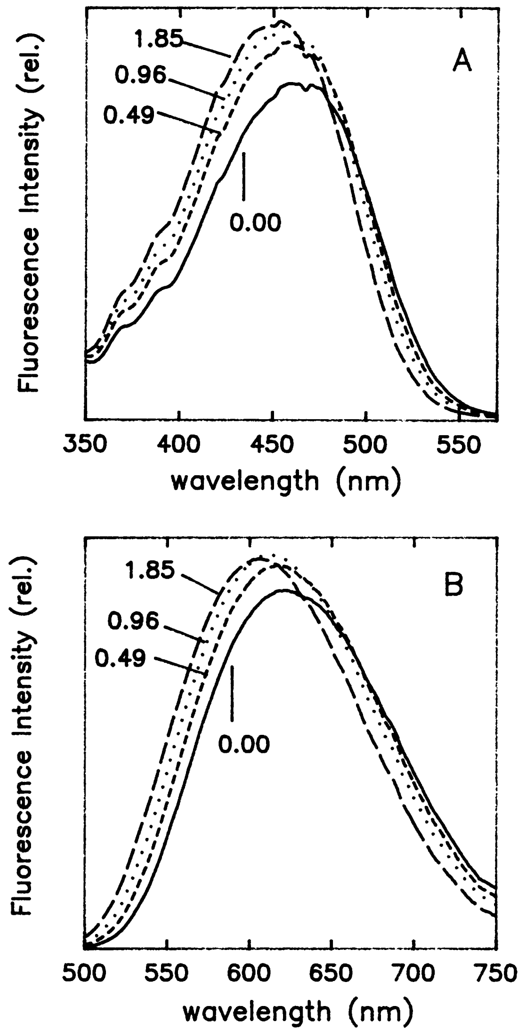

FIGURE 5 Effect of KC on the fluorescence spectra of di-8-ANEPPS-labeled liposomes. (*A*) Excitation spectra, λ_{em} = 645 nm; (*B*) emission spectra, λ_{exc} = 485 nm, of di-8-ANEPPS in the presence of: (*solid line*) no KC; (*short dash*) 0.49 mM; (*dotted line*) 0.96 mM; (*long dash*) 1.85 mM KC in the medium.

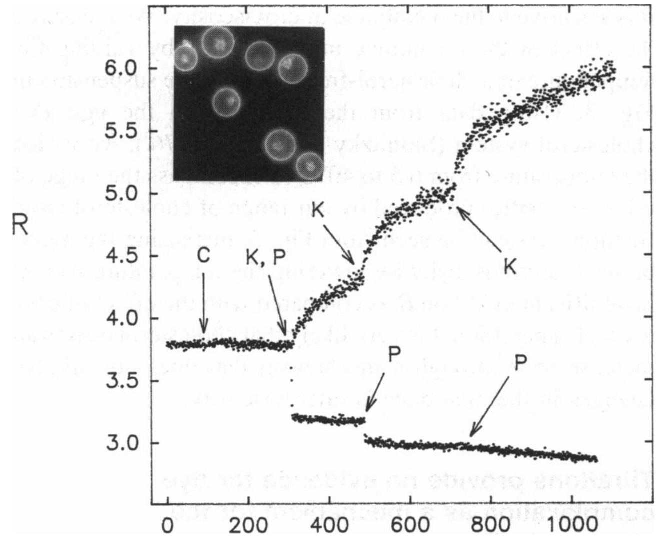

FIGURE 6 Modulation of Ψ_D in cell membranes by KC or phloretin as measured by *R*. Pluronic F127 was used at a higher relative concentration than in Figs. 4 and 5 to facilitate equilibration of KC and phloretin with di-8-ANEPPS-labeled L1210 cells. Three experiments are superimposed, all with the same initial baseline. Additions of KC and phloretin of 10 μM each are indicated by "KC" and "Phl," respectively. Each of these additions included 0.03% Pluronic and 0.4% DMSO. A control experiment in which the cell suspension was exposed to 2% DMSO and 0.15% Pluronic at the point labeled "C" shows no effect on *R* (this tracing was terminated at time 250 s). (*inset*) Fluorescence micrograph of L1210 cells labeled with di-8-ANEPPS.

in the solution, not mole fractions in the liposome membranes. As can be seen from the figures, increasing the concentration of KC in the medium causes a blue-shift in both the excitation and emission spectra. These spectral shifts are the result of KC association with the membrane. There is no isosbestic point in either the fluorescence excitation or the emission spectra. No isosbestic point was observed when the same experiments were repeated with phloretin (not shown). Therefore, the possibility of a simple 1:1 complexation between the dye and KC or the dye and phloretin is unlikely.

The dipole potential of cell membranes can be modulated with KC and phloretin

In Fig. 6 (*inset*), we show a fluorescence micrograph of L1210 cells stained with di-8-ANEPPS. It reveals strong plasma membrane fluorescence with minimal fluorescence from intracellular structures. Therefore, the fluorescence of a L1210 cell suspension reports on the electrical properties of the plasma membrane.

The ability to modulate experimentally the dipole potential of cell membranes offers the prospect of investigating the cell physiological importance of this component of the membrane electrical profile. Pluronic F127 makes this possible, as shown in Fig. 6. Again, the two reagents change *R* in the directions predicted from their known effects on Ψ_D in model membrane systems. For KC, the slower changes in *R* compared with those seen in Fig. 4 are presumably because of the slower rate of insertion into cell membranes compared with the simple lipid bilayer of liposomes. These data were obtained with a ratio of Pluronic:KC 30 times greater than was used in the liposome experiment. The rate of change of *R* is decreased as the Pluronic F127 concentration is decreased; without the catalyst, KC does not change *R* over any timescale that is experimentally accessible. For phloretin, the rate of insertion is fast, but the level of binding saturates after only ~20 μM. This might be because of a competition between weak binding sites on the membrane and on the surfactant for the phloretin molecules. Phloretin is not a lipid, and its structure suggests that it will be weakly bound near the membrane/water interface; KC, on the other hand, would be expected to be fully intercalated into the lipid bilayer.

R is insensitive to surface potential but highly sensitive to dipole potential

To determine whether *R* is sensitive to Ψ_S, we changed Ψ_S by varying the ionic strength of a liposome suspension, con-

taining 20% phosphatidyl serine. The electrostatic potential at the surface of the membrane, because of fixed charges is given to a good first approximation, by the classical Gouy-Chapman equation

$$A\sigma/C^{1/2} = \sinh(ze\Psi_s/2kT), \qquad (1)$$

where σ is the surface charge density, C is the concentration of the electrolyte of charge z; e, k, and T have their usual meanings; and A is a constant equal to 136.4 $M^{1/2}$ at 22°C. Because the area occupied by a phospholipid molecule in a bilayer is ~60 Å^2 (Levine and Wilkins, 1971), the surface potential can be calculated at any electrolyte concentration. For instance, at 20% PS in the bilayer and 10 mM KCl in the solution, σ = 1/300 charges/Å^2 and Ψ_S = −114 mV. Fig. 7. shows R vs. $-\Psi_S$ for PC liposomes containing 20% PS at various dilution factors of a concentrated KCl stock solution corresponding to final KCl concentrations ranging from 1 μM to 1 M. Using Eq. 1 and neglecting atmospheric carbon dioxide solubility on the lower concentration value, this concentration range translates to a surface potential range of approximately −23 to −350 mV. No significant change in R over that range was observed.

We also measured the dependence of R on the surface potential created by charges of opposite signs by incorporating lipids that carry negative and positive charges into the bilayer. Fig. 2 shows the results for PC liposomes with increasing mole fractions of phosphatidyl serine (PS), a negatively charged lipid, and stearylamine (SA), which contains a positively charged amino group attached to a long hydrocarbon chain. As can be seen, no significant change in R was observed.

Studies of the transport of lipophilic ions across bilayers have permitted estimation of the change in the dipole potential as a function of the mole fraction of various membrane

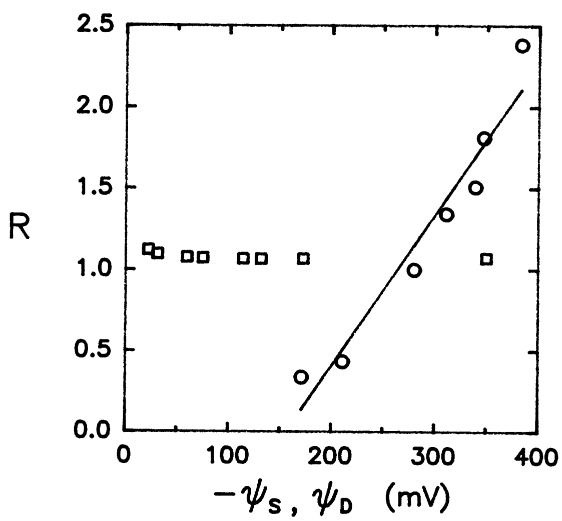

FIGURE 7 The dependence of R on Ψ_S, (□); and on Ψ_D, (○). For the R values, SE < 1%, n = 4. The Ψ_D values have an uncertainty of ca. >±10% as derived from fits of hydrophobic ion permeabilities to a molecular model of a dipolar array (D. Cafiso, personal communication) (Franklin and Cafiso, 1993).

additives. To allow such data to be rationalized in terms of membrane structure, a total potential model was developed in which $\Psi_D(x)$ is explicitly derived from an array of dipoles (Flewelling and Hubbell, 1986). This model was recently extended by Franklin and Cafiso (1993) to analyze their data on the effects of KC and phloretin on the rate of hydrophobic ion spin label transport through PC vesicle membranes. We used the Ψ_D values kindly provided by David Cafiso from this work to plot R as a function of Ψ_D in Fig. 7; Ψ_D for pure PC is taken as the reference point at which R is set to unity by appropriate normalization (or, equivalently, by balancing the dual wavelength optics). The line is the linear least-squares fit to the data. Although over such large changes a linear fit is not strictly theoretically valid, it permits a useful approximate comparison of the sensitivity of R with transmembrane potential. R changes by 0.8 units for a change of 100 mV in Ψ_D, as compared with a change of 0.1 for a 100-mV change in $\Delta\Psi$, determined in previous studies, (Montana et al., 1989; Bedlack et al., 1992). This difference can be understood if the electric field (i.e., voltage gradient) at the location of di-8-ANEPPS is much greater for the dipole potential than for a $\Delta\Psi$ of the same size. Taking the data a step further, they imply that for Ψ_D, the voltage gradient spans a distance ≤1/8 that of the transmembrane potential.

DISCUSSION

The electrical properties of biological membranes are usually studied with intracellular microelectrodes or patch-clamp techniques. These techniques, however, can only be applied on cells or organelles of sufficiently large size. Moreover, they only give information on potential differences between the bulk aqueous phases inside and outside the cell. They cannot be used to obtain information on electric profiles inside membranes. For this reason, we believe, electrophysiologists have not thoroughly investigated the regulatory influence of factors that change the intramembrane electric field without affecting the transmembrane potential. In this study, we show that the dual-wavelength potentiometric fluorescent probe di-8-ANEPPS can be used for measurements of the intra-membrane electric field in lipid vesicles and in cells and that it is particularly sensitive to the dipole potential.

A number of reagents appear to affect the spectral properties of this probe in a manner consistent with their known affects on dipole potential. KC and phloretin are known to increase and decrease the dipole potential in the membrane, respectively (Bechinger and Seelig, 1991; Franklin and Cafiso, 1993). They elicit respective increases and decreases in the dual-wavelength ratio, R, of the membrane-bound dye indicator (Fig. 2); the spectral changes underlying this response are not consistent with simple chemical complexation between the dye and either KC or phloretin (Fig. 5). Similarly, cholesterol is known to increase the dipole potential (Szabo, 1974), but it is also notorious for increasing the microviscosity of the bilayer. Cholesterol increases R in PC vesicles (Fig. 2), but changes in microviscosity elicited by changing the temperature in the same range do not affect R

(Fig. 3). Because di-8-ANEPPS is a well characterized member of a family of potentiometric indicators (Fluhler et al., 1985; Bedlack et al., 1992; Loew et al., 1992), it is not surprising that it is sensitive to this relatively unstudied component of the electrical properties of the membrane; it is fortunate, however, that the probe is apparently insensitive to several likely confounding variables.

R is much more sensitive to dipole potential changes than to changes in transmembrane potential. It is almost completely insensitive to changes in surface potential. This apparent variable sensitivity of R can be explained in terms of a mechanism in which the dye is fundamentally responsive to electric field rather than differences in electrical potential. Several studies from this laboratory have provided evidence that electrochromism is an important component of the dye response to membrane potential (Loew et al., 1979; Loew and Simpson, 1981; Fluhler et al., 1985; Loew et al., 1985, 1992). The spectral shift, $\Delta\nu$, of the chromophore's absorption or emission spectrum in an electric field, according to an electrochromic mechanism, is given by

$$\Delta\nu = (-1/h)\Delta\mu \cdot E - (1/2h)\Delta\alpha E^2, \tag{2}$$

where $\Delta\mu$ is the change in the electric dipole moment of the chromophore upon electronic excitation, $\Delta\alpha$ is the change in polarizability of the chromophore upon excitation and E is the electric field vector at the location of the chromophore. The first term describes frequency changes that depend linearly on the electric field and is generally the dominant contribution for the field strengths that pertain in biological membranes. Referring to Fig. 1., and from the relation $E = -\text{grad } V$, it can be seen that the electric field associated with Ψ_D, which rises steeply within a few Ångstroms, is much larger than the electric field set up by $\Delta\Psi$, which drops uniformly across the entire width of the membrane. Clearly, however, the chromophore must be appropriately located (as depicted in Fig. 1) to be able to experience the intense field associated with the dipole potential. Previous studies have shown that similar styryl dyes are indeed located inside the membrane, but near the membrane/water interface (Loew and Simpson, 1981; Fluhler et al., 1985). On the other hand, this site is inappropriate to measure the smaller field set up by Ψ_S, which produces an electric field in the aqueous phase rather than within the membrane. For each of these contributions to the electrical profile of the membrane, the distance over which the voltage changes is invariant. Therefore, the electric field intensity originating from one component at any point is proportional to the total potential difference of that component; however, the proportionality constant for each of the three potentials is, of course, very different and varies differently from point to point along the width of the membrane. The location of the dye shown in Fig. 1 is appropriate to explain the low sensitivity to Ψ_S, high sensitivity to Ψ_D, and moderate sensitivity to $\Delta\Psi$.

The relationship between the spectral shift and potential is linear, therefore, to the extent that the first term in Eq. 2 is dominant. Further, for small spectral shifts, R should also be linear. Because Ψ_D produces larger electric fields than $\Delta\Psi$, for which the dye had been previously used, it is important to reexamine these assumptions. The measurements for R are taken at the wings of the excitation spectrum where the total fluorescence is low and changes steeply with wavelength. The individual intensities in the blue, I_b, and green, I_g, each are linearly related to potential if the slope, m, of the spectral band is constant over the shifted wavelengths

$$R = I_b/I_g = \frac{m_b\Psi - b_b}{m_g\Psi - b_g}. \tag{3}$$

Fig. 7 was obtained by setting the R to unity at the reference potential taken for pure PC vesicles. This can be achieved either by normalization or, preferably, by balancing the intensities with the optics in the dual wavelength excitation paths. Under these conditions the intercepts $b_b = b_g \equiv b$ and, for small changes, Eq. 3 is approximated by

$$R \simeq 1 + \left(\frac{m_b - m_g}{b}\right)\Psi. \tag{4}$$

Because m_b and m_g have opposite signs, the relative fluorescence changes at each wavelength reinforce to deliver a larger relative change in R for a given change in potential. This equation underlies the calibration of transmembrane potential in the earlier work from this laboratory using ratiometric membrane potential indicators where the slope is typically 0.1/100 mV (Bedlack et al., 1992; Montana et al., 1989). The data in Fig. 7 indicate that Eq. 4 still adequately describes the much larger changes associated with the variations in dipole potential. A 100-mV change in Ψ_D produces a change in R of 0.8. This implies that the dye is sensing a dipole potential gradient at least 8 times steeper than the gradient associated with the transmembrane potential. In other words, if the membrane is ~40 Å wide, the dipole potential drops across a region no wider than 5 Å. It is generally accepted that the dipole potential does not extend out to the aqueous phase at the membrane surface (e.g., Peitzsch and McLaughlin, 1993), but there has been no previous direct evidence concerning its width or placement within the bilayer. Because the total dipole potential for a membrane composed of PC is estimated to be between 275 and 475 mV, the electric field generated across just 5 Å could approach 10^7 V/cm.

An important point to appreciate is that cell-to-cell variations in the dipole potential can change the electric field of the reference potential, and this can change the calibration. A practical implication is that di-8-ANEPPS, as well as other electrochromic potential indicators, cannot be used for measuring absolute potentials if the dipole potential inside the membrane is unknown. Also, if, unlike the situation depicted in Fig. 1, the charge densities at the two surfaces are not identical, there will be a potential gradient within the membrane due to the unsymmetrical Ψ_S at the inner and outer interfaces. Therefore, separate calibrations must be performed for each cell or vesicle preparation to be able to measure changes rather than absolute potentials. A concentrated suspension of PC liposomes labeled with di-8-ANEPPS

could serve as a standard reference sample for both bulk measurements in a fluorometer and single cells in a digital imaging microscope.

The results of this work have provided some new insights into the details of the electrical profile within the membrane and have outlined extensions of optical methods for studying them. Techniques for measuring and modulating dipole potential in cells were described that should permit initiation of studies for the first time into how this relatively obscure, but clearly large, component of the membrane potential profile might affect cell physiology. Is it possible that gradients in dipole potential exist along or across the surface of a cell? Can such gradients affect the conductance of ion channels? Are threshold potentials of gated channels affected by the dipole potential? Approaches toward answering these questions can be developed with combinations of electrophysiological techniques and dual wavelength imaging microscopy.

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