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Effect of lipid structure on the dipole potential of phosphatidylcholine bilayers

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Abstract

A fluorescent ratio method utilizing styrylpyridinium dyes has recently been suggested for the measurement of the membrane dipole potential. Up to now only qualititative measurements have been possible. Here the fluorescence excitation ratio of the dye di-8-ANEPPS has been measured in lipid vesicles composed of a range of saturated and unsaturated phosphatidylcholines. It has been found that the fluorescence ratio is inversely proportional to the surface area occupied by the lipid in its fully hydrated state. This finding allows, by extra- and interpolation, the packing density to be estimated of phosphatidylcholines for which X-ray crystallographic data are not yet available. Comparison of the fluorescence data with literature data of the dipole potential from electrical measurements on monolayers and bilayers allows a calibration curve to be constructed, so that a quantitative determination of the dipole potential using di-8-ANEPPS is possible. It has been found that the value of the dipole potential decreases with increasing unsaturation and, in the case of unsaturated lipids, with increasing length of the hydrocarbon chains. This effect can be explained by the effects of chain packing on the spacing between the headgroups. In addition to the effects of lipid structure on membrane fluidity, these measurements demonstrate the possibility of a direct electrical mechanism for lipid regulation of protein function, in particular of ion transport proteins.

Keywords: Voltage-sensitive dye; Dipole potential; Lipid vesicle; Fluorescence; Lipid packing; Lipid saturation

1. Introduction

The transmembrane electrical potential, $\Delta\psi$, is known to be capable of regulating the function of numerous membrane proteins, e.g. Na^+, K^+ and Ca^{2+} channels [1]. $\Delta\psi$ is defined as the difference in electrical potential in the bulk solution phases on both sides of the membrane. The magnitude and sign of $\Delta\psi$ can, therefore, be experimentally controlled by placing electrodes in the two solution phases. This allows a detailed investigation, for example, of the effect of $\Delta\psi$ on the opening and closing rates of ion channels. The molecular site of action of $\Delta\psi$ is, however, not in the solution phases, rather, it is within the membrane or at the membrane-solution interface, where the electric field created by $\Delta\psi$ can influence the structure of membrane proteins and the distribution of their charged substrates.

The electrical potential, ψ, does not change linearly across a lipid or cell membrane. There are various components which contribute to the value of ψ at any particular point within the membrane or in the adjacent aqueous solutions, leading to a complex

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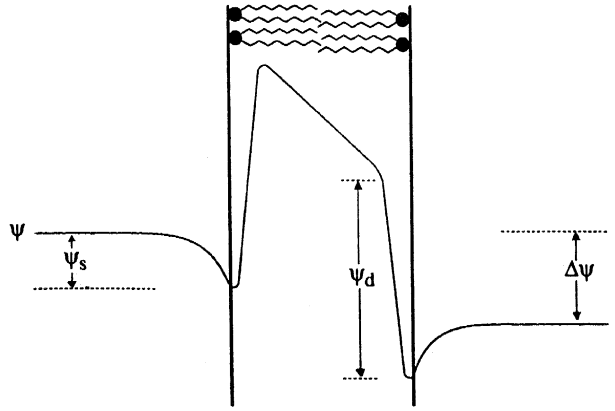

Fig. 1. The electrical potential, ψ, profile across a phospholipid bilayer. The transmembrane potential, Δψ, is due to the differences in anion and cation concentrations between the two bulk aqueous phases. The surface potential, ψ_s, arises from charged residues at the membrane-solution interface. The dipole potential, ψ_d, results from the alignment of dipolar residues of the lipids and associated water molecules within the membrane.

electrical potential profile across the membrane (see Fig. 1). The overall difference in potential, Δψ, between the two bulk phases separated by the membrane is due to the differences in anion and cation concentrations of the two phases. If the membrane contains lipids with charged headgroups, however, this leads to the formation of a surface potential, ψ_s, which controls the concentration of anions and cations at the membrane-solution interface. Furthermore, within the membrane, in the region between the aqueous phase and the hydrocarbon-like interior of the membrane, the alignment of dipolar residues of the lipids as well as water dipoles leads to the formation of a dipole potential, ψ_d [2].

Since the transmembrane potential can be accurately controlled using electrodes, its effect on membrane processes has been examined in great detail. On the other hand, the dipole potential has up to now received relatively little attention. This is certainly not due to its lack of importance, but rather that it is difficult both to control and to measure. Theoretical calculations [3] and kinetic measurements of the rate of transport of hydrophobic ions across bilayer membranes [4,5] and lipid vesicles [6] indicate that the dipole potential of phosphatidylcholine has a value in the range 220–280 mV, positive in the interior of the membrane. Because the dipole potential drops across a small distance within the headgroup region of the membrane, the electric field strength produced is very large; in the range 10^8–10^9 V m^{-1}. Such a high field strength is certainly capable of regulating the orientation of dipolar protein segments which are located in the lipid headgroup region of the membrane, particularly if the dipole moment of the segment is large, i.e., > 10 Debye.

Potential-sensitive fluorescent styryl dyes, such as RH421, RH160 and di-4- and di-8-ANEPPS, originally developed in the laboratories of Grinvald [7,8] and Loew [9–11], are presently attracting great interest as a means of optical imaging of electrical transients in neurons [12–15] and for the investigation of the reaction mechanisms of ion pumps, e.g., the Na^+,K^+-ATPase [16–20]. The dyes respond to a change in the local electric field strength with a shift in their fluorescence excitation spectrum. This has led to the proposal that the dyes might be used as probes of the membrane dipole potential [21–23]. In order to quantify the shift in the fluorescence excitation spectrum, dual wavelength excitation ratiometric fluorescence measurements have been suggested [21,23–28], similar to the method often employed when measuring cellular Ca^{2+} concentrations with the fluorescent indicator fura-2 [29]. In a previous paper [23] experimental conditions were reported under which the dyes RH421 and di-8-ANEPPS (see Fig. 2) are insensitive to changes in membrane fluidity and do not themselves affect the magnitude of the dipole potential. In the present paper, fluorescence ratio measurements have been carried out under these conditions using lipid vesicles made of a range of saturated and unsaturated phosphatidylcholines of varying chain length. In conjunction with X-ray crystallographic data and values of the dipole potential of lipid mono-

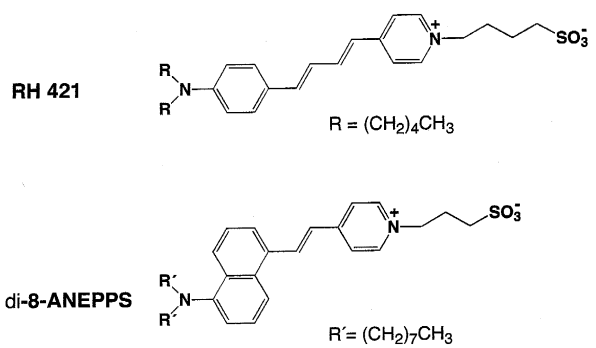

Fig. 2. Structures of RH421 and di-8-ANEPPS.

layers and bilayers from the literature, an analysis of the effect of lipid structure on the packing density and the dipole potential is made possible.

2. Materials and methods

N-(4-Sulphobutyl)-4-(4-(p-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) and 4-(2-(6-(dioctylamino)-2-naphthalenyl)ethenyl)-1-(3-sulphopropyl)-pyridinium inner salt (di-8-ANEPPS) were obtained from Molecular Probes (Eugene, OR, USA). In vesicles as well as in aqueous solution, both dyes showed a single long wavelength fluorescence emission band regardless of the excitation wavelength. A series of stock solutions of the dyes were prepared in ethanol. For spectral measurements, 5 μl of an ethanolic dye solution was added to 1 ml of aqueous solvent. The final solutions thus contained a small and constant percentage of 0.5% ethanol. In the case of experiments in the presence of lipid vesicles, after addition of the dye the solutions were left overnight to allow for dye disaggregation and incorporation into the membrane. The effect of the small volume of ethanol added on the fluorescence spectra of membrane-bound dye was checked in separate control experiments and found to be negligible.

All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere [22,28]. The final vesicle suspension contained no detectable trace of ethanol, i.e. [ethanol] $\leqslant 10$ μM, according to a nicotinamide adenine dinucleotide/alcohol dehydrogenase enzymatic assay (Boehringer, Mannheim). Dialysis tubing was purchased from Medicell International (London, UK). The phospholipid content of the vesicle suspension was determined by the phospholipid B test from Wako (Neuss, Germany).

Measurements with the vesicles were performed in a buffer containing 30 mM Tris, 1 mM EDTA, and 150 mM NaCl. The pH of the buffer was adjusted to 7.2 with HCl. All solutions were prepared using deionised water. The origins of the various reagents used were as follows: Tris-[(hydroxymethyl)amino]methane (99.9%, Sigma Chemical Co., St. Louis, MO, USA), EDTA (99%, Sigma Chemical Co.), NaCl (analytical grade, Merck, Darmstadt, Germany), HCl (1.0 M Titrisol solution, Merck), sodium acetate (99.7%, Sigma), acetic acid (2.0 N solution, Sigma), glycine (99%, Merck) and ethanol (analytical grade, Merck).

Absorbance measurements were performed with an Hitachi (Tokyo, Japan) U-3000 spectrophotometer equipped with a head-on photomultiplier so as to minimize the effects of light scattering. Steady state fluorescence measurements were recorded with an Hitachi F-4500 fluorescence spectrophotometer. To minimize contributions from scattering of the exciting light and higher order wavelengths, glass cut-off filters were used in front of the excitation and emission monochromators where appropriate. The temperature of the cuvette holder was thermostatically controlled.

Measurements of the pH dependence of the absorbance at a wavelength of 460 nm of di-8-ANEPPS were carried out using 0.1 M acetic acid/sodium acetate and 0.05 M glycine buffers of varying pH [30]. In the case of experiments with vesicles, NaCl (1.0 M) was added to the vesicle dialysis medium and to the buffers in order to prevent variations in the ionic strength on either side of the membrane. The determination of the pK_a values was carried out by fitting the data obtained from the pH titrations to the Henderson-Hasselbalch equation [31] using the commercially available nonlinear least-squares program ENZFITTER.

3. Results

3.1. Acid-base properties of the dyes

The dyes RH421 and di-8-ANEPPS possess two nitrogen atoms, one of which could be protonated. It has been observed previously [32] that the sensitivity of the dye RH421 to changes in the local electric field strength in lipid membranes depends on the pH. Therefore, it is important to know the pK_a values of the dyes, so that one can predict at a given pH which form of the dye is predominating.

The apparent pK_a of RH421 has been determined to be 4.9 (± 0.1) in aqueous solution [33], 4.1 (± 0.1) when bound to dioleoylphosphatidylcholine (DOPC) vesicles [33] and 3.1 (± 0.1) when bound to dimyris-

toylphosphatidylcholine (DMPC) vesicles [32]. The apparent pK_a values have been calculated based on the pH measured in the bulk aqueous solution using a glass electrode. The lower apparent pK_a values of RH421 when bound to lipid vesicles in comparison to the value obtained in aqueous solution can be explained by a lower partition coefficient of the protonated form into the membrane than the deprotonated form [34,35]. This would be expected, since the deprotonated form has a positive charge and its transfer into the low dielectric constant medium of the membrane would be energetically unfavourable. The lower apparent pK_a in DMPC vesicles compared to DOPC is likely to be due to the difference in the packing density of the lipids and its effect on the local dielectric constant in the membrane and the dipole potential [22,23].

The variation of the absorbance of the dye di-8-ANEPPS at 460 nm and a constant dye concentration of 0.43 μM was measured in aqueous solution as a function of pH. The data have been fitted to the Henderson-Hassselbalch equation as described in the Materials and Methods section and a pK_a of 3.4 (± 0.1) has been obtained. Similarly, the variation of the absorbance of di-8-ANEPPS at 460 nm and a constant dye concentration of 4.0 μM was measured in the presence of 228 μM of DMPC in the form of vesicles as a function of pH. Down to a pH of 2.5 no significant protonation of the dye could be observed. The titration could not be extended to pH values below 1.8, because of protonation of the phosphate head group of the lipid and the consequent phase transition of the bilayer [36], which causes a significant increase in the turbidity of the suspension. The apparent pK_a of di-8-ANEPPS bound to DMPC vesicles can, therefore, only be estimated to have a value of < 1.8.

At a pH of 7.2, the pH used for the subsequent fluorescence ratio measurements, both RH421 and di-8-ANEPPS can be considered to be in their fully deprotonated states.

3.2. Fluorescence excitation ratios in lipid vesicles

Fluorescence excitation spectra have been recorded of RH421 and di-8-ANEPPS bound to phosphatidylcholine vesicles of differing degrees of saturation and different chain lengths. The observed spectral shifts have been quantified via a ratiometric method, i.e., the ratio of the fluorescence intensities detected at two excitation wavelengths on the blue and red flanks of the excitation spectrum was measured. In the case of RH421, the excitation wavelengths chosen were 440 nm and 540 nm. In the case of di-8-ANEPPS, the excitation wavelengths chosen were 420 nm and 520 nm. For both dyes the ratio was measured at an emission wavelength of 670 nm. Using these wavelengths it has been found [23] that the fluorescence excitation ratio, R, is independent of the fluidity of the membrane and the temperature used for the measurement, as long as the temperature exceeds the gel-to-liquid crystalline phase transition temperature of the lipid, so that the membrane is totally in the liquid crystalline phase. For lipids with a phase transition temperature below or close to 0°C, the experiments were, therefore, performed at a temperature of 20°C. In the case of lipids with higher phase transition temperatures, the experiments were performed at a temperature of at least 2°C above the corresponding phase transition temperature. In order to avoid any dye-induced increases in the magnitude of the dipole potential [23,37], a molar excess of lipid over dye of at least 350 was used in each case. The fluorescence ratios obtained are given in Table 1.

If one compares the R values obtained for RH421 and di-8-ANEPPS, one can see that di-8-ANEPPS shows a greater sensitivity towards the structure of the lipid than RH421. In the case of di-8-ANEPPS, the R value varies between values of 2.025 for dimyristoylphosphatidylcholine and 0.728 for diarachidonoylphosphatidylcholine, i.e., a variation of 178% from the lower value. In the case of RH421, the R value varies between values of 1.674 for dilauroylphosphatidylcholine and 0.903 for diarachidonoylphosphatidylcholine, i.e, a variation of 85%. This agrees with the finding published previously [23] that di-8-ANEPPS is more sensitive than RH421 towards the addition of phloretin and 6-ketocholestanol, substances which have been proposed to decrease [38] and increase [39] the dipole potential, respectively. More importantly, on inspecting the R values given in Table 1 the following points regarding its dependence on the lipid structure become obvious:

1. The value of R decreases with increasing number of double bonds in the chain.

2. For the fully saturated lipids, the value of *R* is almost independent of the chain length, whereas for the unsaturated lipids, the value of *R* decreases with increasing chain length.
3. Unsaturated lipids with a *cis* double bond give a lower *R* value than the corresponding lipids with a *trans* double bond.

All of these observations can be explained on the basis of the packing density of the lipid molecules in the bilayer membrane. Increasing the number of double bonds in the chain would be expected to disturb the packing of the chains, so that each lipid molecule requires more surface area in the membrane. For fully saturated lipids the chain length would not be expected to have a major influence on the packing, because there are no kinks in the chain, whereas for unsaturated lipids the chain length would be expected to have a greater effect, because the hydrocarbon chain after the kink caused by a double bond would tend to force the lipid molecules further apart. A *cis* double bond would also be expected to cause a greater disturbance to the lipid packing than a *trans* double bond, where the kink produced by the double bond is less pronounced. It, therefore, appears that the value of *R* is related to the surface area occupied by the lipid molecule in the plane of the membrane perpendicular to the chains, i.e., *R* decreases with increasing area per lipid molecule.

To test this hypothesis, values of the area per lipid molecule, *A*, based on X-ray crystallographic [40–45] and NMR [46] measurements of phosphatidylcholine membranes in the fully hydrated state have been collected from the literature. Because of the difficulties of such measurements, up to now *A* has only

Table 1
Effect of phosphatidylcholine structure on the fluorescence excitation ratios, *R*

PC	*R* (RH421)	*R* (di-8-ANEPPS)	*T* (°C)	*A* (nm^2)	ψ_d (mV)
12 : 0	1.674 (±0.006)	1.926 (±0.008)	20	0.65 (±0.12)	271 (±108)
13 : 0	1.620 (±0.013)	1.969 (±0.009)	20	0.64 (±0.12)	293 (±116)
14 : 0	1.669 (±0.011)	2.025 (±0.010)	30	0.63 (±0.11)	303 (±118)
15 : 0	1.590 (±0.017)	2.012 (±0.017)	40	0.63 (±0.11)	301 (±118)
16 : 0	1.568 (±0.017)	1.981 (±0.006)	45	0.64 (±0.12)	295 (±116)
17 : 0	1.546 (±0.008)	1.988 (±0.017)	50	0.64 (±0.12)	296 (±117)
18 : 0	1.569 (±0.027)	1.917 (±0.030)	60	0.65 (±0.12)	283 (±113)
14 : 1 tΔ^9	1.485 (±0.013)	1.556 (±0.008)	20	0.75 (±0.15)	216 (± 98)
14 : 1 cΔ^9	1.395 (±0.010)	1.366 (±0.008)	20	0.81 (±0.16)	180 (± 91)
16 : 1 tΔ^9	1.378 (±0.004)	1.496 (±0.006)	20	0.77 (±0.15)	205 (± 96)
16 : 1 cΔ^9	1.319 (±0.003)	1.374 (±0.006)	20	0.81 (±0.16)	182 (± 91)
16 : 0/18 : 1 cΔ^9	1.273 (±0.004)	1.555 (±0.005)	20	0.75 (±0.15)	216 (± 98)
18 : 1 cΔ^9/18:0	1.217 (±0.003)	1.424 (±0.004)	20	0.79 (±0.16)	191 (± 93)
18 : 1 tΔ^9	1.170 (±0.007)	1.373 (±0.002)	20	0.81 (±0.16)	182 (± 91)
18 : 1 cΔ^9	1.129 (±0.007)	1.313 (±0.004)	20	0.83 (±0.17)	171 (± 89)
18:1 cΔ^6	1.100 (±0.005)	1.274 (±0.008)	20	0.84 (±0.18)	163 (± 87)
20 : 1 cΔ^{11}	0.994 (±0.005)	1.087 (±0.004)	20	0.92 (±0.20)	128 (± 81)
22 : 1 cΔ^{13}	0.934 (±0.004)	1.054 (±0.006)	20	0.93 (±0.23)	122 (± 81)
24 : 1 cΔ^{15}	1.041 (±0.006)	1.160 (±0.004)	30	0.89 (±0.19)	142 (± 84)
18 : 2 c$\Delta^{9,12}$	1.095 (±0.010)	0.976 (±0.004)	20	0.97 (±0.22)	108 (± 79)
18 : 3 c$\Delta^{9,12,15}$	0.970 (±0.007)	0.778 (±0.003)	20	1.08 (±0.27)	71 (± 75)
20 : 4 c$\Delta^{5,8,11,14}$	0.903 (±0.014)	0.728 (±0.002)	20	1.11 (±0.28)	62 (± 74)
Dihexadecyl	1.094 (±0.005)	1.059 (±0.005)	50	–	123 (± 81)
Egg	1.372 (±0.018)	1.466 (±0.012)	20	0.78 (±0.15)	199 (± 94)

RH421 (4.25 μM) or di-8-ANEPPS (4.34 μM) were added from ethanolic stock solutions to phosphatidylcholine vesicles ([PC] = 1.5–3.2 mM). In the case of RH421 the fluorescence ratio, *R*, is defined as the fluorescence intensity of the membrane-bound dye measured at an excitation wavelength, λ_{ex}, of 440 nm divided by that measured at 540 nm. For di-8-ANEPPS, *R* is defined as the fluorescence intensity at a λ_{ex} of 420 nm divided by that at a λ_{ex} of 520 nm. In each case the emission wavelength used was 670 nm. *A* is the area per lipid molecule calculated on the basis of Eq. (2). ψ_d is the dipole potential calculated on the basis of Eq. (5). The calculations of *A* and ψ_d are based exclusively on measurements using di-8-ANEPPS. The values of the errors quoted are all standard errors.

been determined for a relatively small number of phosphatidylcholines. The values of the packing densities ($1/A$) and the fluorescence excitation ratios, R, obtained using di-8-ANEPPS are given in Table 2. A linear relationship (correlation coefficient = 0.97) was found between the R values and $1/A$ (see Fig. 3). The straight line is defined by the equation:

$$R = \frac{k}{A} + c \quad (1)$$

where $k = 1.88$ (± 0.25) nm^2 and $c = -0.96$ (± 0.36). Rearranging Eq. (1) gives the equation,

$$A = \frac{1.88}{R + 0.96} \quad (2)$$

which allows the area per lipid molecule in the plane of the membrane, A, in nm^2 to be estimated from the measured R value. In this way the packing densities and the surface areas per lipid molecule can be estimated for phosphatidylcholines for which X-ray crystallographic data is not yet available (see Table 1).

The value of c can be considered to be the hypothetical value of R expected for an infinitely low lipid packing density. Theoretically c can not be less than zero. The fact that the straight line plot of R and $1/A$ yields a negative value for c indicates that at packing densities lower than those of the lipids used in the present study the linear relationship is likely to break down. Presumably, at very low packing densities significant amounts of water would enter between the lipids. This would be expected to alter the dielectric constant of the dye surroundings, so that the value of R would eventually approach a value for dye in pure water.

If the R values of RH421 are plotted against the values of $1/A$ given in Table 2, an approximately linear relationship is also found. However, since the range of R values is significantly greater for di-8-ANEPPS than for RH421, only the di-8-ANEPPS results have been fitted to a straight line and presented in Fig. 3.

Table 2
Comparison of the fluorescence ratio, R, of di-8-ANEPPS with X-ray crystallographic packing densities, 1/A, of various phosphatidylcholines

PC	R	$1/A$ (nm^{-2})	Ref.
12:0	1.926 ($\pm$0.008)	1.456	[40]
14:0	2.025 ($\pm$0.010)	1.608	[41,42]
16:0	1.981 ($\pm$0.006)	1.590	[43,46]
18:0	1.917 ($\pm$0.030)	1.538	[44]
Egg PC	1.466 ($\pm$0.012)	1.323	[45]
18:1 cΔ^9	1.313 ($\pm$0.004)	1.220	[40]

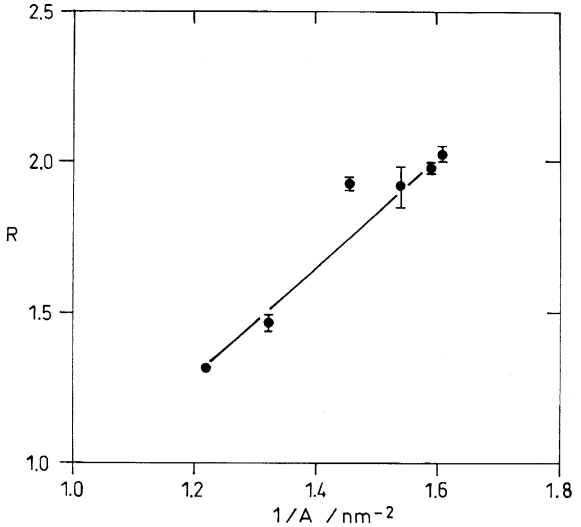

Fig. 3. Correlation between the fluorescence excitation ratio, R, of di-8-ANEPPS and the lipid packing density, $1/A$. R is defined as the fluorescence intensity of membrane-bound dye measured at an excitation wavelength, λ_{ex}, of 420 nm divided by that at a λ_{ex} of 520 nm. The emission wavelength used was 670 nm. The values of $1/A$ have been taken from the literature (see Table 2). The solid line represents a fit of the data to a straight line.

The fact that the fluorescence excitation spectra of RH421 and di-8-ANEPPS depend on the lipid structure indicates that the dyes must be experiencing different electronic environments within the membrane of the various lipids. The fact that the fluorescence ratio, R, of di-8-ANEPPS is directly proportional to the lipid packing density, $1/A$, leads one to suspect that the electronic property being detected by the dye is in fact the dipole potential of the membrane. According to the Helmholtz equation [47–49], which compares a lipid monolayer to a parallel-plate capacitor, the dipole potential, ψ_d, is related to $1/A$ by

$$\psi_d = \frac{\mu_\perp}{A\epsilon_o\epsilon} \quad (3)$$

where $\mu_\perp$ is the average component of the lipid molecular dipole moment (including membrane-asso-

ciated water molecules) perpendicular to the plane of the membrane, $_o$ is the permittivity of free space and is the local dielectric constant. From Eq. (3) it can be seen that ψ_d is expected to be directly proportional to $1/A$, as has been found experimentally for the fluorescence ratio R of di-8-ANEPPS.

3.3. Comparison of R with dipole potential values

In order to test the idea further, that the dye di-8-ANEPPS is responding to changes in the membrane dipole potential, we have collected data of the dipole potential from the literature from direct electrical measurements on monolayers [50] and kinetic measurements using hydrophobic probes on bilayers [4,5] of various phosphatidylcholines. The values of the dipole potential, ψ_d, and the R values of di-8-ANEPPS for the corresponding lipids are given in Table 3. The bilayer measurements of Pickar and Benz [4] and those of Gawrisch et al. [5] are based on the ratio of the transmembrane transport rates of the hydrophobic anion tetraphenylborate and the hydrophobic cation tetraphenylarsonium, which have been measured via the charge pulse [4] and the voltage jump [5] methods. The monolayer measurements of Smaby and Brockman [50] are based on the difference between the electrical potential difference across a lipid monolayer, measured using an ionizing electrode, and the electrical potential difference of the pure air-water interface. Discrepancies have previously been reported between monolayer and bilayer dipole potentials of the same lipid; the bilayer values being 100–150 mV lower than the monolayer values [50]. Smaby and Brockman [2,50] have suggested that these discrepancies may be due to an area-independent contribution to the measured monolayer surface potential, since they observed a non-zero intercept in their plots of surface potential against the lipid packing density. They proposed that the area-independent contribution may come from a reorganization of the water structure by the lipid head groups. Therefore, in order to obtain a meaningful comparison between the bilayer and monolayer dipole potentials given in Table 3, the area-independent contribution has been subtracted from the monolayer data. The monolayer dipole potentials have been calculated for the area per lipid molecule expected from X-ray crystallographic data (see Table 2) according to the Helmholtz equation as given by Smaby and Brockman (see Eq. (1) in [50]).

Table 3
Comparison of the fluorescence ratio, R, of di-8-ANEPPS with electrical bilayer and monolayer measurements of the dipole potential, ψ_d, of various phosphatidylcholines

PC	R	ψ_d (mV)	Ref.
12:0	1.926 (±0.008)	225	[50]
14:0	2.025 (±0.010)	304	[50]
16:0	1.981 (±0.006)	281	[50]
16:0	1.981 (±0.006)	227	[5]
18:1 cΔ^9	1.313 (±0.004)	224	[50]
18:1 cΔ^9	1.313 (±0.004)	213	[50]
18:1 cΔ^9	1.313 (±0.004)	224	[4]
18:1 cΔ^9/18:0	1.424 (±0.004)	197	[4]
Dihexadecyl	1.059 (±0.005)	109	[5]

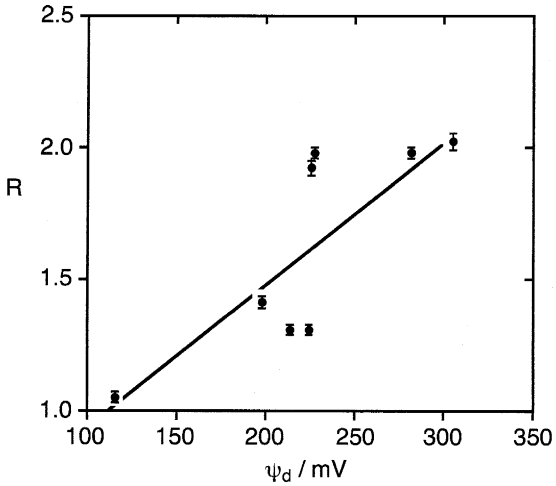

Fig. 4. Correlation between the fluorescence excitation ratio, R, of di-8-ANEPPS and the membrane dipole potential, ψ_d. The wavelengths used for the calculation of R were as given in Fig. 3. The values of ψ_d have been taken from the literature (see Table 3). The solid line represents a fit of the data to a straight line.

If the R values obtained using di-8-ANEPPS are plotted against the literature values of ψ_d an approximately linear relationship (correlation coefficient = 0.77) is obtained (see Fig. 4). Considering the very different electrical methods used and the difficulties and assumptions involved in the determination of ψ_d, a certain degree of scatter of the values of ψ_d is perhaps to be expected. Nevertheless, a correlation between the R value and ψ_d seems to be present. If

the data in Table 3 are fitted to a straight line as defined by the equation:

$$R = m\psi_d + d \quad (4)$$

the slope, m, and the intercept, d, are calculated to be 5.4 $(\pm 1.7) \cdot 10^{-3}$ mV^{-1} and 0.4 (± 0.4), respectively. Rearranging Eq. (4) results in the equation,

$$\psi_d = \frac{R - 0.4}{5.4 \cdot 10^{-3}} \quad (5)$$

which allows the dipole potential of a given phosphatidylcholine to be calculated from the measured R value. Values calculated in this way are given in Table 1. It can be seen that the calculated ψ_d values have large errors. This is not due to the fluorescence measurements, which are very accurate, but rather, due to the scatter of the electrical values of ψ_d taken from the literature, which have been used to calibrate the dye fluorescence ratios. If the calculated values of ψ_d in Table 1 are plotted against the experimentally determined values given in Table 3, one obtains a correlation coefficient of 0.79. Further electrical measurements using a wider range of phosphatidylcholines may be able to provide a more reliable calibration of the dye and hence more confidence in the dipole potential values quoted.

4. Discussion

Summarizing, the fluorescent styrylpyridinium dyes RH421 and di-8-ANEPPS have been found to show shifts in their fluorescence excitation spectra when bound to vesicles composed of phosphatidylcholine of varying structures. The shifts have been quantified by using the ratio, R, of the fluorescence intensity detected at two different excitation wavelengths. Previous work [23] has shown that changes in membrane fluidity can be excluded as a cause of the varying R values. Therefore, under the conditions of the experiments performed here, it can be concluded that the dyes are detecting an electrical property of the membrane.

It has been found that the fluorescence ratio, R, of di-8-ANEPPS is directly proportional to the packing density, $1/A$, of the lipids in the bilayer (see Fig. 3). Therefore, it seems most likely that the property being detected by the dye is the membrane dipole potential, ψ_d, since in this case a linear dependence on $1/A$ would be expected on the basis of the Helmholtz equation (see Eq. (3)). In further agreement with this, a comparison of the R values of di-8-ANEPPS with electrically determined values of ψ_d from the literature indicate an approximately linear correlation (see Fig. 4).

On the basis of the observed correlations of the R value of di-8-ANEPPS with the lipid packing density and the dipole potential, values of the area per lipid molecule in the plane of the membrane, A, and of ψ_d could be estimated for all of the lipids measured, using Eqs. (2) and (5), respectively. These values are given in Table 1. In this way values of A could be estimated for many phosphatidylcholines for which X-ray crystallographic data is not yet available, and likewise, ψ_d could be estimated for many phosphatidylcholines where no electrical measurements have yet been performed.

If one accepts that di-8-ANEPPS is in fact detecting ψ_d, then implicit in the linear relationship between R and $1/A$ is a constant value of the ratio $\mu_\perp/$ for all of the lipids measured (cf. Eq. (3)). For the ether lipid dihexadecylphosphatidylcholine this may not necessarily be true, if the carbonyl group of all of the other lipids is making a significant contribution to the dipole moment in the head group region. For this reason the area per lipid molecule of dihexadecylphosphatidylcholine has been omitted from Table 1. If, in spite of this, a value is calculated using Eq. (2), one arrives at a value for A of 0.93 (± 0.21) nm^2. This is the value one would expect, if the carbonyl group plays no significant role in determining the value of the dipole potential. Unfortunately X-ray crystallographic data on this lipid is not yet available, so that the role of the carbonyl group cannot yet be determined. Nevertheless, it appears that the carbonyl group is not a necessary requirement for the production of a dipole potential, since both ester and ether phosphatidylcholines possess a significant positive value [4,5].

One further point should be made concerning the calibration of the fluorescence ratio of di-8-ANEPPS for the quantitative determination of ψ_d. The calibration is based on electrically determined values of ψ_d, which, in the absence of any transmembrane potential, is the electrical potential which determines the

local electric field strength experienced by the chromophore of the dye in its environment in the head group region of the membrane (see Fig. 1). Previously a quantitative determination of electric field strength in membranes using di-8-ANEPPS was reported by Bedlack et al. [11]. Their calibration procedure was based on the production of transmembrane K^+-diffusion potentials by the addition of valinomycin in the presence of transmembrane K^+ concentration gradients. In order to obtain the intramembrane electric field strength they simply divided the diffusion potential by the thickness of the membrane. This procedure assumes a linear change in the electrical potential across the membrane. As one can see in Fig. 1, this is certainly not the case. Therefore, the electric field strengths quoted by Bedlack et al. [11] are likely to be grossly in error.

The results presented here suggest that the degree of saturation and the chain length of phosphatidylcholines have a major influence on the magnitude of the membrane dipole potential. Based on the data presented in Table 1, ψ_d can vary from a value of around 300 mV for a fully saturated phosphatidylcholine, such as dimyristoylphosphatidylcholine (14:0), to a value of around 60 mV for a highly unsaturated long chain phosphatidylcholine, such as diarachidonoylphosphatidylcholine (20:4 c$\Delta^{5,8,11,14}$). Such a large variation in the dipole potential would produce large differences in the electric field strength in the head group region of the membrane depending on the lipid. Since it is known that the conformational state of ion channels can be regulated by the local electric field strength [1], this suggests a possible direct electrical role of the lipids in controlling the function of ion-transporting membrane proteins. Normally lipid unsaturation is suggested to be an important factor in maintaining membrane fluidity and hence protein function. In addition to its role in fluidity, the results presented here lead one to think that lipid unsaturation may, through its effect in reducing the dipole potential, electrically influence membrane protein structure and hence the kinetics of ion transport. A similar electrical mechanism has been suggested by Bedlack et al. [26] for the role of cholesterol in cell membranes. Now that the dye di-8-ANEPPS is available as an optical probe of dipole potential, these possibilities can be investigated in more detail.

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