Biochimica et Biophysica Acta, 980 (1989) 15–22
Elsevier

BBA 74352

Segregation of anionic lipophiles in bilayers monitored by binding of cationic dye NK-529

Bao-Zhu Yu * and Mahendra Kumar Jain

Department of Chemistry, University of Delaware, Newark (U.S.A.)

(Received 26 October 1988)

Key words: Phase separation; Lipophile; Bilayer; Fluorescence; NK-529; Cyanine dye

Fluorescence emission properties of a cationic indodicarbocyanine dye, NK-529, bound to anionic and zwitterionic vesicles, are examined under a variety of conditions to monitor lateral distribution of anionic amphiphiles in bilayers as a function of their phase properties. The change in the fluorescence properties of NK-529 arises from the binding of the dye to the bilayer that is dominated by ionic interactions when possible, as well as from the self-quenching of the dye bound to bilayers when the surface density of the dye is high. The binding affinity of the dye to anionic interfaces is more than 100-fold higher compared to that in zwitterionic bilayers. The limiting phospholipid/dye ratio in anionic bilayers at low vesicle concentrations is about 3. Thus the density of the bound dye in anionic bilayers can be more than 40-fold higher than that in zwitterionic bilayers, and therefore under such conditions the bound dye is completely self-quenched in vesicles or micelles of anionic phospholipids. The change in the fluorescence emission intensity on incorporation of anionic amphiphiles in zwitterionic bilayers is used to monitor segregation of the anionic amphiphiles. The organizational features of bilayers that cause a change in the fluorescence properties of bound NK-529 show that the lateral distribution of anionic amphiphiles is appreciably influenced not only by the mole fraction of the amphiphile but also in the presence of other additives, and by the gel-fluid thermotropic transition. As shown in the following paper, the fluorescence changes related to self-quenching in anionic bilayers containing NK-529 can be used to understand the organizational changes that occur during the course of interfacial catalysis by phospholipase A_2 on zwitterionic bilayers.

Introduction

Spectral probes are well suited to characterize the changes in the surface charge properties of bilayers [1–3]. In order to understand the biophysical basis of the complex kinetics of the catalytic action of phospholipase A_2 on zwitterionic bilayers [4–9] we searched for a probe that could directly report changes in the surface charge density and distribution occurring at the interface during the interfacial catalysis. Both the products of hydrolysis of phospholipids by phospholipase A_2 remain in the bilayer and the integrity of the bilayer is retained [10–13]. Since one of these is anionic in the pH range under consideration, we found that the fluorescence properties of a cationic indodicarbocyanine dye, NK-529, were sensitive enough to report the changes in the phase properties of the bilayer that influence the distribution of anionic amphiphiles. In this paper we characterize the spectral and equilibrium binding properties of NK-529 in bilayers under conditions that are used for monitoring interfacial catalysis on zwitterionic bilayers. In the next paper we apply these findings to elaborate the changes in the interfacial properties of the bilayer that are responsible for the binding and catalytic action of phospholipase A_2 [14].

Materials and Methods

Phospholipids used in this study were more than 99% pure and synthesized as described elsewhere [11,12] or purchased from Avanti (Birmingham, AL). NK-529 (1,3,3,1′,3′,3′-hexamethylindocarbocyanine) was purchased from Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan). Vesicles were prepared by sonication of a frozen aqueous (10 mM Tris, pH 8.0) suspension of

* Permanent address: Department of Physics, Peking University, Beijing, China.

Abbreviations: LPC, 1-myristoyl-*sn*-glycero-3-phosphoryl choline; DMMe, 1,2-dimyristoyl-*sn*-glycero-3-phosphoryl methanol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphoryl choline; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphoryl choline; NK-529, 1,3,3,1′,3′,3′-hexamethylindocarbocyanine.

Correspondence: M.K. Jain, Department of Chemistry, University of Delaware, Newark, DE 19716, U.S.A.

appropriate lipid, or a dried film of the mixtures of phospholipids, in a bath type sonicator (Sonicor) for 2–5 min till a clear suspension was obtained. The ternary codispersions were prepared from preformed DMPC vesicles by adding the aqueous solution of LPC followed by an ethanol solution of myristic acid in small aliquots. This protocol works reasonably well for incorporation of less than 15 mol% LPC + myristic acid in the vesicles. Under these conditions the additives are presumably present only in the outer monolayer, and their mole fractions are expressed with this assumption.

The experimental conditions used in this and the next paper [14] are essentially identical. All spectroscopic measurements were made in 10 mM Tris chloride at pH 8.0. The buffer used for measurements with DMPC vesicles also contained 5 mM $CaCl_2$. Osmolarity was generally kept constant, although the observations reported in this or the next paper do not appear to be sensitive to such asymmetries. Other conditions, if significantly different, are as given in the figure legends. Unless stated otherwise the concentration of the dye was 5.2 μM (added as 20 μl of ethanol solution to 2 ml of the aqueous buffer) and the concentration of phospholipids was 0.590 mM. Typically, the temperature of the aqueous dye solution was allowed to equilibrate and the lipid dispersions were added after about 5 min. Independent controls with vesicles loaded with potassium sulfate and doped with valinomycin showed that the fluorescence changes described in this and the next paper [14] are not due to changes in transmembrane diffusion potential. Similarly, a direct effect of ionic strength and pH changes is ruled out in order to account for the origin of the fluorescence changes reported in these papers.

Steady-state fluorescence measurements were made in 2 ml Tris-chloride buffer (10 mM) in 1 cm cuvette on SLM 4800S spectrofluorimeter (450W Xenon source) interfaced to a microcomputer to collect and process data. The excitation and emission slitwidths were kept at 4 nm, and all spectra are uncorrected. Polarizers at magic angle were used in the excitation and emission paths to minimize possible contributions from scattering, and control experiments showed that the spectral properties were not noticeably influenced under these conditions. The absorption spectra were obtained on a diode array spectrophotometer (HP8452) equipped with appropriate data processing software for manipulation of spectra.

Results

The fluorescence properties of NK-529 in aqueous phase and zwitterionic vesicles

NK-529 is a water-soluble cationic fluorescent dye. Its excitation maximum is at 620 nm. As shown in Fig. 1, the emission intensity and emission maximum (between 650 and 680 nm) of the dye depends upon the polarity and charge distribution in the microenvironment in which it is localized. The spectral properties in the aqueous phase are not qualitatively influenced by temperature in the 15–35°C range, by the presence of calcium or other salts, or by the presence of fatty acids or other additives in the interface. For example, in dilute aqueous solutions the fluorescence intensity at the emission maximum (660 nm) shows a linear dependence on the dye concentration up to 5 μM, and above 8 μM the fluorescence intensity is self-quenched. The decrease in the fluorescence quantum yield is due to self-quenching in the aqueous phase, and it is most pronounced at the emission maximum of 660 nm. However, as elaborated in this and the next paper, subtle changes in the fluorescence intensity occur under certain conditions that provide insight into the mechanism of binding of phospholipase A_2 to the substrate interface.

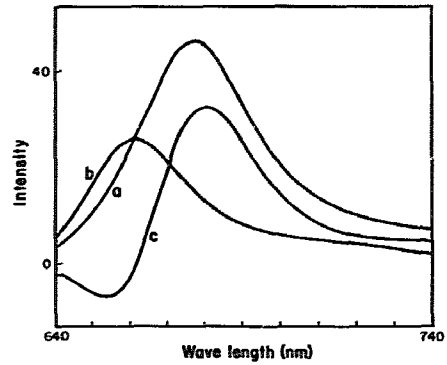

Fig. 1. Fluorescence emission spectra of 5.2 μM NK-529 (curve b) in the aqueous phase, and (curve a) in the presence of 0.59 mM DMPC vesicles in 10 mM Tris chloride and 5 mM $CaCl_2$ at pH 8.0 and 21°C. Curve c is the difference spectrum (a−b), which does not qualitatively change as a function of an increasing concentration of DMPC vesicles.

NK-529 binds to vesicles and micelles of a variety of phospholipids. Binding is rapid, and the bound dye is readily exchangeable with excess vesicles in less than a minute. As shown in Fig. 1, in the presence of DMPC vesicles the fluorescence emission intensity of the dye increases and the maximum is shifted from 660 nm to 677 nm. In the difference spectra the emission maximum remains at 680 nm at all concentrations (0.02 to 2 mM) of vesicles investigated. As shown in Fig. 2 the emission intensity of the dye at 685 nm increases about 4-fold, and reaches a maximum when all the dye is bound to vesicles. The binding curves shown in Fig. 2 are not a simple hyperbolic function. Based on the results described later it is interpreted by assuming that there are two populations of the dye in equilibrium: bound (D_b) and free (D_w), and the fluorescence quan-

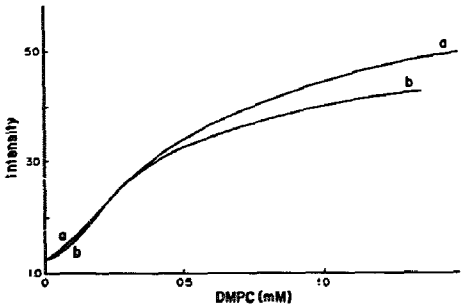

Fig. 2. Dependence of the fluorescence emission intensity of 5.2 μM NK-529 at 685 nm on the total phospholipid concentration for (a) DMPC vesicles and (b) for DMPC vesicles containing 25 mol% LPC + myristic acid (1 : 1) in the outer monolayer. Other conditions as given in the legend to Fig. 1.

tum yield of the bound dye depends upon the lipid/dye ratio. The apparent lipid-water partition coefficient of NK-529 on DMPC vesicles from the linear region of the curve (Fig. 2) is calculated to be about 4000 in the favor of DMPC vesicles on the weight basis according to the protocols outlined elsewhere [11]. The partition coefficient would be about 6000 if it is assumed that the dye binds only to the outer monolayer of vesicles. Similarly, by a linear extrapolation of the middle linear region of the binding curve (Fig. 2) to the maximum change in the intensity it can be calculated that the maximum density is about 1 dye molecule per 100 phospholipid molecules in the outer monolayer of DMPC vesicles. These values of the partition coefficient are also a measure of the affinity of the dye for the interface.

Binding isotherms for NK-529 with aqueous dispersions of several zwitterionic phospholipids were obtained and the characteristic parameters derived from such curves are summarized in Table I. The emission spectra of NK-529 at these interfaces are essentially the same with emission maximum at 677 nm. Similarly, values of the apparent partition coefficient of the dye in these dispersions differ by less than 20%. The results suggest that at high lipid/dye ratios, NK-529 does not appreciably distinguish between the environments of vesicles and micelles. Also, the gel-fluid phase properties of the bilayer or the structure of the zwitterionic phospholipids do not change the steady-state fluorescence spectral properties, although a small change in the partition coefficient is noted.

In the titration curves of the type shown in Fig. 2, at very low lipid concentrations (below 20 μM) the increase in the intensity at 685 nm is somewhat smaller than that is theoretically expected for a simple binding equilibrium which predicts a hyperbolic binding isotherm [15]. If under these conditions the mole fraction of the dye on vesicles is relatively high, there could be self-quenching of the bound dye. Such a behavior is

TABLE I

Parameters for equilibrium binding of NK-529 to vesicles of zwitterionic phospholipids in 10 mM Tris at pH 8.0

All vesicles contained 10 mM Tris chloride except that shown with water alone. The partition coefficients, P, are expressed as (moles of dye per gram of lipid)/(mole of dye per gram of water), and are corrected for the localization of the dye only in the outer monolayer of the vesicle.

Lipid	In	Out	I_m/I_w	L/D ratio	P
DMPC	–	Ca	4.0	93	6000
	Ca	Ca	3.8		
	water	Ca	4.0		
	–	–	4.6		
	Ca	–	4.5		
	water	–	5.0		
DMPC + 25% products			3.2	78	7890
DTPC	water	–	4.4	87	5980
POPC	water	–	4.9	63	6260
HexadecylPC		–	4.0	107	5510
DOPE	water	–	4.6	64	7210

expected if the bound dye promotes binding of excess dye. This would not occur in excess lipid because most of the dye is bound to vesicles.

Since NK-529 has two cationic groups, we examined the binding of the dye to anionic vesicles. As shown in Fig. 2, the binding curve for ternary codispersions containing 25 mol% products in the outer monolayer is different; the apparent affinity is similar but the fluorescence quantum yield of the bound dye is smaller than that in DMPC vesicles. The self-quenching of NK-529 in bilayers is considerably more pronounced in vesicles containing varying mole fractions of the products, 1-myristoylglycerophosphorylcholine + myristic acid, at a constant concentration of DMPC vesicles. As shown in Fig. 3 the fluorescence intensity at 685 nm decreases sharply at mole fraction 0.03 of the products

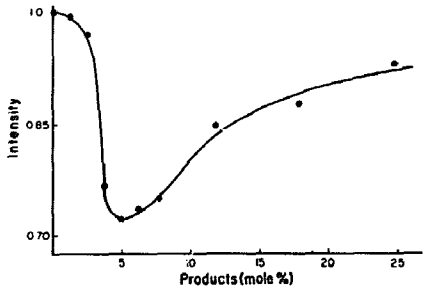

Fig. 3. Relative fluorescence emission intensity of 5.2 μM NK-529 in 0.59 mM DMPC vesicles containing a varying mol% of LPC + myristic acid (in 1 : 1 mole ratios) in the outer monolayers. The additives were incorporated in preformed DMPC vesicles by successively adding small aliquots (typically 2% or less) of LPC followed by equimolar amounts of myristic acid with vigorous mixing.

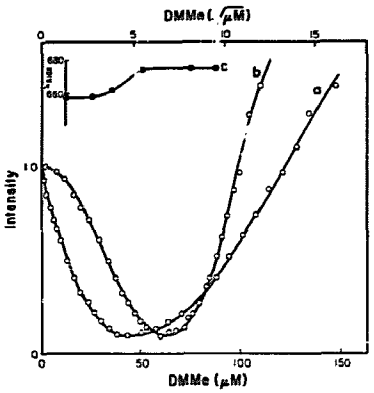

Fig. 4. Dependence of the fluorescence intensity of (curve a) NK-529 at 685 nm on the total DMMe concentration in 10 mM Tris at pH 8.0 and 21°C. Curve b: the same data, as in curve a, plotted as a function of the square root of the concentration of DMMe (upper scale). Curve c: The dependence of the position of the emission maximum on the DMMe concentration (bottom scale).

in the outer monolayer of preformed DMPC vesicles. At higher mole fractions (above 0.05) of the products, the fluorescence intensity increases till it approaches the intensity that is observed in the absence of the additives. One of the most likely interpretations of the biphasic behavior for the fluorescence change under the equilibrium binding conditions (Fig. 3) for NK-529 to the ternary codispersions is that at low mole fractions the fatty acid molecules begin to segregate in the bilayer, and therefore the dye molecules bound to vesicles effectively segregate and self-quench. This hypothesis about the self-quenching of NK-529 bound to anionic vesicles is supported by the studies described below. With appropriate control experiments we have also shown that under these conditions the fluorescence change is not due to a change in the bulk pH, or transmembrane ionic gradients or diffusion potential.

Binding of NK-529 to vesicles of anionic phospholipids

In order to characterize the binding and spectral properties of NK-529 on anionic interfaces we investigated its spectral properties on bilayers of anionic phospholipids. As shown in Fig. 4, the change in the fluorescence intensity at 685 nm as a function of DMMe vesicle concentration is biphasic. The intensity decreases initially to an essentially complete quenching, and then the intensity increases with increasing lipid concentration. The initial decrease in the fluorescence intensity is due to self-quenching of the bound dye, and then the intensity begins to increase as the intermolecular distance of the bound dye molecules increases. As shown in Fig. 4, in the initial linear region only the emission intensity decreases with the lipid concentration, whereas the emission maximum remains unchanged till the fluorescence intensity begins to increase. Also the fluorescence life-time as measured by modulation and phase-shift [16] at 6 mHz remains constant, and these values increase only when the fluorescence intensity begins to increase above the lipid concentration (0.06 mM) where the fluorescence intensity also begins to increase (data not shown).

These results are best explained as follows. At low lipid concentrations the fluorescence is only due to the free dye in the aqueous phase, and therefore the emission maximum and the fluorescence life-times do not change. The bound dye is essentially completely (more than 95%) self-quenched, and therefore it does not contribute to the fluorescence emission. Since this region of the binding curve is essentially linear, only the lower limit of the apparent partition coefficient (affinity) of the dye for the interface of DMMe vesicles can be estimated as more than 38500, as the ratio of the moles of dye per gram of lipid to moles of dye per gram of the aqueous phase. The actual value of the partition coefficient probably approaches 10^6. Near the bottom of the binding profile (Fig. 4) all the dye is bound, and the density of the bound dye is about one dye molecule per 3 phospholipid molecules. An increase in the added lipid disperses the dye molecules farther apart. Under these conditions self-quenching is relieved, and therefore the resulting spectra are due to the dye bound to vesicles where not only the emission intensity increases, the emission maximum is red-shifted, and fluorescence life-time increases.

This interpretation of the genesis of the fluorescence change of NK-529 bound to DMMe vesicles is also consistent with the spectral properties of the dye in the ground state. As shown in Fig. 5 the absorption maximum of the dye shifts to a longer wavelength on binding to DMMe vesicles. As also shown in this figure, while such a shift is observed both at low and high lipid/dye ratios, the difference spectrum at low lipid/dye ratio is noticeably different than it is at

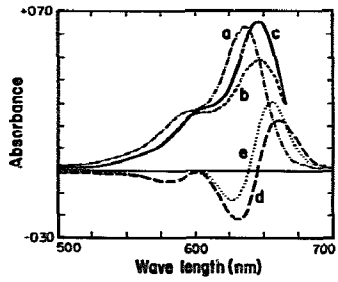

Fig. 5. The absorption spectra of 5.2 μM NK-529 (a) alone, in the presence of (b) 0.035 and (c) 0.6 mM DMMe in 10 mM Tris chloride at pH 8.0 and 21°C. The difference spectra at the (d) lower and (e) higher DMMe concentrations are also shown. The red end of the absorption spectrum (curves b and c) are not shown for clarity.

higher ratios. The difference spectrum in DMPC vesicles is essentially identical to that is observed at high lipid/dye ratios (data not shown). The difference spectrum of the dye bound to vesicles at low lipid/dye ratios also shows that the quenching is due to the formation of a complex in the ground-state, and this does not bring about a change in fluorescence life-time.

The observations described so far show that NK-529 binds to anionic bilayers with a high affinity, and at low lipid/dye ratios substantial quenching is observed. Complex biphasic binding curves of the type shown in Figs. 2 and 4 can be explained in terms of the binding equilibrium:

$$D_w \rightleftharpoons D_q \rightleftharpoons D_b$$

where only the D_b form is fluorescent, and the D_q form of the bound dye is not. Also the fluorescence characteristics of D_b are distinctly different from those of the dye in the aqueous phase D_w. This formalism is supported by the following observations: A biphasic binding curve arises from self-quenching of the dye bound to anionic vesicles with a high affinity where low lipid/dye ratios can be attained as in the beginning parts of the binding curves (Figs. 2 and 4); the increase in the intensity at high vesicle concentration is due to surface dilution on excess vesicles as the equilibrium shifts towards D_b. Based on these considerations the decrease in emission intensity at low concentrations of the aqueous dispersions of a variety of anionic phospholipids (Table II) is due to a high-affinity binding of NK-529. Thus significant self-quenching is seen with low concentrations of the vesicles of anionic but it is not so evident with vesicles of zwitterionic phospholipids (Fig. 2). The emission maximum of the bound dye to anionic vesicles is somewhat lower, in the 673 to 677 nm range compared to 677 for zwitterionic vesicles).

According to these considerations, self-quenching of NK-529 molecules bound to anionic interfaces is related

TABLE II

Binding parameters for NK-529 to vesicles of anionic phospholipids at 21 °C and pH 8.0 in 10 mM Tris chloride

Abbreviations: PC, phosphoryl choline; PE, phosphoryl ethanolamine; Pchol, phosphoryl cholesterol; PG, phosphoryl glycerol; PMe, phosphoryl methanol; PS, phosphoryl serine.

Phospholipids	Lipid/dye ratio
1,2-Dimyristoylglycero-PMe	3.4
1,2-Dimyristoylglycero-PG	4.2
1-Palmitoyl-2-oleoylglycero-PG	1.1
1,2-Dimyristoylglycero-Pchol	2.7
1-Palmitoyl-2-oleoylglycero-PMe	2.7
1,2-Dioleoylglycero-PS	2.0
1,2-Dipalmitoylglycero-PS	3.8

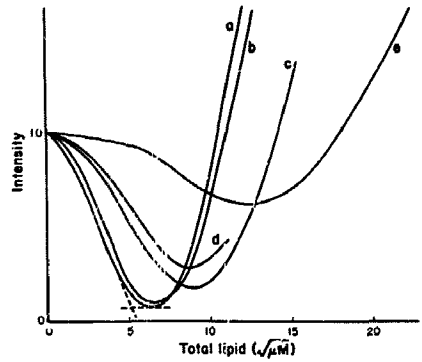

Fig. 6. Dependence of the relative fluorescence emission intensity of 5.2 μM NK-529 at 685 nm in the presence of DMMe vesicles containing varying mole fractions of DMPC: for curves a–e the mole fractions of DMPC are 0, 0.35, 0.5, 0.65 and 0.9. The abscissa is the square root of the total phospholipid concentration. Other conditions as given in Fig. 4.

to the average lateral separation of the dye molecules. Therefore quenching would be proportional to the square root of the area of the interface. A plot of the emission intensity as a function of the square-root of the concentration of the lipid is also shown in Fig. 4. Extrapolation of the initial region of the binding curve to the minimum in the emission intensity, yields a lipid/dye ratio defined as the moles of lipid in the outer monolayer of the vesicle per mole of the bound dye (Table II). These numbers are corrected for the fact that the dye molecules bind only to the outer monolayer of vesicles. For most of the anionic phospholipids the ratio is approximately three, as would be expected if NK-529 covers the area occupied by three phospholipid molecules, however the two positive charges of the dye could bind stoichiometrically only to two phospholipid molecules. The lipid/dye ratio is somewhat smaller for phospholipids with larger areas of cross-section such as dimyristoylglycerophosphoryl cholesterol [17] or the ones with unsaturated acyl chains. This suggests that while the primary driving force for the binding of NK-529 is electrostatic, the binding is of such a high affinity that under limiting conditions the dye virtually completely covers the outer surface of anionic vesicles.

Binding of NK-529 to codispersions of DMPC and DMME

The hypothesis that the falling phase of the binding curve (Fig. 4) is due to the self-quenching, is further supported by the results shown in Fig. 6. The binding curve is appreciably modified in the presence of phosphatidylcholine codispersed in vesicles of DMMe. The fluorescence intensity of bound dye is higher in the presence of DMPC at a mole fraction > 0.3. This suggests that when on the average the dye molecules are farther apart from each other, self-quenching decreases

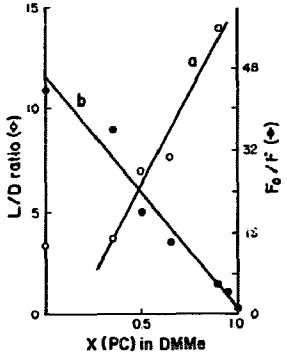

Fig. 7. Dependence of (a, open circles and left ordinate) the lipid/dye ratio and (b, closed circles and right ordinate) ratio of the fluorescence intensity of the dye bound to DMPC to that in the codispersion (F_0/F) as a function of the mole fraction of DMPC in DMMe vesicles. These data are derived from the curves shown in Fig. 6.

as the equilibrium shifts towards the D_b form. The Stern-Volmer plot for the fluorescence intensity of NK-529 at the minimum in the binding curves (obtained from the data shown in Fig. 6) is shown in Fig. 7. The intensity of unquenched bound dye is taken as the intensity at high concentration of DMPC vesicles (cf. Fig. 2), where the lipid/dye ratio is very high and all the dye is bound.

From the binding curves in Fig. 6 we also calculated the lipid/dye ratios when all the dye is bound, and the bound dye is completely quenched. As shown in Fig. 7 the lipid/dye ratio increases with the increasing mole fraction of DMPC in the codispersions, however, this increase is observed only when the mole fraction of DMPC exceeds 0.30. Such a behavior is expected if NK-529 covers three phospholipid molecules out of which only two are anionic. Thus the increase in the fluorescence intensity would be seen only when the separation between the charged phospholipids (two out of three) is increased due to surface dilution. A complete mathematical description is being developed.

Effect of the thermotropic phase transition characteristics

The distribution of anionic charges at the bilayer interface, and therefore the fluorescence characteristics of NK-529, are expected to change with the gel-fluid transition in the bilayer organization. As developed in the next paper for the ternary vesicles, an abrupt decrease in the fluorescence emission intensity occurs at the phase transition temperature. Such a change is considerably more pronounced with codispersions of DTPC containing upto 10 mol% DMMe compared to that with vesicles of only DTPC. Since most of the dye is bound to vesicles under these conditions, the temperature-dependent changes in the fluorescence intensity at the gel-fluid phase transition probably arise from a change in the lateral distribution of the dye bound to anionic lipids, rather than due to a change in the partitioning equilibrium of the dye between the bilayer and the aqueous phase. The temperature-dependent increase in the fluorescence emission of NK-529 is not observed with dispersions containing higher mole fractions of DMMe because the lipid/dye ratio is low and therefore any change in the dye-to-dye distance at the gel-to-fluid transition will not appreciably influence the relative quenching of the dye. It is particularly striking to note that the increase in the fluorescence intensity above the transition temperature is considerably more pronounced in the codispersions of DMPC containing DMMe rather than with DMPC alone. This would be expected if increase in the intensity in the fluid phase is due to ideal mixing of the components, whereas lateral phase separation of the anionic additives occurs in the temperature range where gel-fluid phases coexist [18]. Thus, segregation of anionic lipids would lead to self-quenching of the bound dye. Above the phase transition temperature all the components mix and therefore the effective charge density is lowered and the fluorescence intensity increases. This phenomenon is being investigated further.

The effect of cationic additives

The results described so far emphasize a significant role of the interfacial charges on the binding of NK-529. This is further investigated by monitoring the effect of cationic amphiphiles on the fluorescence intensity of NK-529 in DMMe vesicles. As shown in Fig. 8 the fluorescence intensity of NK-529 in DMMe vesicles increases with increasing concentrations of the various cationic amphiphiles, whereas neutral tetradecanol does not have a noticeable effect. The effect of these amphiphiles is more pronounced in DMMe vesicles than it is in codispersions of equimolar DMMe + DMPC (data not shown). It is interesting to note that the effect

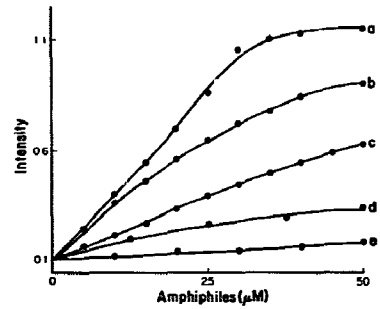

Fig. 8. The change in the fluorescence intensity of NK-529 (5.2 μM) on vesicles of DMMe (42 μM) in the presence of amphiphiles: from top: (a) tetradecylammonium bromide; (b) laurylpyridinium chloride; (c) dibucaine; (d) calcium; and (e) tetradecanol. Other conditions as in Fig. 4.

TABLE III

Effect of salts on the lipid/dye ratio on anionic vesicles

Lipid	Salt	Lipid/dye ratio
DMMe	none	3.4
	NaCl 20 mM	7.6
	Na_2SO_4 10 mM	7.5
	KCl 20 mM	10.4
	LiCl 10 mM	9.1
	$MgCl_2$ 0.5 mM	21.7
	$CaCl_2$ 0.1 mM	15.6
DMMe + DMPC (1 : 1)	none	5.8
	NaCl 10 mM	9.1
	KCl 10 mM	9.1
	LiCl 10 mM	10.4

of the cationic amphiphiles depends not only on their concentration but also on their structure. Low efficacy of dibucaine is probably due to the fact that only a small fraction of dibucaine is bound and of this only a fraction would be in the protonated form. On the other hand a difference in the effect of tetradecylammonium bromide and laurylpyridinium cations must be ascribed to a difference in their sizes.

The effect of salts

Binding of NK-529 to anionic vesicles is dominated by ionic interactions, therefore a shift in the binding equilibrium is expected in the presence of salts. The major effect of salt is to shift the binding curve (cf. Fig. 4) towards a higher substrate concentration. As shown in Fig. 9, increasing calcium concentration leads to an increase in the fluorescence intensity of NK-529 on DMMe vesicles. Also as summarized in Table III the lipid-dye ratio increases significantly in the presence of low concentrations of salts. These effects are concentration dependent, and divalent cations are more effective than monovalent ions. Such a behavior would be expected if these cations compete with NK-529 for binding to the anionic interface.

Discussion

There are few methods available to monitor the distribution of charges on the surface of bilayers. The use of potential-sensitive dyes is based on the principle that the distribution of transbilayer charges is reported by the distribution and orientation of a dye. In this paper we have extended this principle to obtain information about lateral distribution of anionic charges at the surface of bilayers. The results summarized in this paper show that the fluorescence properties of cationic NK-529 are very sensitive to the density and distribution of anionic charges on a bilayer interface. The origin of the fluorescence changes observed in response to changes in the surface charge density is interpreted in terms of the following experimentally verifiable assumptions: NK-529 binds to vesicles with a concomitant increase in the fluorescence intensity if the ratio of lipid to bound-dye is large; the binding of the dye is enhanced considerably at anionic interfaces; a decrease in the ratio of lipid to bound-dye leads to quenching; and the rate of binding and desorption of the dye is rapid. The binding of NK-529 to a bilayer interface is not exclusively but predominantly electrostatic. Thus a change in the distribution of surface charges is reflected in an increase in the fraction of the bound dye which increases the emission intensity, as well as in a decrease in the fluorescence intensity due to self-quenching of segregated of dye molecules. However, based on the considerations outlined in this paper, it is possible to choose appropriate experimental boundary conditions and to manipulate relative contributions of the two opposing processes that determine the fluorescence changes that occur in a variety of experimental conditions leading to changes in surface charge density and distribution. In the next paper [14] we have exploited this situation to monitor changes in the distribution of anionic charges during the time course of interfacial catalysis by phospholipase A_2 on zwitterionic bilayers.

Acknowledgement

This work was supported by a PHS grant (GM29703). We also thank Dr. H.-J. Apell (Konstanz, F.R.G.) for a very stimulating discussion about the properties of NK-529.

References

1 Apell, H.-J., Marcus, M.M., Anner, B.M., Oetliker, H. and Läuger, P. (1985) J. Membr. Biol. 85, 49–63.
2 Ludi, H., Oetliker, H., Brodbeck, U., Ott, P., Schwendiman, B. and Fulpius, B.W. (1983) J. Membr. Biol. 74, 75–84.
3 Waggoner, A.S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–68.
4 Apitz-Castro, R., Jain, M.K. and DeHaas, G.H. (1982) Biochim. Biophys. Acta 688, 349–356.
5 Jain, M.K. and Jahagirdar, D.V. (1985) Biochim. Biophys. Acta 814, 313–318.
6 Upreti, G.C. and Jain, M.K. (1980) J. Membr. Biol. 55, 113–121.
7 Romero, G., Thompson, K. and Biltonen, R.L. (1987) J. Biol. Chem. 262, 13476–13482.
8 Tinker, D.O. and Wei, J. (1979) Can. J. Biochem. 57, 97–106.
9 Tinker, D.O., Purdon, A.D., Wei, J. and Mason, E. (1978) Can. J. Biochem. 56, 552–558.
10 Jain M.K., Van Echteld, C.J.A., Ramirez, F., DeGier, J., DeHaas, G.H. and Van Deenen, L.L.M. (1980) Nature 284, 486–87.
11 Jain, M.K. and DeHaas, G.H. (1983) Biochim. Biophys. Acta 736, 157–162.
12 Klopfenstein, W., De Kruijff, B., Verkleij, A.J., Demel, R.A. and Van Deenen, L.L.M. (1974) Chem. Phys. Lipids 13, 215–222.
13 Wilschut, J.C., Regts, J. and Scherphof, G. (1979) FEBS Lett. 98, 181–186.
14 Jain, M.K., Yu, B.-Z. and Kozubek, A. (1989) Biochim. Biophys. Acta 980, 23–32.

15 Jain, M.K., Rogers, J., Simpson, L. and Gierasch, L. (1985) Biochim. Biophys. Acta 816, 153–162.
16 Lackowicz, J. (1983) Principles of Fluorescence Spectroscopy. Plenum Press, New York.
17 Jain, M.K., Ramirez, F., McCaffrey, T.M., Ioannou, P.V., Marecek, J.F. and Leunissen-Bijvelt, J. (1980) Biochim. Biophys. Acta 600, 678–688.
18 Jain, M.K. (1983) Membr. Fluidity Biol. 1, 1–37.